# CONTRIBUTION OF DYSFERLIN-CONTAINING MEMBRANES TO MEMBRANE REPAIR IN SKELETAL MUSCLE

by

Joel Ryan McDade

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Molecular and Integrative Physiology)
in the University of Michigan
2014

Doctoral Committee:

Associate Professor Daniel E. Michele, Chair
Associate Professor Anatoli Lopatin
Associate Professor Susan Brooks Herzog
Associate Professor Kristen J. Verhey

"Success is not a random act. It arises out of a predictable and powerful set of circumstances and opportunities." -Malcolm Gladwell

This thesis is dedicated to everyone who provided me with the circumstances and opportunities necessary to succeed.

## ACKNOWLEDGEMENTS

First, I would like to thank my mentor Dan, whose tremendous patience and outstanding mentoring made the work within this thesis possible. I am not only speaking for myself when I say that your guidance and support was vital for my success as a graduate student and my vision for the future. I would also like to thank the members of my committee, who have provided me with critical feedback, reagents, and support over the last several years. There are several people with whom I interacted with that were critical for my professional and personal development. When I joined the Physiology Department I was assigned an informal mentor to guide me through the ups and downs of the graduate school experience. My meetings with Ormond ended up teaching me a lot about how I should approach science, and for that I am grateful. The members of the Microscopy and Image Analysis Laboratory have guided me on all matters related to microscopy from the initial experiments in this thesis all the way through to the end. I would especially like to thank Chris Edwards, who, in addition to his knowledge of microscopy was also always good for a laugh. I would like to thank the Molecular and Integrative Physiology administrative staff for all of their assistance, especially Michele Boggs, who is always receptive to frantic emails that require immediate attention. A special thanks to all the past Michele lab members, particularly Matt Campbell and Jessica Gumerson, who came before me and showed me the ropes. Thanks to the current Michele lab members, including Joanne Garbincius, whose optimism and passion for science is contagious. Most importantly, thank you to my wonderful wife Laura, because what goes on at home is just as important as what goes on at school.

## TABLE OF CONTENTS

## LIST OF FIGURES

# CHAPTER 1

## Plasma membrane maintenance in muscular dystrophy and the role of dysferlin-mediated membrane repair in skeletal muscle.

### Muscular dystrophy and plasma membrane integrity:

Muscular dystrophy: The muscular dystrophies are a diverse group of inherited muscle wasting disorders that affect skeletal muscle and/or cardiac muscle resulting from mutations in >30 different genes [1]. Common pathological features include cycles of degeneration and regeneration, infiltration of immune cells, fibrosis, and fatty infiltrate within the muscle; all of which compromise muscle function [1]. Patients with muscular dystrophy typically succumb to respiratory failure as a result of impaired diaphram function or cardiac failure [2]. Proteins implicated in muscular dystrophy play a wide variety of cellular roles, including cell-ECM interactions [3-5], cytoskeletal organization or function [6], sarcomeric organization [7], and membrane trafficking [8]. Mutations in several components of the Dystrophin-glycoprotein complex (DGC), an adhesion complex that mediates the functional linkage between the intracellular actin cytoskeleton and the extracellular matrix, result in muscular dystrophies (Fig 1-1, reviewed in [2]). Although there is no universal cellular mechanism by which genetic mutations result in muscular dystrophy, many muscular dystrophies (particularly those

associated with the DGC) show marked instability of the plasma membrane that renders cells susceptible to cellular wounding [9].

Maintenance of the plasma membrane is critical and loss of DGC components leads to membrane instability and muscular dystrophy: The DGC, is a multi-protein complex that links the intracellular F-actin cytoskeleton through the plasma membrane to laminin present in the basal lamina. The intracellular linkage to F-actin is mediated by dystrophin, a >400 kDa protein that contains an N-terminal actin binding domain, a central rod domain and a C-terminal domain that interacts with β-dystroglycan within the plasma membrane [10]. Dystroglycan is a membrane spanning protein composed of two subunits (α and β) that form as a result of post-translational cleavage of a full length precursor protein [11]. Beta dystroglycan contains the transmembrane domain and interacts with dystrophin, whereas alpha-dystroglycan is entirely extracellular, is highly glycosylated, and mediates the functional linkage with the extracellular matrix through its interaction with laminin [11]. Although the exact mechanism whereby mutations in DGC components cause muscular dystrophy is not entirely clear, a growing body of evidence suggests that loss of the DGC may reduce membrane stability and render cells susceptible to membrane wounding. This assertion is supported by the finding that muscles from several mouse models of DGC deficiency take up more membrane impermeable proteins (IgG) and extracellular dyes (EBD) compared with normal muscle. Furthermore, dye uptake into mutant muscles is markedly increased following exercise [12, 13]. Furthermore, uptake of extracellular macromolecules into muscle fibers is associated with release of muscle-specific cytoplasmic enzyme creatine kinase

into the bloodstream [13]. *In-vitro*, DGC-deficient muscles display reduced force generation capacity following lengthening contraction, which has been associated with increased tearing of myofibers following contraction [14] and uptake of membrane impermeable dyes following contraction induced damage in-vitro [15]. Overall, these data support the assertion that genetic mutations (at least within the DGC) cause muscular dystrophy by compromising plasma membrane integrity, and highlight the critical role for maintenance of the plasma membrane in muscle cells.

**Dysferlinopathy and a novel pathway to muscular dystrophy:**

Dysferlin deficiency results in muscle disease: Dysferlin is expressed in most tissues at low levels, with highest expression in adult skeletal and cardiac muscle [16, 17]. Western blot analysis using an antibody to the dysferlin protein identified a ~220 kDa protein highly enriched in membrane fractions from mature cardiac and skeletal muscle [16, 18]. Antibody labeling of skeletal muscle in cross section reveals that dysferlin is localized to the lateral sarcolemma in muscle biopsies from normal human skeletal muscle and is largely absent in biopsies from patients with Limb-girdle muscular dystrophy 2B (LGMD2B), Myoshi Myopathy (MM) and Distal myopathy (DM) (collectively called dysferlinopathies) [19]. Although secondary loss of dysferlin expression has been noted in LGMD patients with mutations in genes other than dysferlin [20], the vast majority of dysferlinopathy cases can be attributed to homozygous or compound heterozygous mutations spanning the entire dysferlin gene [21], resulting in near complete loss of dysferlin expression. LGMD2B, DM and MM vary in their clinical presentation, with LGMD2B affecting predominantly the muscles in the

limb-girdle, and MM/DM pathology initiating in the distal limb musculature, or a mixed “proximodistal” presentation [22]. In some instances divergent phenotypes can result from identical mutations within the dysferlin gene, which may result from modifier genes or environmental factors such as activity level [19, 23]. Mild cardiac involvement can occasionally be detected in dysferlinopathy patients, but overt cardiomyopathy due to dysferlin-deficiency is rare [24]. Dysferlinopathy patients share several common pathological features with non-dysferlin muscular dystrophies, including, extremely high plasma creatine kinase, marked inflammatory infiltration, and progressive muscle weakness [22]. In some cases, the inflammatory response is so robust that patients are mis-diagnosed as having polymyositis [25]. The age of onset of dysferlinopathy varies considerably in humans ranging from the second to the eighth decade of life, which may be due to environmental factors such as injury due to physical activity [26]. There is currently no effective therapy for dysferlinopathy, as steroid treatments effective in other dystrophies are ineffective in dysferlinopathy [27, 28]. Structure-function studies aimed at finding the minimal structural domains required for functional dysferlin in muscle are currently underway [29], and these functional domains will be described later in this chapter.

Several mouse models serve as important tools for studying the pathogenesis of dysferlinopathy [30]. The two most commonly studied mouse models are the A/J mouse, which is a spontaneous mutation in the dysferlin gene due to a retrotransposon insertion between exons 4 and 5, resulting in loss of both mRNA and protein expression [31]; and the SJL mouse which harbors a splice site mutation, resulting in ~15% dysferlin protein expression in skeletal muscle relative to muscles from wild-type mice

[32]. Loss of dysferlin in the A/J, SJL, and several targeted knockout mice recapitulates several hallmarks of muscle disease in humans including elevated plasma creatine kinase, inflammatory infiltration, centrally nucleated fibers, and a small but significant number of fibers showing uptake of membrane impermeable Evan's blue dye [31]. Analysis of the muscle pathology in SJL compared to A/J mice indicates that the pathology is more severe within the SJL mouse. In the SJL mouse pathology begins in the proximal muscles at 2 months of age, and progresses to both proximal and distal presentation after 5 months of age, whereas presentation of pathology in A/J mice is delayed until 5 months of age [31]. Although the pathogenesis of dysferlin-deficiency is well described within human dysferlinopathy patients [22] and mouse models [31], the exact mechanism by which dysferlin-deficiency results in muscle disease is not clear. Recent studies have begun to address this question, and the findings suggest that the cellular deficit associated with dysferlin-deficiency may be independent of defective membrane stability [18, 33].

Dysferlin deficiency causes muscular dystrophy via a DGC independent pathway: Several independent lines of evidence indicate that dysferlin-deficient muscular dystrophy occurs independently of any effects on the DGC and membrane stability (Fig 1-1). First, dysferlin-deficient mice show normal expression and localization of DGC components, and dysferlin is not an integral component of the DGC in normal muscle, which argues against a role for dysferlin in membrane stability [31]. Although dysferlin-deficiency does not affect the DGC, dysferlin is markedly mislocalized away from the plasma membrane in a subset of muscle fibers from patients and mice with mutations in

DGC components or non-dysferlin LGMDs, indicating that DGC-deficiency or cellular wounding as a consequence of DGC-deficiency may alter dysferlin localization and/or function [20]. Furthermore, combined loss of dysferlin and dystrophin exacerbates the phenotype in dysferlin-null mice, indicating that dysferlin function is critical for minimizing the adverse effect of DGC-deficiency in muscle [34]. Although dysferlin null skeletal muscles contain significantly more Evans blue dye (EBD) positive fibers than control muscle at rest, uptake of EBD is not enhanced by mild exercise in dysferlin-deficient muscle, in contrast to DGC-null muscle [18, 31]. One hallmark of DGC-deficient muscle is the susceptibility to contraction-induced damage following lengthening contractions, which results in reduced maximal force production (termed force deficit) [14, 35]. Dysferlin-deficient muscle does not display a force deficit following lengthening contraction, suggesting that loss of dysferlin expression does not render skeletal muscle susceptible to contraction induced damage, and arguing against a role for dysferlin in membrane stability [33]. However, administration of lengthening contractions sufficient to injure normal muscle results in delayed force recovery in dysferlin-deficient muscle, and recovery of muscle function in dysferlin-null muscle requires myogenesis [36], indicating that dysferlin may play a role in the cellular response to wounding. Overall, these findings indicate that dysferlin-deficiency produces a dystrophic phenotype independent of any effects on the DGC and plasma membrane stability and implies a distinct role for dysferlin in muscle cells.

**What is the role of dysferlin in muscle cells?:**

Ferlins are evolutionarily conserved mediators of membrane fusion: The dysferlin gene encodes a type 2 transmembrane protein with high homology to the ferlin family of proteins (Fig 1-2A) [37]. Ferlin proteins are evolutionary conserved mediators of membrane fusion across a wide variety of species and cell types [38, 39]. For example, mutations in Fer-1, the sole C. Elegans ferlin protein result in sterililty due to impaired fusion of the membranous organelle with the spermatid plasma membrane [40]. Mutations in otoferlin, a ferlin expressed in the inner ear of mammals results in hereditary deafness, due to impaired electrical transmission as a result of impaired fusion of synaptic vesicles within inner hair cells [41]. Dysferlin contains 7 putative C2 domains (C2A-C2F) and a single transmembrane domain toward the extreme C-terminus, resulting in a long cytoplasmic C2-domain containing N-terminal domain, and a short extracellular or luminal domain [38] (Fig 1-2B). The lipid binding ability of C2 domains within dysferlin have been characterized using *in-vitro* lipid binding assays, which revealed that the C2A domain of dysferlin binds lipids in a calcium-dependent manner, while the remaining C2 domains bind lipids independently of calcium [42, 43]. The C2A domains of dysferlin, myoferlin and otoferlin are capable of inducing membrane curvature in liposomes *in-vitro* [43]. Interestingly, the C2 domains of dysferlin show preferential binding with the types of lipids enriched on the inner leaflet of the plasma membrane [42]. Although the exact cellular function that is disrupted in dysferlin-deficient muscle that is causal for muscle disease is not entirely clear, these findings strongly support a role for dysferlin in mediating membrane binding/fusion events in skeletal muscle.

Potential cellular roles for dysferlin: Consistent with structural and biochemical evidence, dysferlin has been implicated in a wide variety of cellular processes related to membrane fusion in both muscle and non-muscle cells. In monocytes, dysferlin expression is increased during differentiation, and dysferlin deficient monocytes display enhanced migration, potentially due to altered integrin trafficking [44]. In endothelial cells, loss of dysferlin leads to altered cell adhesion due to increased degradation of a critical signaling molecule PECAM [45]. Dysferlin-deficient mice show impaired skeletal muscle regeneration following toxin-induced or lengthening contraction-induced wounding, which is consistent with *in-vitro* findings that dysferlin-deficiency impairs cell-cell fusion of myoblasts [33, 46, 47]. Consistent with a role for dysferlin in membrane trafficking, skeletal muscle hypertrophy following insulin-like growth factor (IGF) infusion is impaired in dysferlin-null mice potentially due to defective trafficking of the IGFR in skeletal muscle cells [48]. Dysferlin may play a role in development and maintenance of the transverse tubules, as dysferlin colocalizes with the transverse tubules in developing and regenerating muscle in mice [46], differentiating myotubes *in-vitro* [49], and transverse tubule morphology is altered in dysferlin-deficient mice [50]. Perhaps the most intensively studied cellular role for dysferlin is in the evolutionarily conserved process of membrane repair [18, 49, 51-53]. Given that membrane damage is frequent in mechanically active tissues such as muscle [12], the role of dysferlin in membrane repair in normal muscle and potential adverse effects of dysferlin-deficiency on membrane repair is an area of intense investigation (which will be covered in

subsequent sections), but, at present much of what is known about the mechanism of membrane repair comes from non-muscle model systems.

**Membrane repair in non-muscle model-systems:**

Membrane repair is a critical, evolutionarily conserved process by which cells withstand wounding to the plasma membrane: Membrane repair is a critical process by which cells are able to withstand even the most severe wounds to the plasma membrane (Fig 1-3A) Capacity to reseal the plasma membrane has been documented across a variety of cell types *in-vivo* including epithelial cells [54], intestinal endothelial cells [55], neurons [55-60], cardiomyocytes [61] and skeletal muscle cells [12]. Although the causative agent for membrane damage likely depends on the specific cell type in question, mechanical wounding [58-60], and exposure to bacterial toxins [62] are likely the most common. Several laboratory models of membrane damage have been developed to assess membrane repair *in-vitro*, including detergent permeabilization [51], and electroporation [63]. Currently in the field, mechanical wounding using finely pulled patch pipettes, and laser-induced wounding typically touted in combination with live cell imaging represent the gold standard assays for membrane repair, given that individual cells can be studied in real-time following wounding at a specific location on the plasma membrane [18, 61, 64]. Using these gold standard injury techniques, several independent methods have been employed to quantify membrane resealing *in-vitro*, and these vary from measuring changes in membrane tension [65], and influx of membrane impermeable dyes following wounding [18]. Analyzing uptake of FM1-43 (a membrane impermeable dye that is fluorescent upon contact with membranes but cannot pass

through the plasma membrane) following laser-induced wounds using confocal microscopy has emerged as the most commonly used metric of membrane repair [18, 61, 66, 67] (Fig 1-3B).

Most of the mechanistic understanding of how cells repair wounds in the plasma membrane comes from studies in non-muscle model systems including sea urchin eggs [64, 68-70], neurons [37, 56-60], and fibroblasts [71-73]. Although the mechanism by which various cell types reseal the plasma membrane may vary, resealing in most cell types requires directed transport of intracellular vesicles toward lesions and calcium-induced vesicle-vesicle and vesicle-membrane fusion.

Exocytosis, endocytosis, and vesicle-vesicle fusion is critical for membrane resealing: Early studies into membrane resealing were prompted by the finding that erythrocytes spontaneously reseal following osmotic shock [74]. In erythrocytes, "resealing" occurs due to passive interactions between the hydrophobic membrane and aqueous solution [74]. In nucleated cells, which have a more elaborate cytoskeletal architecture and endure more severe physiological wounds, resealing requires calcium activated vesicle-vesicle and vesicle-membrane fusion [60, 68, 69, 75, 76]. A role for damage-induced exocytosis in membrane resealing was definitively shown in early studies using the sea urchin egg, which contain thousands of vesicles docked at the plasma membrane [64]. Following wounding, these "docked" vesicles fuse with each other and remaining portions of intact plasma membrane to facilitate membrane resealing [64]. Sea urchin eggs undergoing larger lesions (~400 $um^2$) reseal due to a dramatic vesicle-vesicle and vesicle-plasma membrane fusion response near the lesion [70]. Electron micrographs of

sea urchin eggs immediately following large wounds confirmed the presence of a dense network of vesicles undergoing vesicle-vesicle fusion, which, at later time points appear as a large relatively protein free "plug" spanning the entire lesion [75]. Damage-induced exocytosis of docked vesicles in sea urchin eggs is depends on SNARE complexes, as cleavage with toxins specific for these interactions impair exocytosis and inhibit resealing [64]. Although studies in the urchin egg directly demonstrates a role for exocytosis of vesicles in membrane resealing, virtually all cell types lack cortically docked vesicles, calling into question whether all cell types utilize an analogous mechanism. Subsequent analysis of resealing in fertilized urchin embryos (which lose the docked vesicle population as a result of fertilization) have shown that membrane wounding activates transport of intracellular vesicles toward the membrane lesion via the cytoskeleton and can be separated into kinetically distinct waves of exocytosis [77]. Vesicles are delivered along microtubules by kinesin motors until the vesicles reach cortical actin, whereby non-muscle myosin-II transports the vesicles toward the plasma membrane for their subsequent fusion; disruption of either step reduces damage-induced exocytosis and impairs resealing [77]. Fibroblasts and other mammalian cell types reseal the plasma membrane via an exocytotic mechanism, and the intracellular membranes primarily involved appear to be lysosomes [72]. Pharmacological or genetic inhibition of lysosomal function impairs membrane resealing in fibroblasts, and knockout of synaptotagmin-VII (a dysferlin homolog present on lysosomes) impairs lysosomal exocytosis and inhibits membrane resealing in fibroblasts [71]. Several studies have also implicated endocytosis in membrane resealing. In NRK cells treated with SLO toxin (which forms transmembrane pores) enzyme release due to lysosomal-exocytosis

facilitates rapid endocytosis of toxin pores and this coupling may be required for membrane resealing at least in this model of toxin-induced wounding [78]. In giant crayfish neurons or cockroach neurons, endocytic vesicles directly contribute to membrane resealing following axotomy by forming a vesicular plug at the membrane lesion [79]. In Aplysia neurons, axotomy induces microtubule based transport of vesicles toward the cut end of the axon, indicating that microtubule-based transport of vesicles may contribute to membrane repair [80]. Although there is no unifying mechanism of membrane resealing, and the cellular components involved appear to vary, these studies highlight the critical role for vesicle-vesicle and vesicle-membrane interactions during membrane resealing, and provide clues for the potential role for dysferlin in membrane resealing in muscle.

The cytoskeleton is a critical regulator of membrane resealing in non-muscle cells: The cytoskeleton is a critical regulator of membrane remodeling and membrane transport processes across a wide variety of cell types [81]. In sea urchin embryos, delivery of vesicles to membrane lesions is mediated by microtubules, and disruption of microtubules or kinesin-motors (the major outwardly directed motor along microtubules) inhibits membrane resealing [77]. In fibroblasts, while microtubules are not required for resealing initial wounds, disruption of microtubules impairs membrane resealing following multiple wounds [82]. This can be explained, in part, by the fact that microtubules undergo dramatic re-orientation toward membrane wounds, and deliver newly synthesized Golgi-derived vesicles to replenish those used during resealing the initial wound [82]. The sub-membrane actin cytoskeleton is critical for membrane

resealing in sea urchin embryos, as disruption of actin filaments or inhibition of myosin motors inhibits damage-induced exocytosis and membrane resealing [77]. A role for actin-dependent non-muscle myosin motors in membrane repair has also been confirmed in fibroblasts [83] and potentially muscle cells [67]. Furthermore, In drosophila embryos and *Xenopus* Oocytes, membrane lesions are repaired via the formation of an actin and myosin-II "contractile ring" which, through direct interactions with cadherin, generates a pulling force to "close" lesions in the plasma membrane [84, 85]. Subsequent studies have also determined that proteins that regulate actin dynamics, such as Rho-kinase, are activated in response to wounding [86]. F-actin accumulates at membrane lesions in primary human myotubes [87], but whether an analogous mechanism of F-actin contractile ring formation contributes to membrane resealing in muscle cells remains to be explored.

**Membrane repair in muscle and the role of dysferlin:**

Membrane damage and repair in muscle: Plasma membrane disruption is a common occurrence in dystrophic muscle, and normal muscle following injurious contractions or prolonged endurance exercise, and often can be marked pathologically by staining muscle sections for infused vital dyes, or by staining for intracellular accumulation of normally non-permant extracellular soluble proteins [13]. Although dysferlin-deficient muscle does not appear to be more susceptible to membrane damage than normal muscle, several lines of evidence indicate that membrane resealing may be impaired in dysferlin deficient muscle [18]. Dysferlin-null muscle fibers show increased uptake of membrane impermeable FM1-43 dye following laser-induced wounding, indicating that

dysferlin-null muscle cells do not repair the membrane as efficiently as normal muscle (Fig 1-3B and C). Furthermore, dysferlin is enriched at sites of cellular wounding in adult skeletal muscle fibers, indicating that recruitment of dysferlin at membrane lesions is critical for membrane resealing [18]. Electron micrographs of dysferlin-null muscle show dramatic accumulation of vesicles under the plasma membrane near putative membrane lesions, which supports the model that vesicle-vesicle or vesicle-PM fusion of dysferlin-containing vesicles may be critical for resealing in muscle cells [18]. Recently, mutations within several additional proteins have been shown to impair membrane resealing in muscle cells, and their potential interaction with dysferlin, as well as their contribution to membrane resealing is currently being addressed [52, 53]. MG53 is a tri-partate motif protein that is highly expressed in muscle, and MG53 knockout mice display a dystrophic phenotype characterized by high CK, centrally nucleated fibers, muscle weakness and inflammatory infiltrate [51]. MG53 accumulates at membrane lesions via non-muscle myosin-II (NMII), and inhibition of NMII with blebbistatin decreases MG53 accumulation and impairs membrane resealing [67], consistent with a role for the actin cytoskeleton in membrane resealing in muscle. MG53 accumulation may represent an initial activating event in membrane repair, as MG53 accumulates at membrane lesions prior to dysferlin, and dysferlin accumulation at lesions may be dependent on MG53 expression [52]. Strikingly, MG53 accumulation is not calcium-dependent, instead MG53 undergoes oxidation-dependent oligomerization at membrane lesions following exposure to the oxidative extracellular environment [51]. Caveolins are critical components of caveolae, which regulate endocytosis across a wide variety of cell types [88]. Mutations in a muscle specific caveolin, Caveolin-3, result

in a LGMD phenotype in humans [8], and over-expression of P140L mutant caveolin-3 in mice causes muscle disease, as a result of impaired membrane resealing. Furthermore, resealing deficits in this model appear to be due to altered interactions between MG53 and dysferlin [52]. Caveolin positive vesicles form following membrane wounding, which may contribute to membrane resealing, but whether these vesicles contain critical repair proteins has not been explored [89]. Annexins accumulate at membrane lesions in both non-muscle (annexin 1, 5), and muscle cells (annexin A6, A2a, A1a) [53, 90], and recruitment of annexin A2 and A1 is dependent on dysferlin in zebrafish muscle [90]. These studies indicate that the interaction between dysferlin and proteins involved in membrane trafficking may underlie the role of dysferlin in membrane repair, and this is supported by a growing body of biochemical evidence that dysferlin may mediate critical membrane fusion events during membrane repair in skeletal muscle.

Dysferlin as a critical regulator of membrane fusion during membrane resealing in adult skeletal muscle: The C2A domain of dysferlin is capable of binding lipids in a calcium dependent manner, consistent with a role for dysferlin in the calcium activated fusion events that are required for membrane resealing [42, 91]. The additional C2 domains (C2B-F) bind lipids independently of calicum, which indicates that these domains may be constitutively bound to lipids, however the relevance of the lipid binding capacity of these domains to membrane resealing is not clear [42]. In addition to lipid binding, the C2 domains of dysferlin are thought to play a critical role in regulating protein-protein interactions and these interactions may be critical for resealing. For example, the

interaction between dysferlin and MG53 is mediated by the C2A domain in-vitro [92]. Recently, structure-function analyses have demonstrated that a mini-dysferlin containing only the C2A, C2F and transmembrane domain is sufficient to restore membrane repair capacity in dysferlin-deficient myotubes, but whether mini-dysferlin is sufficient to restore muscle function in dysferlin-deficient mouse models is not known [29]. Therefore, recruitment of dysferlin to membrane lesions could facilitate membrane repair by enhancing vesicle-vesicle and vesicle-membrane fusion specifically at membrane lesions.

The mechanism by which dysferlin-containing compartments contribute to membrane resealing is not clear: Although the resealing defect associated with dysferlin-deficiency is well described, there is still a fundamental lack of understanding as to how dysferlin-containing membranes contribute to membrane resealing in adult skeletal muscle. Figure 1-4 outlines the currently hypothesized model for dysferlin-mediated membrane resealing, which indicates that sarcolemmal wounding leads to localized calcium influx, subsequent vesicle-vesicle and vesicle-sarcolemma fusion of dysferlin-containing vesicles to form a dysferlin-rich "patch" over the membrane lesion [18] (Fig 1-4). However, there is little direct evidence in support of this model. Dysferlin localizes to intracellular vesicles in differentiating myotubes [93], and adult dysferlin-null muscle shows accumulation of sub-sarcolemmal vesicles of unknown composition [18], but a study that examined dysferlin localization using electron microscopy of normal skeletal muscle immunolabeled for dysferlin concluded that dysferlin is restricted to the plasma membrane [16]. Furthermore, the biochemical composition of dysferlin-containing

vesicles has not been defined [18]. Furthermore, a recent study examining the contribution of dysferlin to membrane repair in zebrafish muscle concluded that dysferlin expression may be restricted to the plasma membrane [90]. There have also been very few studies attempting to examine the behavior of dysferlin-containing membranes following membrane wounding. Therefore, a more thorough examination of dysferlin-containing compartments and development of novel reagents to examine the behavior of those compartments following wounding is critical to understanding the function of dysferlin-containing membranes in membrane resealing. There is some evidence to suggest that dysferlin-containing membranes interact with the cytoskeleton in differentiated myotubes [94], however, the relevance of these interactions to dysferlin-mediated membrane resealing is not clear.

**Rationale and Approach:**

Membrane repair is critical for muscle function but the exact contribution of dysferlin-containing membranes to resealing is not clear: Mutations in several proteins involved in membrane repair result in muscle disease [18, 51], and enhancing membrane repair capacity may be a viable therapeutic approach for muscle diseases [95]. However, a more thorough understanding of membrane resealing in normal muscle is critical in order to design novel therapeutics aimed at enhancing membrane repair. Dysferlin-containing membranes are thought to contribute to membrane repair, but the exact function of dysferlin-containing membranes in membrane repair in muscle is not clear. Furthermore, whether the cytoskeleton is required for the function of dysferlin-containing membranes during membrane repair in muscle is not clear. Therefore, the overall goal

of the work in this thesis was to examine the dynamic behavior of dysferlin-containing membranes and examine the role of the cytoskeleton in dysferlin-mediated membrane repair in skeletal muscle. **In this thesis, I tested the overall hypothesis that dynamic interactions between the cytoskeleton and dysferlin-containing membranes are critical for membrane resealing in skeletal muscle.** Live-cell imaging based approaches were developed to investigate the behavior of dysferlin-containing membranes in differentiated myotubes expressing dysferlin-eGFP, or adult skeletal muscle fibers isolated from a muscle specific transgenic reporter mouse expressing dysferlin-pHGFP. Genetic or pharmacological disruption of the cytoskeleton or motor proteins was used to examine the role of the cytoskeleton in dysferlin-mediated membrane repair.

**Specific Aim 1:** Examine the behavior of dysferlin-containing membranes following wounding and determine the role of the cytoskeleton in dysferlin-mediated membrane repair in differentiating myotubes.

The purpose of the experiments outlined in Aim 1 was to examine the behavior of dysferlin-containing vesicles following wounding and the role of the cytoskeleton in regulating that behavior in differentiated myotubes. Although dysferlin-containing vesicles have been implied as critical mediators of membrane repair in muscle, there is actually limited evidence that this is the case. L6 myotubes expressing fluorophore labeled dysferlin molecules were subjected to live-cell imaging to examine the behavior of dysferlin-containing compartments prior to and following membrane wounding with or

without pharmacological or genetic disruption of the cytoskeleton/motor proteins. Our data indicates that dysferlin-containing vesicles undergo vesicle-vesicle fusion following membrane disruption which generates large dysferlin-containing vesicles that may act as a vesicular plug to reseal membrane lesions. Pharmacological disruption of microtubules or genetic inhibition of kinesin motors impairs large vesicle formation likely through direct inhibition of vesicle function following wounding. This data supports the overall model that microtubule-dependent vesicle-vesicle fusion of dysferlin-containing vesicles is critical for membrane resealing in differentiated myotubes.

**Specific Aim 2:** Examine the behavior of dysferlin-containing membranes following wounding and determine the role of the cytoskeleton in dysferlin-mediated membrane repair in adult skeletal muscle.

The purpose of the experiments outlined in Aim 2 was to examine the contribution of dysferlin-containing membranes to membrane repair in adult skeletal muscle. A novel muscle-specific transgenic mouse expressing dysferlin-pHluorin GFP was generated and used to examine the behavior of dysf-pHGFP prior to and following laser-induced wounding using confocal microscopy. The data indicate that dysferlin is restricted to the sarcolemma and t-tubules, with minimal dysferlin within vesicles at rest. Following wounding, adjacent sarcolemma-derived dysferlin is rapidly pulled into stable dysferlin-rich structures surrounding the lesion. Interestingly, membrane damage also induces the formation of endocytic dysferlin containing vesicles, which may contribute to membrane repair by plugging wounds in the plasma membrane. Disruption of the actin

cytoskeleton impairs accumulation of sarcolemma derived dysferlin as well as membrane resealing in adult skeletal muscle fibers with no effect on endocytosis. These findings support the overall model that actin-dependent recruitment of sarcolemma-derived dysferlin contributes to membrane resealing by creating an "active-zone" of high lipid binding activity to facilitate interaction with dysferlin-containing or non-dysferlin repair vesicles specifically at membrane lesions.

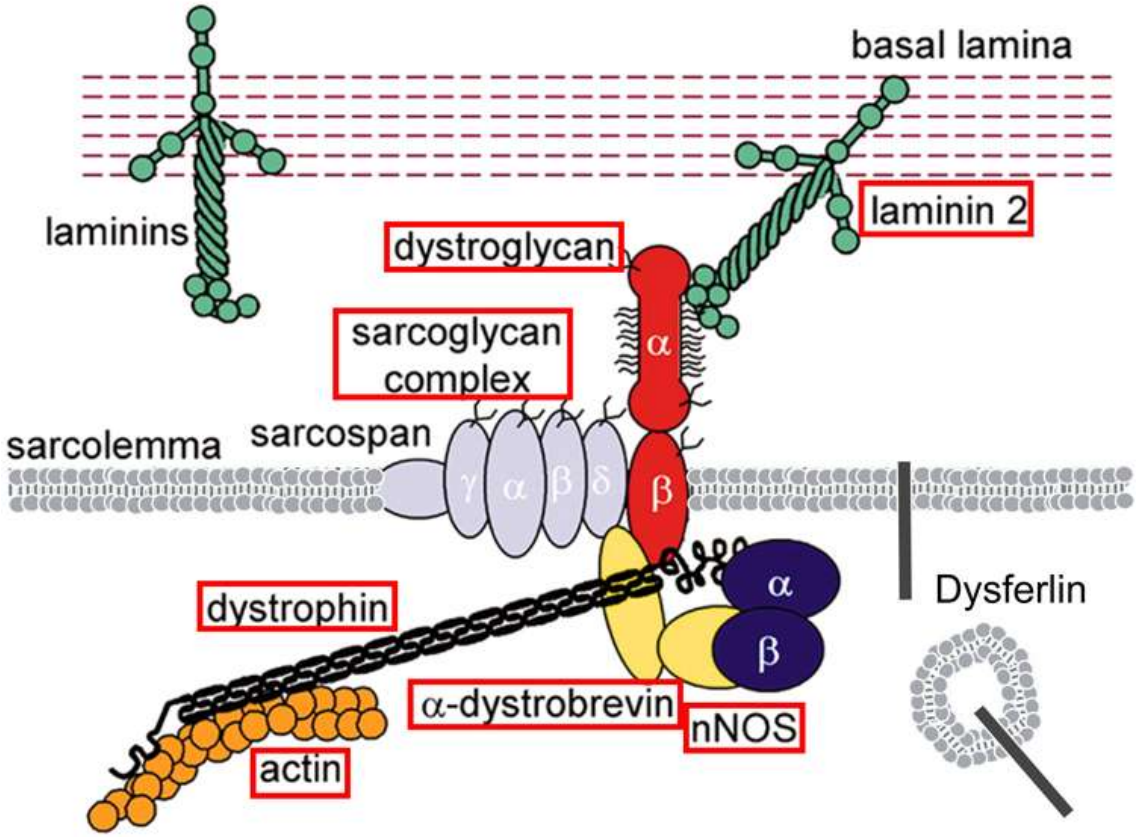



**Figure 1-1.** Loss of dysferlin results in muscular dystrophy independently of any effects on the DGC or membrane stability. The protein names in red boxes are components or interact directly with proteins in the dystrophin-glycoprotein complex (DGC). The DGC acts as a structural link between the actin-cortex (via dystrophin), through the plasma membrane (via dystroglycan) and the basal lamina (through laminin). Mutations in any component of the DGC are thought to destabilize the plasma membrane and result in susceptibility to contraction-induced sarcolemma wounding. Dysferlin is enriched in membrane fractions from muscle, but is not an integral component of the DGC. Loss of dysferlin does not affect DGC components, and does not alter membrane stability, which highlights an independent function for dysferlin in skeletal muscle cells. (Figure modified from [96])

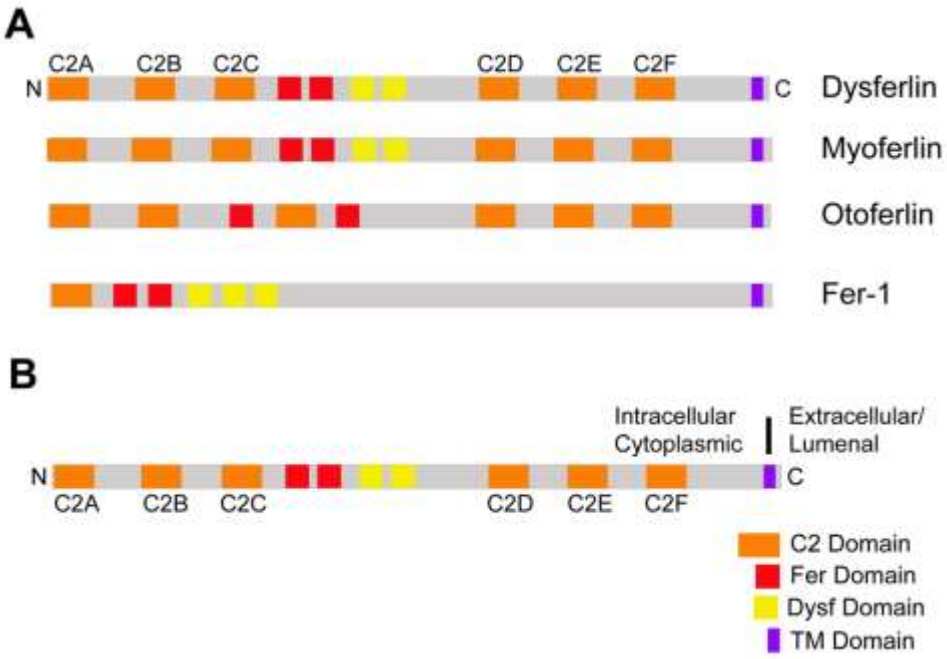



**Figure 1-2. A)** Phylogenic analysis of and structure of ferlin proteins. Ferlins have a highly conserved structure highlighted by multiple tandem containing C2 domains (with the exception of Fer-1, which contains 1), ferlin and dysf-domains, and an extreme C-terminal transmembrane domain. Ferlin proteins regulate a wide variety of membrane fusion events across several species and cell types, indicating that dysferlin may regulate membrane fusion in muscle. **B)** Orientation of dysferlin within the plasma membrane and/or intracellular vesicles. The long cytoplasmic N-terminal domain contains the C2 domains. The short c-terminal domain localizes to the lumen of intracellular vesicles or extracellular surface of the plasma membrane or t-tubules.

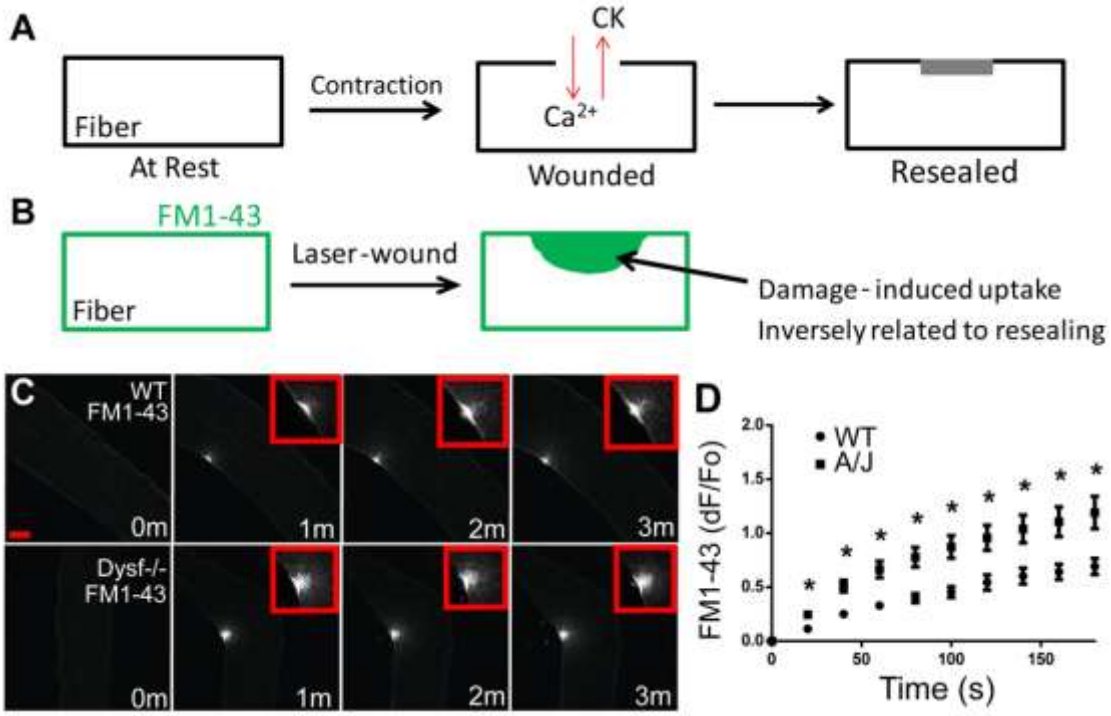



**Figure 1-3.** Impaired membrane resealing in dysferlin-deficient muscular dystrophy. **A)** Simple schematic of membrane resealing. Contraction is thought to induce tears in the plasma membrane, particularly when genetic mutations in DGC components render the sarcolemma unstable. Lesions result in the leakage of intracellular molecules (Creatine Kinase, CK), and the uptake of extracellular ions/molecules (calcium and impermeable dyes), and subsequently cell death unless the cell repairs the lesion. Resealing is thought to require recruitment of intracellular vesicles to lesions in non-muscle cells, but the mechanism by which muscle fibers reseal the sarcolemma is largely unknown. **B)** Membrane repair is assayed in-vitro by incubating adult skeletal muscle fibers in membrane impermeable fluorescent FM1-43 dye and measuring the uptake of dye following laser-induced injury. **C)** Dysferlin-deficient muscle fibers take up more FM1-43 dye than wild-type fibers following laser induced injury (quantification in **D**), indicating that membrane repair is defective in dysferlin-deficient muscle (representative data shown from Chapter 3 and consistent with [18]).

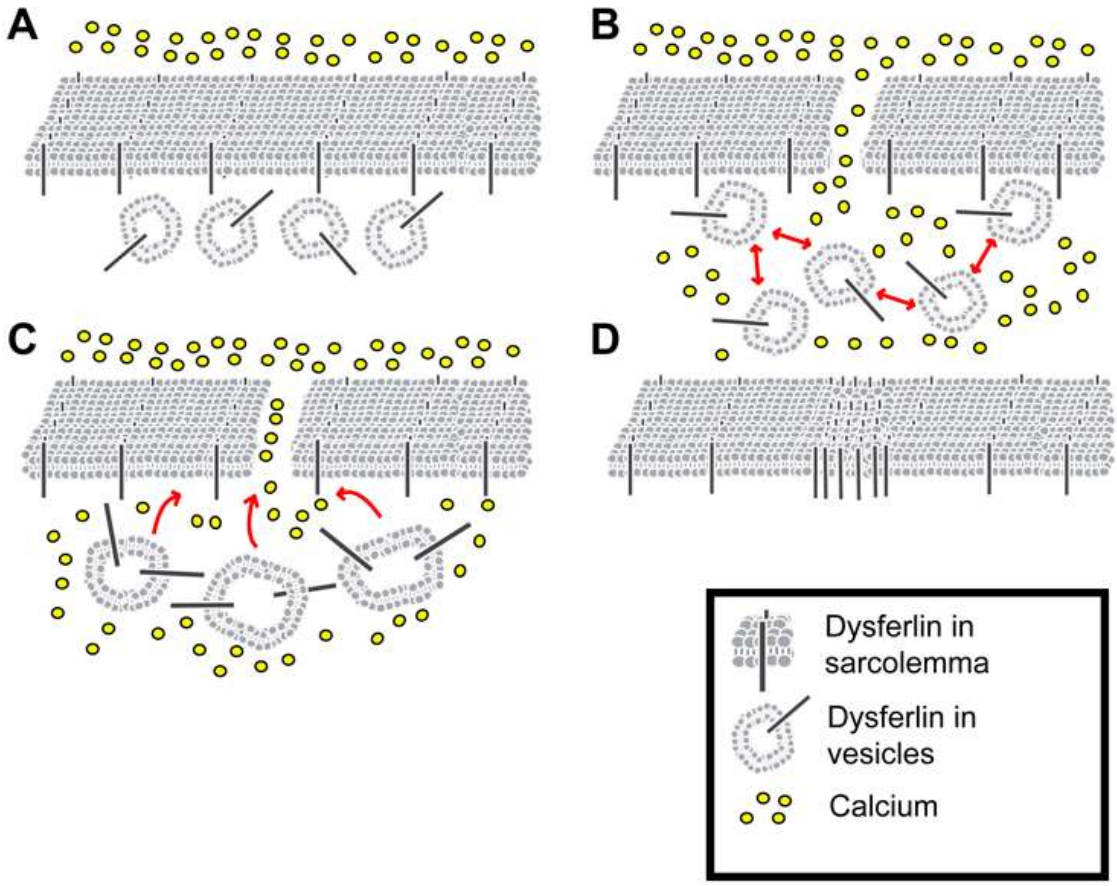



**Figure 1-4.** Hypothesized model for dysferlin-mediated membrane repair in skeletal muscle. **A)** Dysferlin-containing vesicles are present under the sarcolemma of resting skeletal muscle fibers. Physical disruption of the sarcolemma results in a localized influx of extracellular calcium, which triggers dysferlin-dependent vesicle-vesicle **(B)** and vesicle-sarcolemma **(C)** fusion of dysferlin-containing vesicles. Fusion of dysferlin-containing vesicles with the sarcolemma results in formation of a dysferlin-rich "patch" structure at the lesion, which restores the physical barrier between the cytosol and extracellular space **(D)**. Therefore, dysferlin-deficiency is hypothesized to impair membrane resealing by impairing damage-induced vesicle fusion, but damage-induced fusion of dysferlin-containing vesicles and the dysferlin-containing compartments critical for membrane repair have not been explored.

## CHAPTER 2

# Membrane damage induced vesicle-vesicle fusion of dysferlin-containing vesicles in muscle cells requires microtubules and kinesin.


## ABSTRACT

Mutations in the dysferlin gene resulting in dysferlin-deficiency lead to Limb-girdle Muscular Dystrophy 2B and Myoshi Myopathy in humans. Dysferlin has been proposed as a critical regulator of vesicle mediated membrane resealing in muscle fibers, and localizes to muscle fiber wounds following sarcolemma damage. Studies in fibroblasts and urchin eggs suggest that trafficking and fusion of intracellular vesicles with the plasma membrane during resealing requires the intracellular cytoskeleton. However, the contribution of dysferlin-containing vesicles to resealing in muscle and the role of the cytoskeleton in regulating dysferlin-containing vesicle biology is unclear. Here, live-cell imaging was used to examine the behavior of dysferlin-containing vesicles following cellular wounding in muscle cells and examine the role of microtubules and kinesin in dysferlin-containing vesicle behavior following wounding. Our data indicate that dysferlin-containing vesicles move along microtubules via the kinesin motor KIF5B in muscle cells. Membrane wounding induces dysferlin-containing vesicle-vesicle fusion and the formation of extremely large cytoplasmic vesicles, and this response depends on both microtubules and functional KIF5B. In non-muscle cell types, lysosomes are

critical mediators of membrane resealing, and our data indicate that dysferlin-containing vesicles are capable of fusing with lysosomes following wounding which may contribute to formation of large wound sealing vesicles in muscle cells. Overall, our data provide mechanistic evidence that microtubule-based transport of dysferlin-containing vesicles may be critical for resealing, and highlight a critical role for dysferlin-containing vesicle-vesicle and vesicle-organelle fusion in response to wounding in muscle cells.

## INTRODUCTION

Membrane damage is a frequent occurrence in mechanically active tissues, such as skeletal and cardiac muscle, and successful repair of the cell membrane following disruption is critical to muscle function [12, 18, 32]. Mutations within a critical membrane-repair protein, dysferlin, leads to two mild but progressive forms of muscular dystrophy termed Limb-Girdle Muscular Dystrophy 2B (LGMD2B) and Myoshi Myopathy (MM)[23, 97]. Dysferlin is a single-pass transmembrane protein containing multiple cytoplasmic C2 domains, and is a member of the evolutionarily conserved ferlin family of proteins. Ferlin proteins play a critical role in membrane fusion across multiple species and cell types [38]. Dysferlin appears to have evolved in higher order vertebrates and is relatively muscle specific, with the highest expression in mature skeletal and cardiac muscle [17]. Dysferlin is expressed predominantly at or near the plasma membrane in normal adult skeletal muscle, and is markedly mislocalized into the cytoplasm in skeletal muscle from patients with Duchenne muscular dystrophy, as well as Limb-girdle muscular dystrophies caused by genetic mutations in genes other than dysferlin [20]. Furthermore, combined deficiency of dystrophin and dysferlin

worsens disease progression in mice, highlighting the critical role for dysferlin both in normal, and diseased skeletal muscle [34]. While the functions of dysferlin in muscle that are important for its causal role in muscular dystrophy and the mechanisms of those functions are still unclear, dysferlin has been suggested to play a role in a wide variety of processes related to membrane fusion, including t-tubule biogenesis and maintenance [46], cell-cell fusion [47], cell adhesion [45], and muscle growth [98]. Perhaps the most intensively studied role for dysferlin is in the process of membrane resealing. Membrane resealing is a conserved process by which cells are able to survive mechanical disruption of the plasma membrane [64, 76, 77, 83]. Dysferlin-null skeletal and cardiac muscle show enhanced uptake of membrane impermeable dye following laser-induced wounding suggesting dysferlin may play a role in membrane resealing [18, 61]. Dysferlin is enriched at sites of cellular damage, and these "patches" are devoid of plasma membrane proteins, indicating dysferlin may have been delivered from an intracellular membrane source [18, 61]. Electron micrographs of dysferlin-deficient muscle fibers show robust accumulation of vesicles under the sarcolemma, suggesting that in wild-type muscle, dysferlin may play a role in fusion of sub sarcolemma vesicles with the plasma membrane and that these vesicles may be critical to repairing the membrane following wounding [18]. Although these data are consistent with a role for dysferlin-containing vesicles in membrane resealing in skeletal muscle, exactly how dysferlin containing vesicles contribute to membrane resealing in muscle cells is not clear.

Current knowledge of membrane resealing is largely derived from studies in the sea urchin egg and fibroblast model systems, which demonstrated that fusion of

intracellular vesicles with the plasma membrane is critical for resealing [76, 77, 83, 99]. Dysferlin localizes to the plasma membrane and intracellular vesicles in developing myotubes, and interacts with numerous proteins involved in membrane transport, including caveolin-3 [93], annexin-4 [97], annexin-6 [90], enlargeosomal marker AHNAK [100], and tubulin [94], but the exact contribution of dysferlin-containing vesicles to resealing following wounding remains elusive, as few studies have examined the behavior of dysferlin-containing vesicles in live cells following cellular wounding. Therefore, the behavior of dysferlin-containing vesicles in live muscle cells prior to and following wounding, the role of kinesin and microtubules in dysferlin-vesicle biology was examined.

Live-cell imaging of L6 myotubes expressing fluorescently-tagged dysferlin molecules was used to study the real time dynamic behavior dysferlin-containing vesicles prior to, and following mechanical membrane disruption. These data demonstrate that dysferlin-containing vesicles interact with microtubules via plus-end directed kinesin heavy chain motor, KIF5B. In response to membrane damage, dysferlin-containing vesicles undergo rapid vesicle-vesicle and vesicle-organelle fusion with lysosomes to form extremely large cytoplasmic vesicles, in a manner that is dependent on both microtubules and functional KIF5B. These data support the overall hypothesis that the interaction of dysferlin-containing vesicles with microtubules is critical for dysferlin-vesicle function following cellular wounding, and implicate lysosomal membranes as potential partners for dysferlin-containing vesicle fusion events following cellular wounding in skeletal muscle.

## METHODS

Mouse dysferlin isoform-1 (Gen Bank: NM_021469) was obtained as a kind gift from the Jain Foundation. A C-terminal fragment of dysferlin was isolated using a BSTBI-NOT1 fragment, and sub cloned into a TOPO PCR 2.1 shuttle vector. The remaining N-terminal portion of dysferlin was excised using a KPNI-BSTBI digestion and inserted into the dysferlin C-terminus PCR2.1 vector. An eGFP fragment was generated using custom primers which generated a 5'SACII-eGFP-NOTI 3' fragment in PCR 2.1. The GFP fragment was then excised using a SACII-NOTI digest, and inserted into the dysferlin containing vector at the C-terminus. The entire dysferlin-GFP construct was then excised using a KPNI-NOTI digestion and inserted into a PCDNA 3.1+ vector for mammalian expression. Dysferlin-mCherry was generated using PCR amplification of mCherry with custom primers to generate a 5'SACII-mCherry-NOTI fragment. This fragment was then inserted into the aforementioned Dysferlin-eGFP construct using a SACII-NOTI digest. All dysferlin constructs were sequenced by the University of Michigan Sequencing Core. Motorless kinesins (containing the C-terminal stalk-tail region) are generally used as dominant negative inhibitors of kinesin function [101]. Constructs encoding GFP or mCherry labeled full-length rat KIF5 and dominant negative KIF5B (amino acids 568-964) were obtained as a kind gift from Dr. Kristen Verhey. Previous work has shown that KIF5B and KIF5C interact, and that KIF5B can compensate for the loss of KIF5C [102], as such these constructs were used interchangeably throughout this manuscript. Thanks to Dr. Kristen Verhey for the fluorophore labeled kinesin and LGP120 constructs used in this study. Rat anti-α tubulin polyclonal antibody (AB6161) was obtained from ABCAM. Rabbit polyclonal antibody to

Protein disulfide isomerase (PDI) was obtained from Sigma. Mouse monoclonal antibody to GM130 was obtained from BD transduction labs. Nocodazole (Methyl *N*-(5-thenoyl-2-benzimidazolyl)carbamate) was obtained from Sigma Aldrich. Blebbistatin (1,2,3,3a-Tetrahydro-3a-hydroxy-6-methyl-1-phenyl-4H-pyrrolo[2,3-b]quinolin-4-one) was obtained from Toronto Research Chemicals Inc. Alexa 488-conjugated phalloidin was obtained from Life Technologies. Anti Lamp-1 antibody was obtained from ABCAM (ab24170). Lipofectamine™ 2000 reagent was used in all transfection experiments.

Cell Culture and Transfection: L6 myocytes were grown on 100mm dishes under standard conditions at 37C + 5% $CO_2$ in the presence of DMEM+10% FBS +1% P/S. Cells were sub-cultured using PBS wash followed by treatment with 0.25% trypsin-EDTA and plated on either 100mm dishes for continued sub-culturing or 35mm glass bottom dishes (MatTek) for live-cell imaging, or glass coverslips contained in 6-well dishes for immunofluorescence. L6 myocytes were transfected or co-transfected with cDNA(s) of interest using Lipofectamine 2000, according to the manufacturer's protocol. Following transfection, cells were switched to DMEM+2% Horse Serum and allowed to differentiate for 4-8 d until cells formed elongated myotubes.

Immunofluorescence and Image Analysis: Differentiated L6 myotubes were fixed for 15min in 3% paraformaldehyde, and permeabilized for 1h in block solution containing 5% Bovine Serum Albumin and 0.5% triton X-100. Following blocking, cells were incubated in block solution containing the appropriate titer of antibody at room temperature for 1.5h. Following incubation in primary antibody, cells were washed and

incubated in block solution containing the appropriate secondary antibody for 1h. DAPI was used to stain nuclei in all immunofluorescence experiments. Images were obtained on the Deltavision ® system using standard filter sets or a Leica SP8 confocal microscope with a 63x objective where indicated. For deltavision imaging, optical sections were generated at a thickness of 0.2 um for each channel and the resulting raw data was used to create projection images in SoftWoRx 1.3.0. Adobe Photoshop CS2 and Adobe Illustrator were used to compile images.

Live-cell Imaging and Quantification of Dysferlin-containing vesicle Transport: Dysferlin-eGFP expressing L6 myotubes were switched from differentiation media to PBS + $Ca^{2+}$ prior to imaging. Imaging was carried out on an Olympus BX-71 Deltavision® microscope, equipped with a climate control chamber to maintain 37ºC and 5% $CO_2$. All imaging data was obtained on the Deltavision® live-cell system with an Olympus 60x, 1.4 numerical aperture objective, equipped with a PhotometricsCoolSnap HQ monochromatic camera. SoftWoRx Explorer 1.3 ® imaging software was used in all experiments to analyze raw time-lapse data. SoftWoRx 3.5.0 ® software was used to deconvolve time-lapse data when indicated, and was carried out using enhanced ratio with medium noise filtering unless otherwise noted. Quicktime movies or individual image files were generated from raw time-lapse data. Dysferlin-containing vesicle movement and nocodazole treatment: For analysis of dysferlin-containing vesicle movement, dysferlin-eGFP expressing L6 myotubes were imaged at 250ms exposure for 2m prior to nocodazole treatment. To determine the effect of microtubule disruption on dysferlin-containing vesicle transport, cells were incubated in 750mM nocodazole for

30m and subjected to a second round of live-cell imaging for 2min. Raw time-lapse images were then analyzed for dysferlin-containing vesicle movements >2um in length using Softworx ® Particle Tracking software by an observer blind to treatment group. Each individual motile vesicle was tracked through time and the total distance traveled was determined by manually assigning XY-coordinates to each motile vesicle at time-point. Each vesicle moving greater than 2um was counted as a movement, and the total number of movements was compared for each cell, prior to, and after treatment with nocodazole. Significance was determined using a student's T-test. Velocities of individual dysferlin containing vesicles (24 movements from 10 cells) were calculated by tracking individual motile vesicles and calculating the change in xy distance over time. This data should be taken as a close approximation, as our methodology does not account for displacement of vesicles within the z-plane.

Live-cell imaging of co-transfected L6 myotubes: To study multiple fluorescently tagged molecules, in single cells, experiments were designed to alternate imaging between each channel at 250msec exposure for 2min. The raw data for each channel was analyzed independently and in combination for vesicle motility and colabeling of dysferlin vesicles with molecules of interest. Movie files and images of individual and merged time-lapse data were generated for further analysis. Raw data and quicktime files were analyzed for colocalization, as well as the dynamic movement of both labels within the cell.

Live-cell Membrane Damage Assay: To study the behavior of dysferlin-containing vesicles following membrane disruption, L6 myotubes expressing dysferlin-eGFP were subjected to live-cell imaging. Each cell was imaged in the GFP channel at 250msec exposure for 2min prior to membrane damage. Following initial imaging, each cell was re-visited and cells were wounded using a glass pulled micropipette and imaged for 4-5min following wounding. To damage myotubes, an MX140-R manual micromanipulator was mounted to the stage of an Olympus BX-71 Deltavision® microscope and equipped with a finely pulled glass pipette. Wounding was confirmed visually by the absence of fluorescence in the affected area, as well as hypercontracture of the myotube. Using these criteria over 90 percent of the cells that were assayed were characterized as wounded. Cells that did not hypercontract were re-wounded or discarded. Due to movement artifact following membrane wounding, the z-axis was adjusted when necessary to highlight structures of interest. To quantify the vesicle formation response of dysferlin-eGFP expressing myotubes to membrane disruption, raw time lapse (.dv) files were analyzed using SoftWorx Explorer 1.3 for the formation of large dysferlin-vesicles following wounding. Any cell that formed dysferlin-containing vesicles with a visible lumen that was not present prior to wounding was characterized as a "responder," and any cell that did not form vesicles was excluded from the analysis. For each responding cell, the total number of large (>2um) damage-induced dysferlin-containing vesicles was quantified at a single time point after vesicle formation had subsided. For nocodazole experiments, cells were incubated with DMSO or DMSO+ 750nM nocodazole for ≥ 30min prior to wounding, imaged in the GFP channel at 250 msec exposure for 2min prior to wounding, and 4-5 min following wounding. For

dominant negative kinesin experiments, imaging alternated between GFP and dsRed channels at 250 msec exposure for 2min to confirm expression of both constructs. Following initial imaging, each cell was wounded and imaged in the GFP channel alone at 250msec exposure for 4-5min. To determine the effect of nocodazole or dominant negative KIF5 on large vesicle formation, the average number of large vesicles (>2µm) was quantified and compared to the respective control (DMSO or wt-KIF5, respectively). The data is presented as a bar graph in figure 5B and 6C, and represents data collected from multiple independent experiments. Data is presented as mean +/- SE and statistical analysis were carried out using a two-tailed t-test with significance set at $p<0.05$.

## RESULTS

**Dysferlin-eGFP localizes to distinct cytoplasmic vesicles in differentiated L6 myotubes.** Fluorescently-tagged dysferlin fusion constructs were generated to study the localization and behavior of dysferlin-containing vesicles in cultured skeletal muscle cells. Deconvolution imaging of L6 myotubes transiently expressing dysferlin-eGFP revealed that dysferlin-containing membranes and vesicles were present in linearly arranged clusters throughout the length of the myotube (Fig 2-1 A). The exact composition of dysferlin-containing vesicles, and whether these vesicles are derived from any known membrane compartments remains to be determined. Antibody labeling of endoplasmic reticulum marker, PDI (Fig 2-1B), as well as Golgi marker GM130 ( Fig 2-1 C), in dysferlin-eGFP expressing myotubes was used to examine the relationship between dysferlin-containing vesicles and the secretory pathway in skeletal muscle

cells. Dysferlin-eGFP showed minimal overlap with either GM130, or PDI, indicating that dysferlin-containing vesicles are distinct from the secretory pathway. Dysferlin-eGFP and LGP120-mCherry localize to distinct vesicular structures when coexpressed in L6 myotubes, indicating that dysferlin-eGFP does not localize to lysosomes in skeletal muscle cells (Fig 2-1D).

**Dysferlin-containing vesicles move along microtubules via KIF5B motors in differentiated L6 myotubes.** Live-cell imaging of fluorophore labeled dysferlin expressing L6 myotubes was used to explore the dynamic behavior of dysferlin-containing vesicles, and the relationship between dysferlin-containing vesicles and microtubules as well as kinesin motors in cultured L6 myotubes. The majority of dysferlin-containing vesicles, particularly those clustered within the cytoplasm or near the membrane, were non-motile over the course of imaging (~2 min) except for occasional back-and-forth movements. However, a portion of dysferlin-containing vesicles moved in linear paths along the longitudinal axis of the myotube up to tens of microns in length at an approximate average rate of 0.63 +/- 0.045 μm/sec (Fig 2-2A). Due to the remarkable length and linearity of dysferlin-containing vesicle movements, we proposed that microtubules may serve as tracks for dysferlin-containing vesicle transport in skeletal muscle. Microtubules were antibody labeled in fixed dysferlin-eGFP expressing L6 myotubes in order to explore the relationship between dysferlin-containing vesicles and microtubules in skeletal muscle. Microtubule structures arranged in a dense longitudinally oriented lattice that extended throughout the entire myotube, and a portion of dysferlin-containing vesicles were associated with

microtubules (Fig 2-2B, see inset). To directly assess whether microtubules are required for dysferlin-containing vesicle transport in L6 myotubes, movement of dysferlin-vesicles was analyzed for long-range (>2µm) vesicle movements in dysferlin-eGFP expressing L6 myotubes prior to, and following treatment with nocodazole. As shown in figure 2-2C, treatment with 750nM nocodazole clearly disrupted the microtubule lattice and significantly reduced vesicle movements >2µm in differentiated L6 myotubes (Fig 2-2D). We examined whether dysferlin-containing vesicles labeled with ubiquitously expressed kinesin motor KIF5B by imaging L6 myotubes co-expressing dysferlin-mCherry and GFP-KIF5B and analyzed the dynamic movement of each label relative to the other in live skeletal muscle myotubes. Motile and non-motile cytoplasmic dysferlin-containing vesicles are labeled by GFP-KIF5B in L6 myotubes (Fig 2-2E), but not neuron specific isoform KIF5C (Fig 2-7), indicating that dysferlin-containing vesicles contain KIF5B motors in L6 myotubes.

**Membrane disruption induces formation of extremely large dysferlin-containing vesicles in L6 myotubes.** There have been few studies attempting to directly examine damage-induced fusion of dysferlin-containing vesicles following wounding in living skeletal muscle cells. Therefore, to characterize the response of dysferlin-containing vesicles to membrane disruption in skeletal muscle cells, we used in-vitro mechanical wounding of dysferlin-eGFP expressing L6 myotubes in conjunction with live-cell imaging to track the dynamic responses of dysferlin-containing vesicles. Mechanical wounding has been used previously to study the behavior of other repair proteins in muscle cells [51], but the response of dysferlin-containing vesicles to mechanical

wounding has not been fully characterized. Prior to wounding, dysferlin-containing vesicles were distributed throughout the cytoplasm of the myotube (Fig 2-3A, left). Cellular damage was elicited using a glass pulled micropipette guided to a precise location on the myotube, and allowed to puncture the membrane. Damage was indicated by: (1) loss of GFP-fluorescence in the affected area, and (2) noticeable tactile response, including membrane tearing and subsequent retraction of the myotube. Immediately following disruption, dysferlin-containing vesicles combine to form extremely large cytoplasmic vesicles adjacent to the lesion, and throughout the cytoplasm (Fig 2-3A, white arrows, right panel). The resulting vesicles varied in size from 1µm to greater than 10µm. All vesicles forming following wounding had an apparent lumen, which was not the case for dysferlin-containing vesicles prior to membrane disruption. The time-course of large-vesicle formation was rapid, beginning as rapidly as 1 sec post-wound and completely formed around 1 min post wound. Representative time-lapse images of large vesicle formation following wounding is presented in high magnification in figure 2-3B, where multiple small vesicles are incorporated into a large vesicle within a time-frame of 1 sec to form an incrementally larger vesicle (white arrows, right panel). In some instances, damage-induced vesicles collapsed into the adjacent membrane (Fig 2-3C), while the vast majority persist many minutes following wounding (Fig 2-3D).

**Disruption of microtubules but not inhibition of actin-myosin interaction diminishes formation of large dysferlin-containing vesicles following membrane disruption in L6 myotubes.** Our data suggests that dysferlin-containing vesicles

interact with microtubules and microtubule disruption inhibits vesicle movement. Therefore, the requirement of microtubules for fusion of dysferlin-containing vesicles following wounding in L6 myotubes was examined. Dysferlin-eGFP expressing L6 myotubes were pre-treated with either DMSO or DMSO + 750nM nocodazole for ≥ 30m and subjected to live-cell imaging prior to, and following membrane disruption. Time-lapse data was analyzed for fusion of dysferlin-containing vesicles and the resulting formation of large-vesicles with a visible lumen. Dysferlin-eGFP expressing L6 myotubes were not affected by pre-treatment with DMSO (Fig 2-4A, top-left), and mechanical wounding induced formation of extremely large vesicles in DMSO treated cells (Fig 2-4A, top-right, supplementary movie 6). Treatment with nocodazole did not affect dysferlin-containing vesicle localization within the cell prior to wounding (Fig 2-4A, middle-left panel), but the formation of large dysferlin-containing vesicles following wounding was significantly reduced (Fig 2-4A middle-right panel, quantified in Fig 2-4B, supplementary movie 7). To determine whether the inhibitory effect of nocodazole treatment on formation of large dysferlin-containing vesicles was specific for microtubule disruption or due to indirect disruption of actin-based transport, dysferlin-eGFP expressing L6 cells were acutely treated with a potent and specific non-muscle myosin-II inhibitor, blebbistatin, and assayed for fusion following mechanical wounding. Treatment with 25uM blebbistatin did not markedly alter dysferlin-containing vesicle localization and did not affect the formation of large dysferlin-containing vesicles following membrane wounding (Fig 2-8).

**Functional KIF5B is required for formation of large dysferlin-containing vesicles following membrane disruption.** Our data indicates that KIF5B labels dysferlin-containing vesicles and may be a critical motor protein for movement of dysferlin-containing vesicles along microtubules in skeletal muscle cells. Therefore, over-expression of a dominant negative KIF5B construct was used to examine the role of KIF5B in the formation of large dysferlin-containing vesicles following mechanical wounding in L6 myotubes. The dominant negative KIF5B encodes a “headless” truncation mutant of KIF5B, which contains half of the stalk domain and cargo-binding domain, but lacks a functional motor domain. Deconvolution imaging of fixed L6 myotubes expressing dysferlin-eGFP and either wild-type mCherry-labeled KIF5C (mCherry-wtKIF5) or mCherry-labeled dominant negative mutant of KIF5B (mCherry-dnKIF5), revealed that localization of dysferlin-containing vesicles was not markedly affected by expression of either construct (Fig 2-5A). Genomic deletion of KIF5B has been reported to disrupt organization of the Golgi complex, actin cytoskeleton, lysosomes in KIF5B-null myoblasts [102, 103]. Therefore, we examined whether expression of dnKIF5 affected the Golgi complex, actin cytoskeleton, and lysosomes in differentiated L6 myotubes. Over-expression of dnKIF5 did not disrupt the actin-cytoskeleton or the Golgi complex in L6 myotubes (Fig 2-9A, B). Lysosomal organization was not affected by expression of dnKIF5, as most lamp-1 positive structures were dispersed throughout the cytoplasm of wtKIF5 and dnKIF5 expressing cells (Fig 2-9C). To examine the role of KIF5B in the formation of large dysferlin-vesicles, myotubes co-expressing either dysferlin-eGFP and mCherry-wtKIF5 (Fig 2-5B, top-left panel) or dysferlin-eGFP and mCherry-dnKIF5 (Fig 2-5B, bottom-left panel)

were subjected to mechanical wounding and assayed for the formation of large dysferlin-containing vesicles. Expression of mCherry-wtKIF5 did not affect the behavior of dysferlin-containing vesicles following wounding as membrane disruption induced the formation of large dysferlin vesicles adjacent to the lesion site (Fig 2-5B, top-right). Expression of mCherry-dnKIF5 significantly reduced the formation of large dysferlin-containing vesicles following wounding, indicating that functional KIF5B is required for formation of large dysferlin-containing vesicles following wounding in skeletal muscle cells (Fig 2-5B, bottom-right panel, quantified in Fig 2-5C).

**Dysferlin-containing vesicles undergo heterotypic fusion with lysosomes following mechanical wounding in differentiated L6 myotubes.** Lysosomes are thought to undergo exocytosis in response to membrane wounding, and have been implicated in membrane resealing in non-muscle cell systems [72]. Interestingly, lysosomal marker LAMP-2 is mislocalized in dysferlin-null myoblasts indicating a possible defect in lysosomal transport [98], but whether lysosomal compartments interact with dysferlin vesicles in muscle cells is not known. To address this, L6 myotubes co-expressing dysferlin-eGFP and LGP120-mCherry (LGP120-mCh) were subjected to live-cell imaging prior to, and following mechanical wounding and the extent of co-labeling was analyzed. Prior to membrane wounding, dysferlin-eGFP and LGP120-mCh label independent vesicle populations with minimal overlap (Fig 2-6A, top panels). Large co-labeled structures form following mechanical wounding, indicating that dysferlin-containing vesicles are capable of fusion with lysosomal compartments in L6 myotubes following cellular wounding (Fig 2-6A, bottom panels). High magnification

time-lapse images of heterotypic dysferlin/lysosome vesicle formation are shown in figure 2-6B.

## DISCUSSION

Dysferlin is a critical component of the membrane repair machinery in both skeletal and cardiac muscle [18, 61], and loss of this protein leads to muscular dystrophy [18, 37, 104]. Dysferlin-containing vesicles are thought to play a critical role in muscle resealing, but there have been very few studies attempting to directly examine the behavior of dysferlin-containing vesicles in live muscle cells in the context of cellular wounding. Studies from non-muscle model systems suggest that microtubule-based transport of intracellular vesicles is critical for resealing [77, 82], but the role of microtubules and kinesin motors in dysferlin-mediated membrane repair in muscle remains unexplored. Therefore, the aim of this study was to examine the behavior of dysferlin-containing vesicles under normal conditions and following cellular wounding in muscle cells, and test the hypothesis that microtubules and kinesin are required for dysferlin-containing vesicle function in muscle cells. Due to the dynamic nature of vesicle transport/fusion and membrane resealing, we used a live-cell imaging approach to study dysferlin-containing vesicles in unperturbed and mechanically wounded myotubes. This approach allowed for the resolution of individual vesicle movements and direct visualization of vesicle fusion events following cellular wounding in live-muscle cells. Our data show that dysferlin-containing vesicles are transported along microtubules via kinesin heavy chain isoform KIF5B, and undergo rapid microtubule- and KIF5B-dependent fusion to form extremely large vesicles following cellular wounding in skeletal muscle cells.

Furthermore, we identify lysosomal compartments as potential interacting partners of dysferlin-containing vesicles following cellular wounding in skeletal muscle cells.

**Dysferlin-containing vesicles move along microtubules via KIF5B in skeletal muscle cells.** Antibody labeling of microtubules in fixed dysferlin-eGFP expressing L6 myotubes revealed that dysferlin-containing vesicles are arranged along longitudinally oriented microtubule structures. Live-cell imaging of dysferlin-eGFP expressing L6 myotubes showed that the majority of dysferlin-containing membranes are arranged in non-motile vesicle clusters, with a small fraction of dysferlin-containing vesicles undergoing microtubule-dependent long range movement. Kinesin heavy chain has been implicated in damage-induced exocytosis of vesicles in other model systems [77, 83], and a study that screened for dysferlin interacting proteins using mass spectrometry identified ubiquitous kinesin motor KIF5B [105]. Consistent with a role for KIF5B on dysferlin-containing vesicles, live-cell imaging of L6 myotubes expressing dysferlin-mCh and eGFP-KIF5B revealed that dysferlin-containing vesicles are labeled by KIF5B, and co-labeled vesicles are capable of long-range movements. Furthermore, the measured velocity of motile dysferlin-containing vesicles is in the range of velocities reported for mitochondria in neurons [106], and kinesin motors in COS cells [107]. Interestingly, most KIF5B labeled dysferlin-containing vesicles did not show long-range processive movement and are limited to local movements in resting L6 myotubes. These findings are consistent with reports describing processive and non-processive movements of kinesin bound cargoes [108, 109]. Possible explanations for the non-processive behavior of a subpopulation of kinesin containing dysferlin vesicles are that

KIF5B may be bound to dysferlin-containing vesicles without sufficiently activating kinesin motor activity or without actively engaging microtubules [108] or perhaps the clustering of vesicles limits their motility or anchors them to another sub cellular structure.

**Dysferlin-containing vesicles undergo fusion to form large cytoplasmic vesicles following cellular wounding in L6 myotubes.** L6 myotubes expressing dysferlin-eGFP were subjected to mechanical wounding using a finely pulled glass pipette and analyzed for fusion of dysferlin-containing vesicles following wounding. Mechanical wounding reproducibly led to contracture and ultimately retraction of the myotube away from the wound site. This model of injury, in contrast to single point laser injury, is more analogous to the process of contraction induced injury that occurs in muscle in vivo [14]. In dystrophin-deficient dystrophic muscle, injurious lengthening contractions result in complete fiber tearing and the formation of contraction clots at the site of tearing [14]. Interestingly, rather than accumulate specifically at membrane lesions in the L6 injury model, dysferlin-containing vesicles undergo rapid vesicle-vesicle fusion to form a population of extremely large dysferlin-containing vesicles throughout the cytoplasm of the myotube. In some cases the vesicles collapse on the membrane, but the majority of large vesicles remain in the cytoplasm and are stable for minutes following injury. This finding was somewhat surprising as our expectation was that dysferlin-containing vesicles would accumulate specifically at membrane lesions and fuse with the plasma membrane. The vesicle formation response was reminiscent of the "vesicular plug" model of membrane resealing as has been previously documented in the urchin egg

and crayfish medial giant axon, whereby endocytic and exocytic vesicles undergo fusion adjacent to membrane lesions and plug the lesion with minimal fusion with the plasma membrane [57, 75]. Therefore, we propose that formation of large dysferlin vesicles may act as a vesicular plug which is, in itself, acting as a cellular “contraction clot” capable of plugging large wounds in the plasma membrane of skeletal muscle cells. Additionally, it is interesting to speculate that cytoplasmic dysferlin-containing vesicles could play a different yet unappreciated role in response to wounding in muscle cells such as sequestering cytoplasmic components or damaged organelles that would otherwise harm neighboring cells. In fact, this idea is supported by recent evidence that the complement system is activated in dysferlin-null muscle, which contributes directly to disease progression, and may result from excess leakage of cellular contents following wounding [110].

**Disruption of the functional linkage between dysferlin-containing vesicles and microtubules inhibits formation of large dysferlin-containing vesicles following wounding in L6 myotubes.** Due to the presence of KIF5B on dysferlin-containing vesicles and the requirement of microtubules for dysferlin-containing vesicle movement, we hypothesized that disruption of microtubules and/or kinesin motors would inhibit formation of large dysferlin-containing vesicles following wounding in L6 myotubes. We addressed this hypothesis by examining the effect of pharmacological disruption of microtubules or dominant negative inhibition of KIF5 motors on the formation of large dysferlin-vesicles following wounding in L6 myotubes. Both microtubule disruption and expression of dominant negative KIF5B significantly reduced the formation of large

dysferlin-containing vesicles following wounding in differentiated L6 myotubes. These findings taken together with the fact that KIF5B is present on dysferlin-containing vesicles indicates that the functional linkage between KIF5B on dysferlin-containing vesicles and the microtubule lattice is critical for formation of large dysferlin vesicles following cellular wounding in L6 myotubes. There are several ways in which disrupting the link between microtubules and dysferlin-containing vesicles could inhibit large vesicle formation in muscle cells. It is possible that dysferlin-containing vesicles are transported throughout the cytoplasm via KIF5B prior to wounding and inhibition of KIF5B inhibits proper trafficking of dysferlin. However, the finding that dominant negative KIF5B does not markedly alter dysferlin localization indicates that transport of dysferlin-containing vesicles off of the Golgi apparatus is intact, and cytoplasmic targeting of dysferlin-containing vesicles does not require KIF5B. One possible explanation for the dominant negative effect of KIF5B on vesicle formation is that KIF5B is required for movement of dysferlin-containing vesicles along microtubules following wounding, and this movement facilitates coalescence of adjacent dysferlin-containing vesicles. In this scenario, inhibition of KIF5B could impair large vesicle formation by disrupting proper movement of dysferlin-containing vesicles along microtubules following wounding. Unfortunately, analysis of motility following wounding in our assay was limited by the fact that wounding caused dramatic contracture of the myotube, resulting in movement of most cellular components. Regardless, our data indicate that the interaction between functional KIF5B motors on dysferlin-containing vesicles and microtubules is critical for formation of large vesicles following wounding.

**Dysferlin-containing vesicles fuse with lysosomal compartments following wounding in L6 myotubes.** The exact composition of dysferlin-containing vesicles, and membrane compartments involved in membrane repair in muscle have not been fully examined. Lysosomes have been implicated in membrane resealing across a variety of non-muscle cell types [72], but whether lysosomes are involved in dysferlin-mediated membrane resealing in skeletal muscle cells is not known. Lysosomal marker Lamp-2 accumulates around the nucleus of dysferlin-null myoblasts [48], indicating that loss of dysferlin may adversely affect lysosomal function. Furthermore, dysferlin is required for Fas-L induced lipid raft clustering in endothelial cells, which is thought to be dependent on lysosomal fusion [111]. Therefore, we sought to directly examine whether dysferlin-containing vesicles interact with lysosomal compartments following wounding in skeletal muscle cells. Live cell imaging of L6 cells co-expressing dysferlin-eGFP and LGP120-mCh shows that dysferlin-eGFP and LGP120-mCh label independent populations of vesicles prior to wounding. Following wounding, dysferlin-containing vesicles undergo fusion with LGP120-mCh labeled compartments to form large vesicles in L6 myotubes. A previous study reported that lysosomal dispersion is impaired in cells isolated from KIF5B knockout mice, indicating that KIF5B may be required for motility of lysosomes [102]. Our data indicates that lysosomal organization is not dramatically altered by dnKIF5B expression in differentiated L6 myotubes. This finding, taken together with the fact that KIF5B is present on dysferlin-containing vesicles prior to wounding indicates that the dominant negative effect on large-vesicle formation is likely through direct impairment of dysferlin-containing vesicle function rather than an indirect effect on lysosomal function. These data support the novel assertion dysferlin-containing vesicles

may interact to form large-vesicles following wounding which may contribute to membrane repair in L6 myotubes. Interestingly, knockout of synaptotagmin-VII, a dysferlin homolog necessary for lysosomal fusion, displays an inflammatory muscle myopathy [71], but whether loss of lysosomal function impairs resealing in muscle cells is not known. Furthermore, additional studies are needed to examine the exact contribution of lysosomal compartments to resealing in muscle and whether lysosomal behavior following wounding is impaired in dysferlin-deficient muscle cells.

## ACKNOWLEDGEMENTS

We would like to thank the members of the University of Michigan Microscopy and Image Analysis Laboratory (MIL) for all of its help with live-cell imaging. We would like to thank Dr Kristen Verhey for her generosity with the wild-type and dominant negative KIF5 constructs, as well as the construct encoding LGP120.

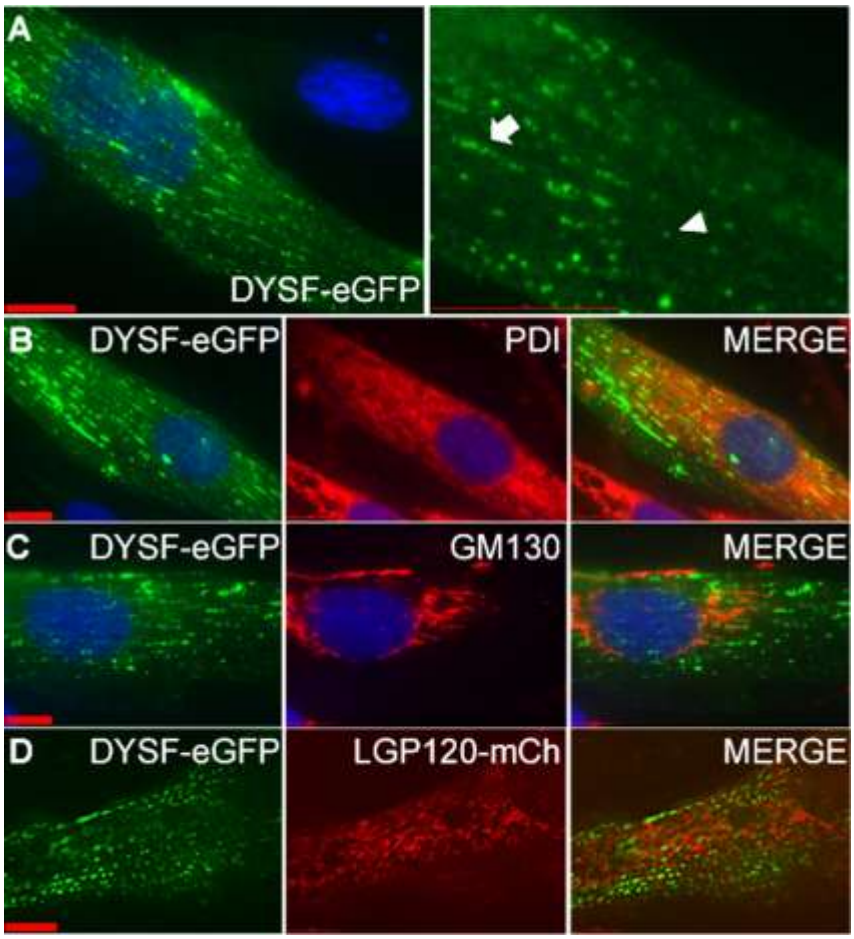



**Figure 2-1. Dysferlin-eGFP localizes to a population of distinct vesicles in differentiated L6 myotubes. A**) Dysferlin-containing vesicles are arranged in linear arrays throughout the cytoplasm of L6 myotubes. High magnification of (**A**) shows that dysferlin-containing vesicles are isolated (arrowhead, right panel), and also accumulate in regularly distributed vesicle clusters (arrow, right panel). Antibody labeling of fixed L6 myotubes shows that cytoplasmic dysferlin-containing vesicles do not colocalize with endogenous ER marker PDI (**B**), or Golgi marker GM130 (**C**). **D**) Dysferlin-eGFP and lysosomal marker mCherry-LGP120 localize to distinct vesicle populations in differentiated L6 myotubes. Scale bar =10 um.

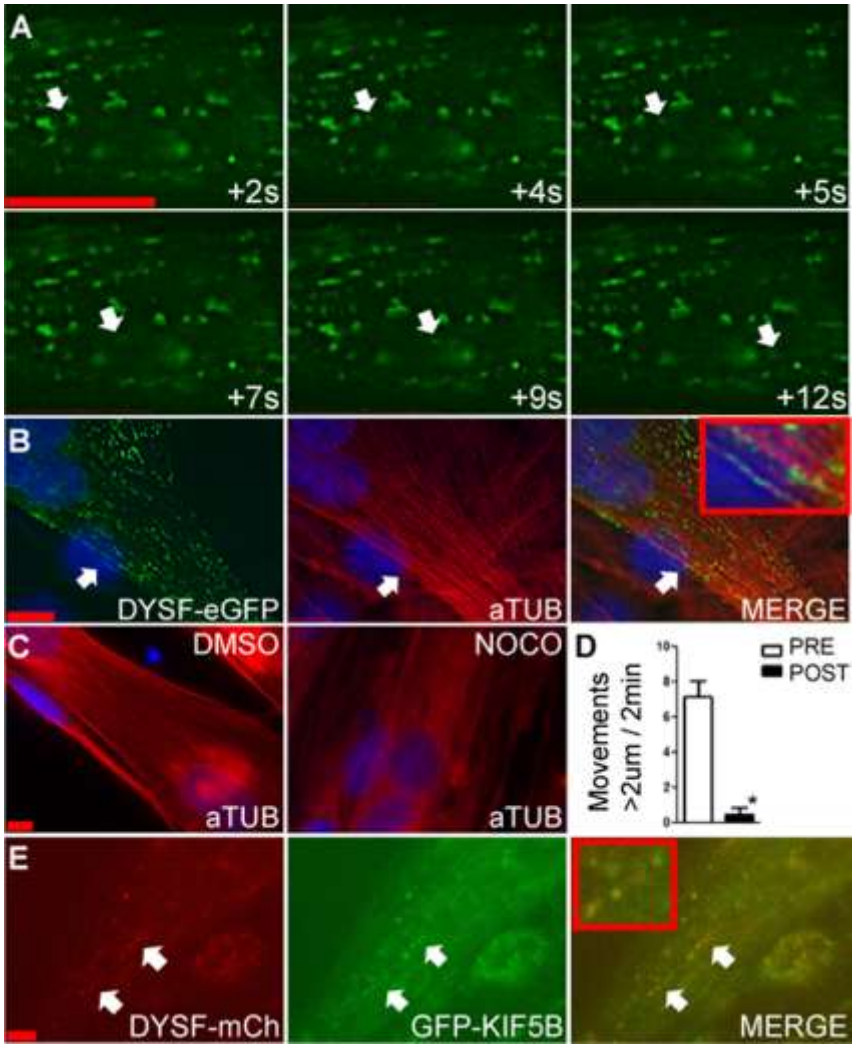



**Figure 2-2. Dysferlin-containing vesicles move along microtubules via KIF5B motors in differentiated L6 myotubes. A**) Live-cell imaging of Dysferlin-eGFP expressing L6 myotubes shows that dysferlin-containing vesicles undergo long-range movements along the longitudinal axis of L6 myotubes (Supplementary movie 1). **B**) Antibody labeling of α -tubulin in dysferlin-eGFP expressing L6 myotubes reveals that dysferlin-containing vesicles colocalize with microtubules in L6 myotubes; DAPI=blue. **C**) Antibody labeling of α-tubulin in differentiated L6 myotubes shows that microtubules form a dense, longitudinally oriented lattice in L6 myotubes (**C**, left-panel) and are disrupted following treatment with microtubule depolymerizing agent, nocodazole (**C**, right-panel)**D**) Microtubule disruption inhibits long-range movement of dysferlin-containing vesicles in skeletal muscle myotubes. Dysferlin-eGFP expressing myotubes were imaged 2min prior to, and following treatment with nocodazole, and vesicle movements greater than 2um were quantified for each condition (n=13, $p < 0.001$). **E**) Dysferlin-containing vesicles label with KIF5B motors in skeletal muscle myotubes. Data are representative of 16 co-transfected myotubes (Supplementary movie 2). Scale bar=10 um.

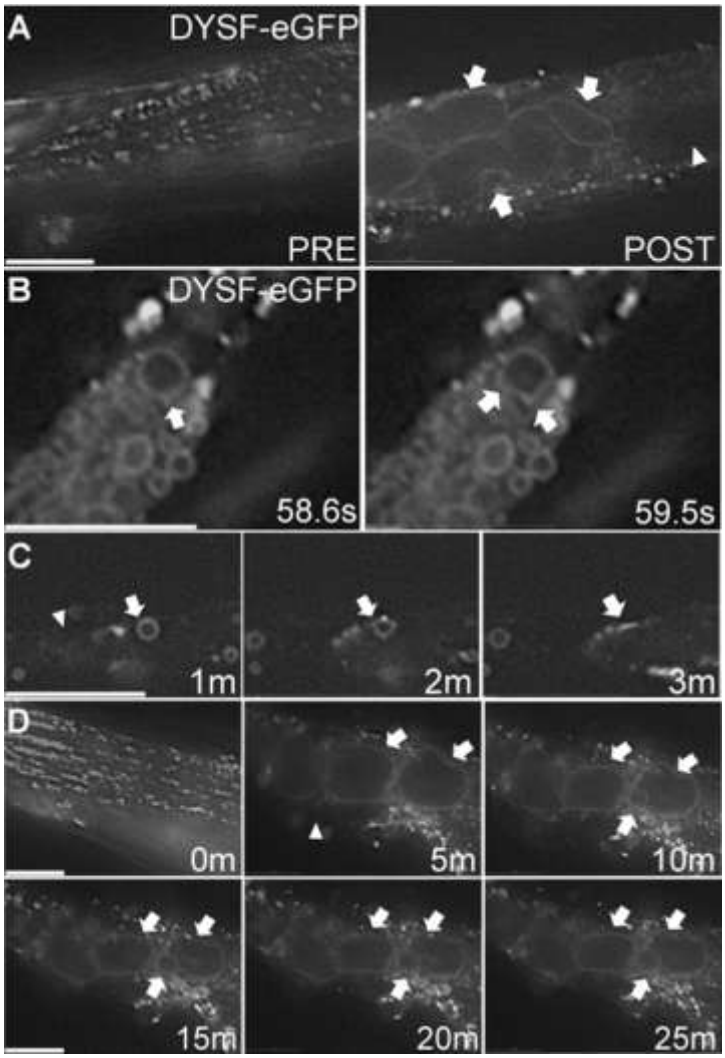



**Figure 2-3. Membrane damage induces fusion of dysferlin-containing vesicles leading to the formation of extremely large vesicles in L6 myotubes. A**) Membrane disruption induces the formation of large dysferlin-containing vesicles in L6 myotubes. L6 myotubes expressing dysferlin-eGFP were subjected to live-cell imaging prior to and after membrane disruption with a glass micropipette. Prior to damage, dysferlin-containing vesicles appear regularly distributed throughout the cytoplasm (left-panel). Following damage (arrowhead), dysferlin-containing vesicles (shown in left panel) undergo rapid vesicle-vesicle fusion to form extremely large vesicles adjacent to membrane lesion (white arrows), and throughout the cytoplasm (Supplementary movie 3). **B**) Large damage-induced dysferlin-containing vesicles result from fusion of smaller dysferlin-containing vesicles in L6 myotubes. Analysis of high magnification, consecutive live-cell images shows small vesicles (arrowheads) undergo fusion with a large vesicle to form an incrementally larger vesicle (Supplementary movie 4). **C**) Example of large dysferlin-containing vesicle collapsing with the plasma membrane following membrane disruption (Supplementary movie 5). **D**) The majority of damage-induced dysferlin-containing vesicles persist for many minutes following membrane disruption. Scale bar=10 um.

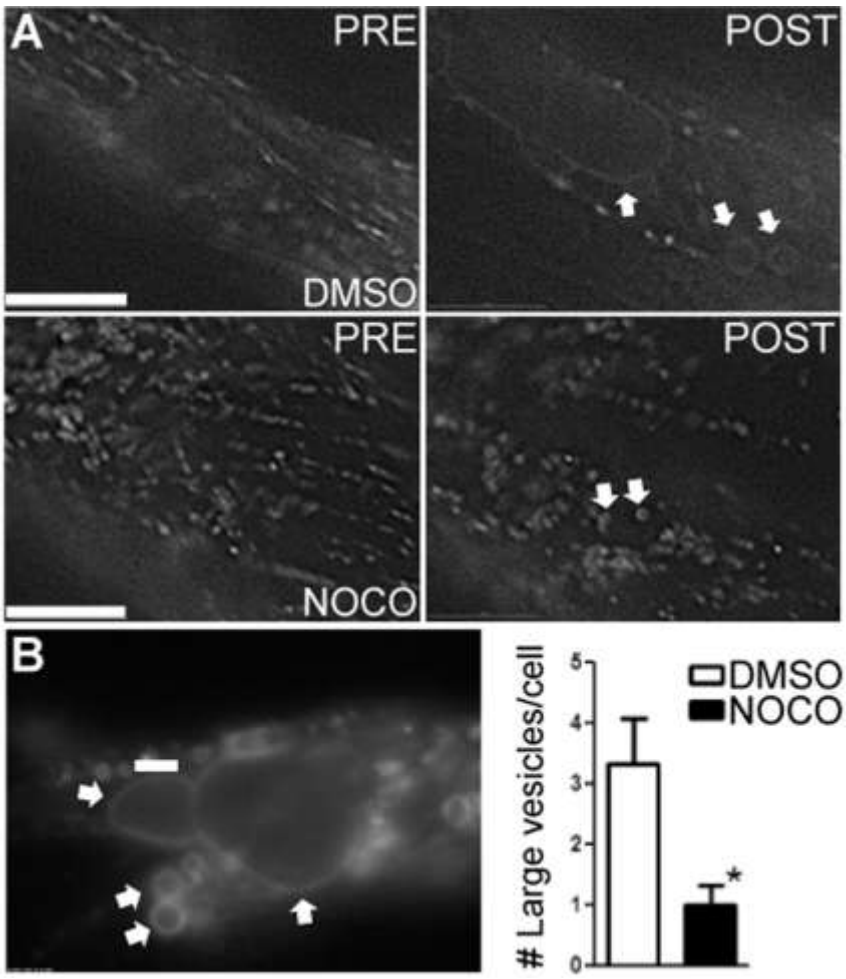



**Figure 2-4. Disruption of microtubules reduces the formation of large dysferlin-containing vesicles following membrane wounding in L6 myotubes. A**) Effect of pharmacological inhibition of microtubules on large vesicle formation following wounding in L6 myotubes. Prior to damage, dysferlin-containing vesicles show typical arrangement in linear arrays throughout the cytoplasm of differentiated L6 muscle cells (left-panels). In DMSO treated control cells dysferlin-containing vesicles fuse to form large vesicles following membrane disruption (arrowheads, top-right panel). Disruption of microtubules by pre-treatment with nocodazole markedly reduces the formation of large dysferlin-containing vesicles following wounding (middle-panels). **B**) Quantification of large dysferlin-containing vesicle formation. The left panel is a representative image showing multiple vesicles (arrowheads) greater than 2um in size (white bar). The total number of large vesicles per cell was quantified for DMSO and Nocodazole treatment (bar graph). Treatment with nocodazole significantly reduced the number of large vesicles formed following wounding compared to DMSO control (n=15 cells for DMSO; n=9 cells for Nocodazole; $p<0.05$). See supplementary movie 6 (DMSO), and 7 (Nocodazole); Scale bar=10 um.

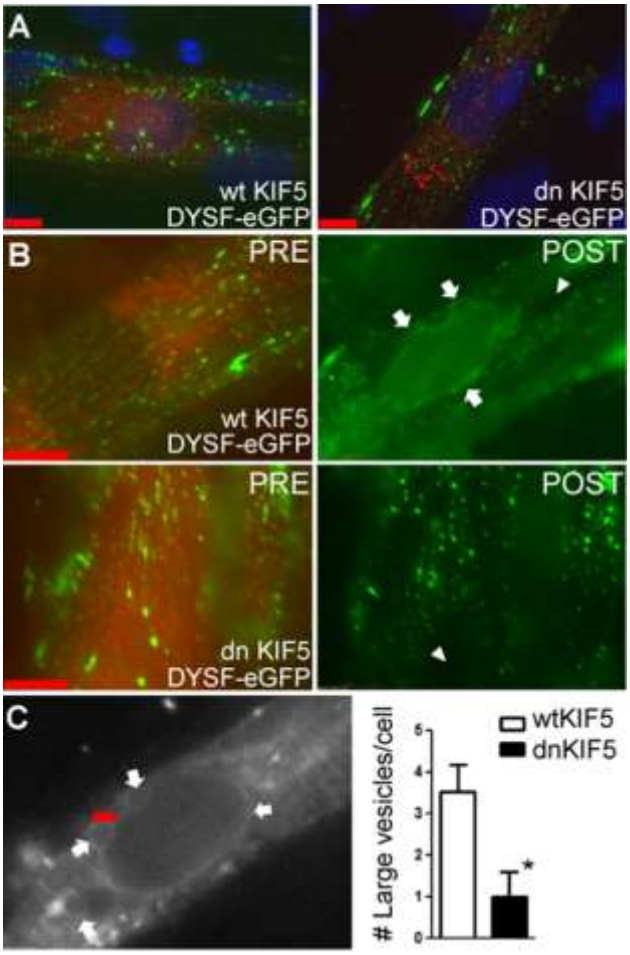



**Figure 2-5. Expression of dnKIF5 inhibits formation of large dysferlin-containing vesicles following membrane wounding in L6 myotubes. A**) Expression of dominant negative KIF5 does not dramatically alter dysferlin-containing vesicle localization in L6 myotubes. Representative deconvolved images of fixed L6 myotubes co-expressing dysferlin-eGFP (green) and either mCherry labeled wtKIF5 (left, red) or mCherry labeled dnKIF5 (right, red); DAPI=blue. **B**) Dominant negative KIF5 inhibits formation of large dysferlin-containing vesicles following membrane disruption in L6 myotubes. L6 myotubes expressing dysferlin-eGFP and mCherry-wtKIF5 control or mCherry-dnKIF5 constructs were analyzed prior to (left panels) and following mechanical wounding (right-panels) using live cell imaging. Formation of damage-induced large dysferlin vesicles is diminished in dnKIF5 expressing (bottom-right) but not wtKIF5 expressing myotubes (arrows, top-right). **C**) Quantification of large dysferlin-containing vesicle formation. The left panel is a representative image showing multiple vesicles (arrows) greater than 2um in size (red bar). The total number of large vesicles per cell was quantified for myotubes expressing dysferlin-eGFP with mCherry-wtKIF5 or mCherry-dnKIF5 (bar graph). Expression of dnKIF5 significantly reduces the formation of large dysferlin-containing vesicles following mechanical disruption in L6 myotubes. wtKIF5 n=13 cells, dnKIF5 n=10 cells; $p<0.05$. Supplementary movie 8 (wtKIF5) and 9 (dnKIF5); Scale bar=10 um.

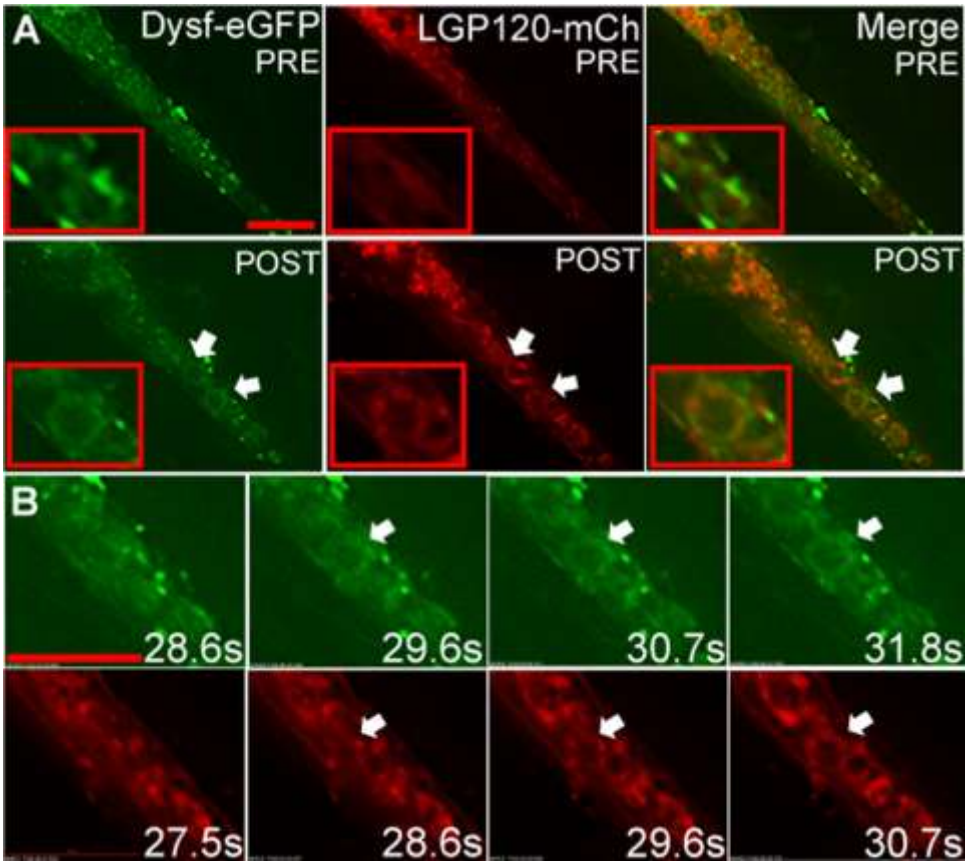



**Figure 2-6. Dysferlin-eGFP interacts with lysosomal membranes following mechanical wounding in differentiated L6 cells. A**) Dysferlin-eGFP and LGP120-mCh occupy distinct compartments in unwounded L6 myotubes (top panels), and form large colabeled cytosolic vesicles following mechanical wounding (bottom panels). **B**) High magnification time-lapse imaging of dysferlin-eGFP (top panels, starting from left) and LGP120-mCh (bottom panels, starting from left) from cell shown in panel (**A**). Small interspersed dysferlin-containing vesicles and LGP120 containing membranes undergo heterotypic fusion to form a large vesicle adjacent to the membrane lesion (time indicated is time post-wounding). See supplementary movie 10. Scale bar=10 um.

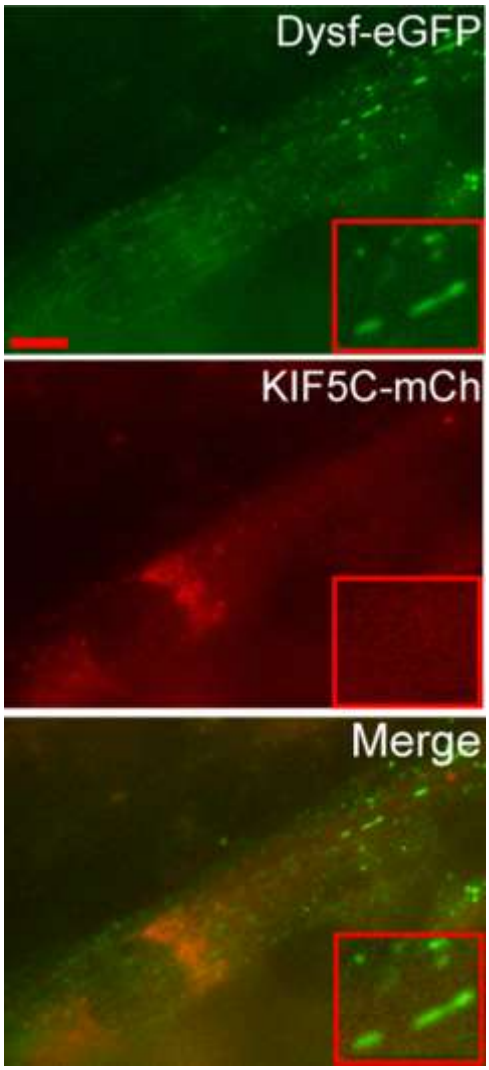



**Figure 2-7.** Dysferlin-containing vesicles do not label with KIF5C in differentiated L6 myotubes. L6 myotubes co-expressing dysferlin-eGFP (top panel) and mCherry-Kif5C (middle panel) were subjected to live cell imaging to examine the relationship between dysferlin-containing vesicles and mcherry-KIF5C motors in muscle cells. Cytoplasmic dysferlin-containing vesicles do not label with KIF5C (insets), indicating that dysferlin-containing vesicles do not contain KIF5C motors in differentiated L6 myotubes. Scale bar = 10 um.

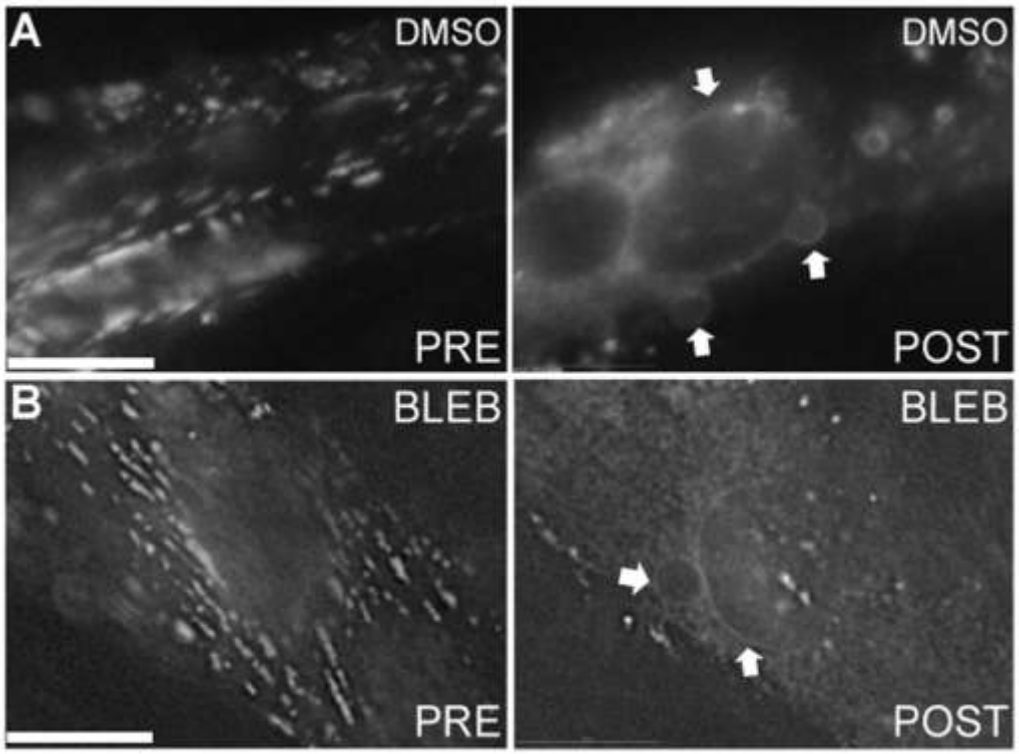



**Figure 2-8.** Pharmacological inhibition of actomyosin interaction does not affect formation of large dysferlin-containing vesicles following wounding in differentiated L6 myotubes. Dysferlin-eGFP expressing L6 myotubes were treated with DMSO (**A**) or DMSO +/- 25uM Blebbistatin (**B**), subjected to mechanical wounding and analyzed for large vesicle formation. Treatment with blebbistatin did not alter the localization of dysferlin-containing structures within the cell (**B**, left) and large dysferlin-containing vesicles formed following wounding in blebbistatin treated cells (**B**, right). Scale bar = 10 um.

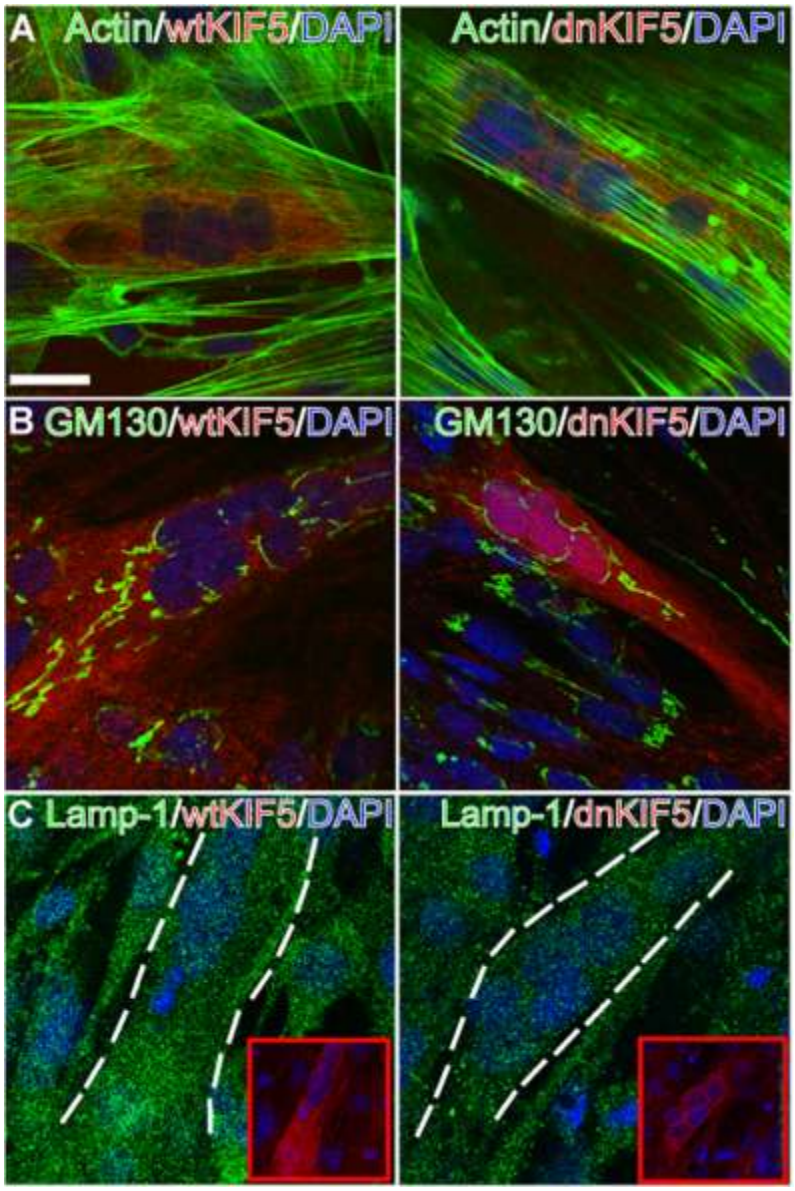



**Figure 2-9.** Effect of dominant negative KIF5 on the organization of the actin-cytoskeleton, Golgi-complex, and lysosomes in differentiated L6 myotubes. L6 myotubes expressing either wtKIF5 (left panels) or dnKIF5 (right panels) were fixed and labeled for actin (**A**), Golgi marker GM130 (**B**) or lysosomal marker lamp-1 (**C**), and examined by confocal microscopy. There were no obvious differences in the organization of the actin cytoskeleton (**A**), or Golgi Apparatus (**B**). Lamp-1 positive structures were dispersed throughout the cytoplasm of wtKIF5 and dnKIF5 expressing myotubes (**C**). For clarity, wtKIF5 and dnKIF5 expression is shown in the insets in panel C. Scale bar = 20 um.

## CHAPTER 3

# Rapid actin cytoskeleton dependent recruitment of plasma membrane-derived dysferlin at wounds is critical for muscle membrane repair.


## ABSTRACT

Deficits in membrane repair may contribute to disease progression in dysferlin-deficient muscular dystrophy. Dysferlin, a type-II transmembrane phospholipid binding protein, is hypothesized to regulate fusion of repair vesicles with the sarcolemma to facilitate membrane repair, but the dysferlin-containing compartments involved in membrane repair and the mechanism by which these compartments contribute to resealing is unclear. A dysferlin-pHluorin (dysf-pHGFP) muscle-specific transgenic mouse was developed to examine the dynamic behavior and sub-cellular localization of dysferlin during membrane repair in adult skeletal muscle fibers. Live-cell confocal microscopy of uninjured adult dysf-pHGFP muscle fibers revealed that dysferlin is highly enriched in the sarcolemma and t-tubules. Laser-wounding induced rapid recruitment of ~30μm of local dysferlin-containing sarcolemma, leading to formation of stable dysferlin accumulations surrounding lesions, and endocytosis of dysferlin and formation of large cytoplasmic vesicles from distal regions of the fiber. Disruption of the actin-cytoskeleton decreased recruitment of sarcolemma-derived dysferlin to lesions in dysf-pHGFP fibers

without affecting endocytosis and impaired membrane resealing in wild-type fibers similar to dysferlin-deficiency (2-fold increase in FM1-43 uptake). Our data support a new mechanism whereby recruitment of sarcolemma-derived dysferlin creates an “active-zone” of high lipid-binding activity at wounds to interact with repair vesicles and facilitate membrane resealing in skeletal muscle.

## INTRODUCTION

Dysferlin is ~220kDa type-II transmembrane protein that is highly expressed in adult skeletal and cardiac muscle [17]. Loss of dysferlin expression leads to a delayed but progressive muscle disease, presenting either in the limb-girdle (LGMD2B) or in distal muscles (Myoshi Myopathy) [23]. Although the pathology of dysferlin-deficiency is well described, the exact role of dysferlin within muscle cells is largely unknown. Dysferlin has been implicated in various cellular processes such as muscle cell-cell fusion during regeneration [33], muscle growth [48], and cell adhesion [44, 45]. Perhaps the most intensively studied cellular role for dysferlin is as a critical component for membrane resealing in skeletal muscle and cardiac muscle cells [18, 61]. Membrane resealing is a critical and evolutionarily conserved mechanism by which cells are able to withstand transient disruptions of the plasma membrane [62, 64]. Although the exact mechanism by which cells reseal the plasma membrane may vary depending on cell type, most cells utilize recruitment of intracellular vesicles capable of fusing with each other, and or the plasma membrane to repair the membrane lesion [70].

Muscle cells are thought to undergo frequent membrane disruption as a result of mechanical activity, particularly when mutations in other important cell adhesion

proteins causing human muscular dystrophies render the sarcolemma susceptible to mechanical injury, and in such cases, resealing may be critical for cell survival [112]. The proposed role of dysferlin in membrane resealing is based on the finding that dysferlin-deficient muscle fibers take up membrane impermeable FM1-43 dye to a greater extent than wild-type fibers following laser-induced sarcolemma wounding [18]. Furthermore, dysferlin accumulates at membrane lesions in resealed skeletal muscle fibers, along with other proteins proposed to be involved in resealing including MG53 and annexin-VI [51, 53], and recruitment of additional repair proteins may be impaired in dysferlin-deficient muscle [90]. Dysferlin-containing vesicles are hypothesized to play a role in resealing due to the dramatic accumulation of vesicles under the sarcolemma of dysferlin-deficient muscle [18], but the composition of dysferlin-containing vesicles and the involvement of dysferlin-containing vesicles in resealing has not been definitively shown in adult skeletal muscle fibers. Mechanistic analysis of resealing in non-muscle cell types supports a role for the cytoskeleton and exocytosis of intracellular vesicles in membrane resealing [62, 64, 76], but whether the cytoskeleton plays a role in dysferlin-mediated resealing in adult muscle fibers is not known. Therefore, the goal of our study was to examine the behavior of dysferlin-containing membranes during membrane repair, and examine the role of the cytoskeleton in dysferlin recruitment and membrane resealing in adult skeletal muscle.

We generated a novel transgenic mouse (termed dysf-pHGFP TG) expressing a dysferlin-pHluorin GFP fusion protein (dysf-pHGFP) specifically in muscle cells, and used this model to study the dynamic behavior and of dysferlin-containing membranes in response to laser-induced membrane disruption in live adult skeletal muscle cells *in-*

*vitro*. Our data indicate that dysferlin is enriched in the sarcolemma and transverse tubules (t-tubules) in resting adult skeletal muscle fibers, with no detectable dysf-pHGFP in vesicles in resting fibers. During membrane repair, sarcolemma-derived dysferlin is rapidly pulled into the lesion where it forms a stable dysferlin-rich structure at the sarcolemma which may be critical for membrane resealing in adult skeletal muscle fibers. Additionally, wounding induces endocytosis and formation of large dysferlin-containing vesicles which may contribute to act as vesicular plugs to repair membrane lesions. Pharmacological disruption of the actin-cytoskeleton blocks the recruitment of sarcolemma-derived dysferlin at lesions and impairs membrane resealing without disrupting dysferlin endocytosis and formation of dysferlin-containing intracellular vesicles. Therefore, our data supports a new model that the dysferlin-containing membrane required for membrane resealing is actually derived from the sarcolemma, and that rapid, actin-cytoskeleton dependent recruitment of dysferlin-containing membrane into stable enrichments at the wound site represents a critical step in the membrane resealing process.

## METHODS

*Generation of dysf-pHGFP transgenic mice:* Murine dysferlin isoform 1 with a C-terminal pHGFP [113] tag was cloned into PBSII SK+ vector downstream of the muscle creatine kinase promoter [114], and used to generate a transgenic mouse on an inbred C57/Bl6 background by the University of Michigan Transgenic Core (described in detail below). A C-terminal fragment of murine dysferlin isoform-1 was sub-cloned from a pDNOR vector, resulting in a BSTBI-NotI fragment within PCR 2.1 shuttle vector. The remaining

N-terminal dysferlin sequence was inserted using a KPNI-BSTBI digest resulting in full length dysferlin within PCR2.1. A fragment containing pHGFP [113] flanked by a 5' SacII and 3' NotI was generated using PCR and inserted into the Dysferlin-PCR2.1 plasmid using a SacII-NotI digest. The resulting dysf-pHGFP fragment was isolated and inserted into PCDNA 3.1 for mammalian expression. A poly-A sequence was inserted on the 3' end of the coding sequence by cloning a pA fragment containing a 5' EAGI site and 3' SPEI, KPNI, and NOTI sites. The pA sequence was inserted into dysf-pHGFP PCDNA 3.1 using a NotI digest. The full length MCK promoter [114] was inserted 5' to the dysf-pHGFP-pA PCDNA 3.1 construct with a KPNI-NOTI digest. The entire cDNA was excised and linearized using a NotI-SpeI digest. Purified DNA was microinjected into fertilized eggs obtained by mating C57BL/6J female mice with C57BL/6J male mice. Pronuclear microinjection was performed as described[115].

*Western blotting:* Skeletal and cardiac muscle KCl-washed microsomes were isolated from adult wild-type and dysf-pHGFP TG mice and analyzed by SDS-PAGE and Western Blotting as described [116]. Membranes were blotted for anti-dysferlin (NCL-Hamlet, Novacastra/Leica, Buffalo Grove, IL, USA) or anti-GFP (AB13970, Abcam, Cambridge, MA, USA) followed by secondary HRP-conjugated antibodies (Jackson Immunoresearch, West Grove, PA, USA) and chemiluminescence detection.

*Muscle fiber isolation, imaging, and staining:* Single muscle fibers were isolated from adult mouse flexor digitorum brevis muscles as described [117]. Briefly, FDB muscles were isolated from anaesthetized adult wild-type or dysf-pHGFP TG mice and incubated

in MEM + 0.2% collagenase for 4 hr at 37ºC with gentle shaking. Following incubation, muscles were switched to MEM+10% FBS, residual connective tissue was removed using forceps, and the muscle was triturated using progressively smaller diameter glass pipettes to liberate single muscle fibers. Isolated muscle fibers were allowed to adhere for 20 minutes on glass-bottom cover dishes pre-plated with 10% matrigel in MEM + 10% FBS. Live fibers were imaged on a Leica SP8 Confocal microscope at 37ºC using 63x oil objective at 1.5x zoom at 512x512 resolution using an argon laser at excitation: 488nm and detection at 498-525nm (GFP) or 580-620nm (FM1-43). For live-cell experiments, cells were imaged in a physiological saline solution (PSS) pH 7.4 containing (in mM) 15 Hepes, 145 NaCl, 5.6 KCl, 2.2 CaCl, 0.5 MgCl, 5.6 Dextrose. To quench the pHGFP oriented toward the extracellular buffer, citrate buffer was used in place of HEPES with pH adjusted to 5.5 using concentrated HCl. To alkalinize intracellular vesicles, NaCl was reduced 95 mM and 50mM $NH_4Cl$ was added to physiological saline solution [118]. Quantification of fluorescence intensity prior to, and following acid wash (or $NH_4Cl$ treatment) was carried out by quantifying mean fluorescence intensity within the entire visible region of the muscle fiber and using raw intensity data to generate percent change values. For staining experiments, cells were fixed for 15min in 3% paraformaldahyde, and permeabilized for 1hr in block solution containing 5% BSA and 0.1% triton-x. Cells were stained with Romeo anti-dysferlin (Abcam) at 1:100 dilution for 1.5hr followed by labeling with 1:200 goat-anti rabbit Cy3 (Jackson Immunoresearch).For staining experiments, cy3 channel was imaged using white light laser at 550nm excitation and detection from 560-595, and all images were collected at 1024x1024 resolution and 2x zoom.

*Membrane injury experiments:* Membrane injury protocols were performed similar to previous studies measuring laser-wounding induced FM1-43 uptake in adult skeletal muscle fibers [18]. Cells were imaged for 3 frames prior to wounding, and every 1.5s for 2-5 minutes after the onset of membrane damage. Membrane damage was elicited by exposing a 2 x 2 μm ROI at the lateral membrane to a multiphoton laser at a fixed intensity, tuned to 890nm for ~2 sec. Recruitment of dysferlin at membrane lesions was analyzed by quantifying $dF/F_o$ (which is $(Ft-F_o)/F_o$, where Ft equals mean fluorescence at time=t, and $F_o$ equals initial fluorescence) within a 10 x 5 μm ROI surrounding the lesion at 10s intervals following wounding. Quantification of "total" cellular pHGFP fluorescence was carried out by quantifying $dF/F_o$ within an ROI spanning the entire cell. Quantification of FM1-43 dye uptake was carried out by quantifying mean $dF/F_o$ following wounding within a 40x40 μm ROI. For cytochalasin D experiments cells were pre-treated with either 0.1% DMSO or 50μm Cytochalasin D for 1.5 h and cytochalasin D was included in the imaging solution. In experiments where high resolution images of wounded fibers are presented, high resolution images (1024x1024 resolution, line average 3) were taken following completion of the wounding protocol.

*FRAP experiments:* To bleach specific populations of dysf-pHGFP within muscle fibers, a 30x5μm ROI at the plasma membrane, or 30x10μm ROI within the transverse tubules was bleached for 3 frames at 3 separate z-planes (+/-1μm) using an argon laser at 488nm. Successful photobleaching was confirmed visually, and lack of photobleach induced wounding was confirmed using DIC optics. Images were taken prior to

bleaching, post-bleach, and for 2-5 minutes following a laser-induced wound delivered in the geographical center of the pre-bleached region.

*Statistics:* All values are presented as Mean +/- SE. Significance was determined based on a Student's t-test, significance was set at $p<0.05$.

## RESULTS

***Generation of dysf-pHGFP TG reporter mice.*** We generated a cDNA encoding murine dysferlin with a C-terminal pHluorin GFP tag (dysf-pHGFP), and generated an MCK-driven muscle-specific transgenic mouse (dysf-pHGFP TG) expressing the dysf-pHGFP transgene in striated muscle (Fig 3-1A). Based on the topology of dysferlin as a type-II transmembrane protein, the dysf-pHGFP reporter molecule places a pH-sensitive GFP on the extracellular face of the plasma membrane and transverse tubules or the lumen of dysferlin-containing vesicles (Fig 3-1B). GFP fluorescence from dysf-pHGFP is visible around the periphery of skeletal muscle fibers within transverse sections of dysf-pHGFP TG skeletal muscle (Fig 3-1C, right) but not wild-type muscle (Fig 3-1C, left). The peripheral localization of dysf-pHGFP near or in the sarcolemma is similar to what has been previously reported for endogenous dysferlin using antibody labeling in adult skeletal muscle [18]. Western blotting for dysferlin with the NCL-hamlet antibody identified a prominent band (~240 kDa) in wild-type skeletal muscle microsomes and an upward shifted band corresponding to dysf-pHGFP specifically in transgenic skeletal (Fig 3-1D, top) and cardiac muscle microsomes (Fig 3-8, top). Western blot using an anti-GFP antibody revealed a strong band at ~260 kDa corresponding to dysf-pHGFP

specifically in transgenic skeletal (Fig 3-1D, bottom) and cardiac microsomes (Fig 3-8, bottom). Interestingly, we identified several lower molecular weight fragments of dysferlin expressed at lower levels detected by both anti-GFP and anti-dysferlin antibodies in transgenic microsomes, indicating the presence of C-terminal dysferlin cleavage products (Fig 3-9A). However, a similar pattern of low molecular weight dysferlin antibody immunoreactive proteins was observed in wild-type skeletal muscle microsomes using the hamlet antibody at longer exposures, suggesting that both endogenous dysferlin and the dysferlin-pHGFP reporter undergo cleavage (Fig 3-9B, right panel). Western blotting for dysferlin expression in wild-type and transgenic skeletal muscle microsomes at various dilutions (1:1 - 1:40) showed that the MCK dysferlin-pHGFP transgene results between 2.5 and 5-fold over-expression of dysferlin in skeletal muscles of dysf-pHGFP TG mice (Fig 3-1E). Dysf-pHGFP TG skeletal muscle is morphologically normal based on comparative analysis of H & E staining of gastrocnemius muscles (Fig 3-1F), quantification of central fiber nucleation (Fig 3-1G), and measurments of plasma creatine kinase (not shown) in dysf-pHGFP-TG and age matched wild-type mice, indicating that dysf-pHGFP TG mice are free of muscle pathology into adulthood.

***Dysf-pHGFP is enriched in the plasma membrane and transverse tubules of adult skeletal muscle fibers and cardiac myocytes.*** The exact localization of dysferlin in adult muscle fibers and the composition of dysferlin-containing membrane compartments remain elusive. GFP fluorescence in fixed adult skeletal muscle fibers isolated from dysf-pHGFP transgenic mice localized to the lateral sarcolemma and in a

striated internal membrane compartment, similar to immunofluorescence labeling of endogenous dysferlin in wild-type muscle fibers (Fig 3-2A and B). In addition, dysf-pHGFP fluorescence colocalizes extensively with immunofluorescence signal from "total" dysferlin with dysferlin antibodies in isolated adult transgenic skeletal muscle fibers (Fig 3-2C). Given that pHGFP fluorescence is quenched at low pH and fluorescence is high at high pH [119], our dysf-pHGFP reporter allows for the selective visualization of GFP signal based on surrounding pH (model shown in Fig 3-1B). Confocal imaging of live adult dysf-pHGFP TG skeletal muscle fibers (Fig 3-3A) and cardiac myocytes (Fig 3-8, left panels) at pH 7.4 revealed that dysf-pHGFP is visible at the lateral sarcolemma and a striated internal membrane compartment. Changing the extracellular solution from physiological saline at pH 7.4 to physiological saline pH 5.5 led to a significant reduction in fluorescence from live dysf-pHGFP TG skeletal (Fig 3-3B, quantified in C) and cardiac myocytes (Fig 3-8B), demonstrating that dysferlin is highly enriched in the plasma membrane and t-tubules in adult muscle cells. Ammonium chloride ($NH_4Cl$) is commonly used to alkalinize all cellular compartments and reveal pHluorin molecules within intracellular vesicles with an acidic luminal pH [119]. Surprisingly, treatment with 50mM ammonium chloride did not reveal additional internal dysf-pHGFP fluorescence in resting dysf-pHGFP TG skeletal muscle fibers (Fig 3-3D, quantified in E), or cardiac myocytes (Fig 3-8C). These data indicates that dysferlin is not primarily an intracellular vesicular protein but instead is primarily localized to the sarcolemma and t-tubules in resting adult muscle fibers.

***Adjacent sarcolemma-derived dysferlin is recruited to membrane lesions following wounding in adult skeletal muscle fibers.*** There have been very few studies examining the dynamic behavior of dysferlin in live adult muscle cells following wounding, and the dysferlin-containing membranes involved in membrane repair are not well defined. To address this, adult skeletal muscle fibers isolated from dysf-pHGFP TG mice were imaged via confocal microscopy and the behavior of dysf-pHGFP was analyzed prior to and following laser-induced plasma membrane wounding. Prior to membrane disruption, dysf-pHGFP localizes to the sarcolemma and t-tubules (Fig 3-4A, left). Analysis of dysf-pHGFP localization during repair shows that the fluorescence signal rapidly increases at membrane lesions and results in a stable dysf-pHGFP-rich structure at the lesion (Fig 3-4A, B red arrows, quantified in 3-4C) consistent with either recruitment of sarcolemmal dysf-pHGFP or exposure of new pHGFP to the extracellular surface as a result of dysferlin-vesicle fusion with the plasma membrane. Interestingly, pHGFP signal from the sarcolemma and t-tubule components in regions of the fiber considerably distant from the wound itself including the sarcolemma on the side opposite the wound, is rapidly quenched following wounding (Fig 3-4B white arrows, quantified in Fig 3-4D), indicating that dysferlin may also be rapidly endocytosed into acidic compartments following wounding.

Given the rapid increase of dysf-pHGFP fluorescence at membrane lesions and our data indicating that dysferlin is restricted to the sarcolemma in resting fibers (Fig 3-3), we tested the hypothesis that the dysferlin-containing membrane recruited to membrane lesions is derived from the adjacent sarcolemma in adult skeletal muscle fibers, rather than fusion of pre-existing dysferlin containing vesicles at the wound site.

To address this, the dysf-pHGFP fluorescence within a 30μm wide ROI at the plasma membrane was photobleached (pre-bleach shown in 3-4Ea, post-bleach in 3-4Eb) and movement of the adjacent GFP-positive dysferlin in the lateral sarcolemma were tracked following a 2μm wound delivered in the center of the pre-bleached ROI (Fig 3-4Ec). Adjacent GFP-positive dysferlin containing sarcolemma is rapidly pulled toward the lesion and constricts the size of the pre-bleached regions without any evidence of new dysf-pHGFP appearing at the wound prior to lateral accumulation (Fig 3-4E). Representative line plots of GFP intensity spanning the sarcolemmal regions prior to wounding (Fig 3-4F), and post wound (Fig 3-4G) show that the photobleached region of plasma membrane is reduced following wounding due to recruitment of adjacent GFP-positive sarcolemma. Conversely, dysf-pHGFP still accumulated at membrane lesions after laser-wounding in fibers subjected to protocols where the local t-tubule GFP fluorescence was photobleached prior to wounding (Fig 3-10). This latter result indicates the t-tubule localized dysferlin does not appear to markedly contribute to the accumulation of dysferlin at membrane wounds.

***Membrane damage induces formation of endocytic dysferlin-containing vesicles in adult skeletal muscle fibers.*** Based on the finding that dysf-pHGFP fluorescence is reduced in regions of the sarcolemma and t-tubules distant from the wound, we proposed that dysferlin may also be endocytosed into acidic membrane compartments following wounding in adult muscle fibers. To test this hypothesis, dysf-pHGFP TG muscle fibers were imaged at rest and for 1 min following wounding in physiological saline (PSS) at pH 7.4, and then switched to excess PSS +/- 50mM $NH_4Cl$ to reveal any

dysf-pHGFP residing in acidic compartments. Switching wounded cells from PSS to PSS without $NH_4Cl$ had no effect on cellular fluorescence (Fig 3-5A) and did not reveal vesicle populations in high resolution images post-wounding (Fig 3-5B). Switching wounded cells from PSS to PSS + 50mM $NH_4Cl$ led to a significant increase in cellular fluorescence back toward baseline (Fig 3-5C), which was associated with the presence of large dysferlin-containing vesicles throughout the cytoplasm of wounded fibers in high resolution images (Fig 3-5D). Rapid wound-induced formation of large dysferlin containing vesicles is similar to what we have observed and recently reported in mechanically wounded dysferlin-GFP expressing myotubes [120].

***Pharmacological disruption of the actin-cytoskeleton impairs recruitment of sarcolemma-derived dysferlin without affecting formation of dysferlin-containing vesicles.*** The subcortical actin cytoskeleton plays an important role in membrane remodeling in many types of motile and mechanically active cells [81, 84]. Given the rapid formation and stability of dysferlin-rich structures at nascent membrane lesions in adult skeletal muscle fibers, we examined whether the subcortical actin cytoskeleton was required for recruitment of plasma membrane derived dysferlin in adult skeletal muscle fibers. Dysf-pHGFP TG skeletal muscle fibers were pre-treated for 1.5 hr with either DMSO (Fig 3-6A) or DMSO + 50μM cytochalasin D (Fig 3-6B) assayed for recruitment of sarcolemma-derived dysferlin at laser-induced membrane lesions following wounding. Treatment with cytochalasin D significantly reduced dysferlin recruitment following laser induced wounding compared to DMSO control (Fig 3-6B, quantified in Fig 3-6C), without markedly affecting damage-induced endocytosis of

dysferlin (Fig 3-6D). Fibers wounded in the presence of either DMSO or DMSO + Cytochalasin D (shown in Fig 3-6A and B) were subsequently treated with physiological solution containing 50mM $NH_4Cl$ and analyzed at high resolution for the presence of dysferlin-containing vesicles. Intracellular dysferlin-containing vesicles were visible in wounded DMSO treated (Fig 3-6A') and wounded cytochalasin D treated fibers (Fig 3-6B') in the presence of $NH_4Cl$. These findings demonstrate that the recruitment of sarcolemma-derived dysferlin at membrane lesions requires the actin cytoskeleton, whereas endocytosis and formation of large dysferlin-containing vesicles following wounding occur independently of the actin cytoskeleton.

*Disruption of cytoskeletal actin impairs membrane resealing in adult skeletal muscle fibers.* Consistent with previous studies examining the effect of dysferlin-deficiency on membrane resealing in adult skeletal muscle [18], dysferlin-deficient muscle fibers from the A/J mouse strain show increased uptake of FM1-43 following laser-induced wounding (Fig 3-7A, quantified in 7B). To determine whether cytoskeleton-mediated delivery of sarcolemmal dysferlin is required for efficient membrane repair in adult skeletal muscle fibers, wild-type muscle fibers were pre-treated for 1.5 hr with either DMSO or 50μM cytochalasin D (which was sufficient to impair dysf-pHGFP recruitment to membrane lesions in dysf-pHGFP skeletal muscle fibers) and assayed for uptake of FM1-43 dye following laser-induced wounding. Treatment with cytochalasin D resulted in a 2-fold increase in FM1-43 uptake following wounding in adult skeletal muscle fibers (Fig 3-7C), comparable to that of dysferlin deficiency (Fig 3-7B) suggesting that

cytoskeleton-dependent recruitment of sarcolemma derived dysferlin at wounds is required for resealing in muscle fibers.

## DISCUSSION

We generated a novel transgenic reporter mouse expressing dysf-pHGFP specifically in mature striated muscle cells, in order to examine the dynamic behavior of dysferlin following sarcolemma wounding and determine the role of the cytoskeleton in regulating dysferlin-mediated membrane repair in adult skeletal muscle *in-vitro*. The dysf-pHGFP reporter molecule is expressed primarily as a full-length high molecular weight protein (~260 kDa) in transgenic skeletal muscle membranes detected by both anti-dysferlin and anti-GFP antibodies. The isolation and identification of dysferlin containing membrane compartments has proven challenging with traditional biochemical techniques, and definitive evidence whether dysferlin is localized in the sarcolemma or in subsarcolemmal vesicles was still lacking [18]. Our dysf-pHGFP transgenic mouse is ideally suited to examine dysferlin localization in adult skeletal muscle cells given that it facilitates selective visualization of dysferlin localization and orientation in adult muscle fibers based on the ability to experimentally manipulate the surrounding pH. The observed quenching dysf-pHGFP fluorescence in dysf-pHGFP TG fibers by lowering the extracellular pH indicates the C-terminal pHGFP is exposed to the extracellular environment at the sarcolemma and in t-tubules. Furthermore, exposure of dysf-pHGFP TG fibers to $NH_4Cl$, which is commonly used to reveal pHluorin reporter proteins sequestered in acidic intracellular vesicles [118, 119], does not increase cellular fluorescence. Together this shows that dysferlin expression is largely restricted

to the sarcolemma and t-tubules in resting adult muscle fibers, and argues against the presence of an intracellular vesicle pool containing dysferlin in resting adult skeletal muscle cells, which has been reported previously in developing myotubes [52, 93, 117, 121]. This assertion is further supported by previous reports and data presented here showing that endogenous dysferlin localizes at or near the sarcolemma and t-tubules using antibody labeling of dysferlin in wild-type skeletal muscle [16, 122-124]. In addition to the predominant high molecular weight dysf-pHGFP protein, we also identified several minor lower molecular weight proteins in muscle using C-terminally directed antibodies to dysferlin or GFP, indicating the presence of C-terminal cleavage products. A similar pattern of low molecular weight fragments of endogenous dysferlin were detected in wild-type muscle using a C-terminal dysferlin antibody. C-terminal fragments of dysferlin containing only the terminal C2 and transmembrane domains have been previously shown to be present and localize at lesions in wounded human myotubes [125]. These findings indicate that although a minor portion of dysf-pHGFP detected in transgenic fibers may be present as low molecular weight C-terminal fragments, the presence of full-length protein and the C-terminal fragments likely represent endogenous dysferlin processing. The dysf-pHGFP reporter is expressed in skeletal muscle at levels that are well below those previously shown to induce toxicity in muscle [126], and we did not observe any evidence of skeletal muscle disease in the dysf-pHGFP TG mice. These findings confirm that dysf-pHGFP reporter molecule is expressed in adult striated muscle from dysf-pHGFP TG mice, behaves similarly to wild type dysferlin in its processing and localization, and support a primary functional role for dysferlin in the sarcolemma and/or in t-tubule membranes.

Dysferlin has been implicated in membrane repair, in part, due to the finding that it is enriched at potential membrane lesions in fixed adult skeletal muscle fibers following mechanical wounding [18]. Our data extend on these findings by showing with live-cell microscopy that dysferlin recruitment to membrane lesions occurs within seconds following wounding, and results in remarkably stable structures surrounding the lesion (that last >20 min post wounding, the longest time points measured). Although it has been suggested previously that the dysferlin accumulating at membrane wounds is derived from intracellular vesicles [18], our data showing that dysferlin is enriched in both the plasma membrane and the transverse tubules in resting skeletal muscle fibers prompted us to investigate which populations of dysferlin-containing membranes contribute to membrane repair in adult skeletal muscle. Selective bleaching of dysf-pHGFP at the plasma membrane or t-tubules demonstrated that the majority of dysferlin recruited to membrane lesions is derived from the sarcolemma immediately adjacent to the wound, and argues against rapid fusion of pre-existing quenched intracellular dysferlin-containing vesicles to reseal the plasma membrane in adult skeletal muscle. Our data also show that pharmacological disruption of the subcortical actin cytoskeleton impairs recruitment of sarcolemma-derived dysferlin at membrane lesions, consistent with the hypothesis that the actin cytoskeleton facilitates rapid recruitment of sarcolemma-derived dysferlin to membrane lesions in adult skeletal muscle fibers. Previous studies in *Xenopus* oocytes showed that the actin cytoskeleton plays an active role in wound-closure by organizing contractile actin-containing “rings” around lesions to constrict the wound [127]. This raises the intriguing possibility that cortical actin may facilitate membrane repair in adult skeletal muscle by generating the

force required to coalesce sarcolemma-derived repair proteins at membrane lesions. Although cytochalasin D at similar doses does not markedly impair the organization of sarcomeric actin in adult isolated muscle cells [128], we cannot rule out that cytochalasin D may also have some effects on sarcomeric actin. However, the recruitment of nearly ~30μm of dysferlin-containing sarcolemma at membrane lesions appears to far exceed the capacity for local sarcomere shortening. Membrane resealing was directly examined to determine whether cytoskeleton-dependent recruitment of dysferlin to membrane lesions was required for efficient membrane repair in adult skeletal muscle fibers. Increased uptake of membrane impermeant FM1-43 dye following laser-induced wounding is commonly used as an indicator of impaired membrane resealing in various cell types [18]. Under identical treatment conditions used to inhibit dysferlin recruitment to lesions, FM1-43 dye uptake was significantly increased in wild-type muscle fibers treated with cytochalasin D compared to DMSO control, and the magnitude of the deficit in resealing with cytochalasin D treatment is comparable to that observed in dysferlin-deficient muscle fibers using the same assay conditions. This indicates that actin-dependent recruitment of sarcolemma-derived dysferlin to membrane lesions is likely critical for functional contribution of dysferlin to membrane resealing in adult skeletal muscle.

Interestingly, although dysf-pHGFP fluorescent signal is elevated at membrane lesions, the signal from dysf-pHGFP in the distant t-tubules and sarcolemma is rapidly reduced after wounding, which suggests that membrane damage may also induce endocytosis of dysferlin into an acidic vesicular compartment. Consistent with this interpretation, increasing intracellular pH in laser-wounded dysf-pHGFP TG skeletal

muscle fibers using $NH_4Cl$ increased dysf-pHGFP fluorescence back toward the initial value prior to wounding, and revealed a population of heterogeneous large cytoplasmic dysferlin-containing vesicles, which were not detectable in non-wounded fibers, or wounded fibers in the absence of $NH_4Cl$. These data support the overall model that dysferlin resides in the sarcolemma and t-tubules prior to wounding, but in regions of the fiber distant from the wound, is rapidly incorporated into cytoplasmic vesicles following membrane disruption. Formation of wound-induced dysferlin vesicles is reminiscent of wound-induced vesicle formation described previously in crayfish giant axons following mechanical wounding [57], where endocytic vesicles accumulate and coalesce to form a “vesicular plug” to reseal the lesion. Formation of large dysferlin-containing vesicles in adult muscle fibers following laser-wounding is also consistent with the formation of large cytoplasmic dysferlin-containing vesicles in dysferlin-eGFP expressing myotubes following mechanical wounding, which result from homotypic fusion of dysferlin-containing vesicles, and heterotypic fusion of dysferlin-containing vesicles with lysosomes [120]. It is reasonable to suggest that membrane wounding could stimulate endocytosis of dysferlin in adult muscle fibers, as dysferlin interacts directly with sarcolemmal proteins that regulate endocytosis such as Caveolin-3 [52], and caveolin-mediated endocytosis may be activated in response to wounding in adult muscle fibers [89]. This finding may also explain why dysferlin is mislocalized to the cytoplasm in a subset of muscle fibers from patients with Duchenne muscular dystrophy or non-dysferlin Limb-Girdle muscular dystrophies, whose muscle fibers may be susceptible to sarcolemma wounding [20]. While our data is consistent with endocytosis of dysferlin following wounding, we cannot completely rule out the

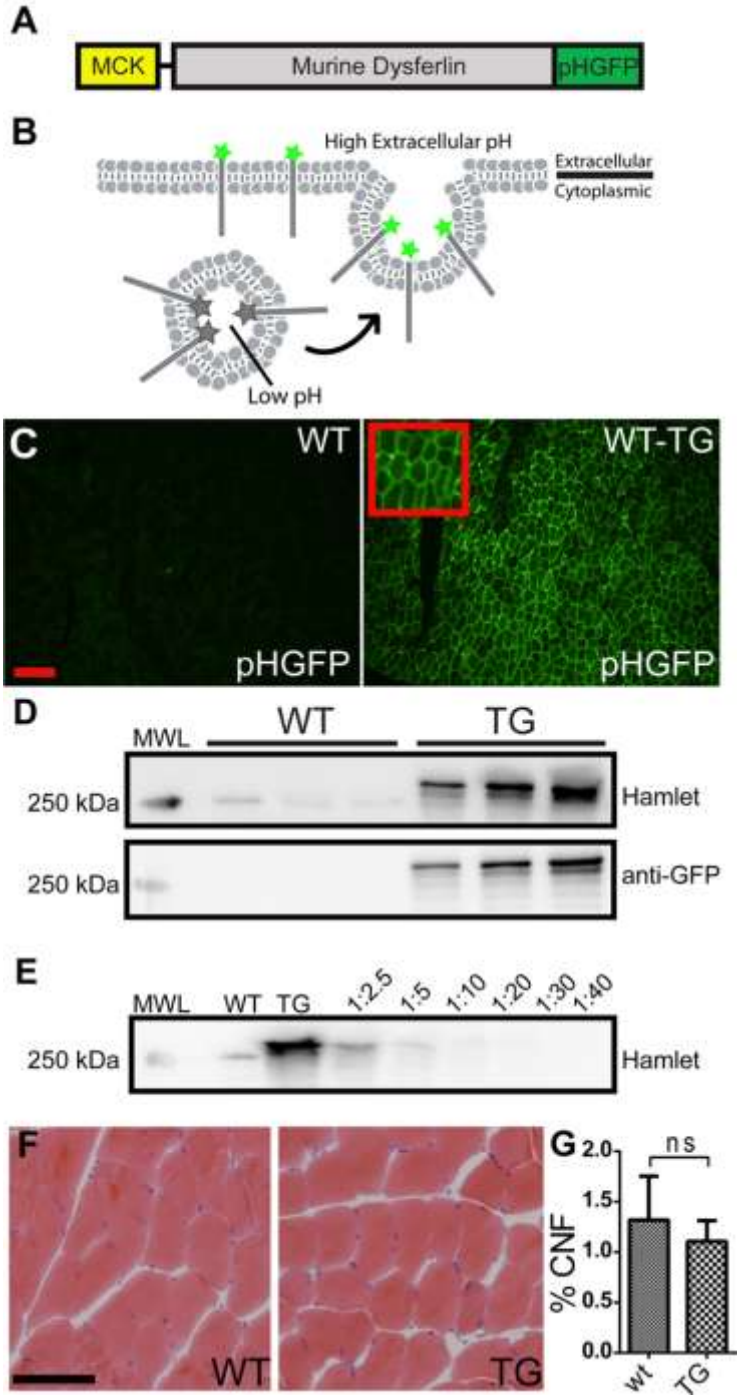
alternative hypothesis that dysferlin-containing vesicles form due to vesicularization of the transverse tubules, which has been described in cardiac myocytes following osmotic shock [129] and is consistent with the presence of several putative membrane repair proteins within the t-tubules [130]. Notably, photobleaching of dysf-pHGFP within the t-tubules adjacent to the wound did not prevent the observed recruitment of dysf-pHGFP fluorescence at wounds; supporting our hypothesis that sarcolemma-derived dysferlin is the primary source of dysferlin accumulated at membrane wounds. Whether dysferlin-containing vesicles formed by endocytosis following wounding contribute directly to membrane repair in skeletal muscle is not clear at present. Our findings that cytochalasin D impairs membrane resealing (as measured by FM1-43 uptake) similar to dysferlin deficiency, without markedly affecting dysferlin endocytosis, suggests that the cytoskeletal recruitment of dysferlin-containing sarcolemma to wounds is a critical primary step in membrane resealing resealing, and endocytosis of dysferlin may play a secondary role in the resealing process.

While the use of the dysf-pHGFP reporter does not directly examine the role of dysferlin in resealing sarcolemmal lesions, independent biochemical studies indicate that dysferlin is capable of calcium-dependent lipid binding [29, 42, 91]. The findings reported here with the dysf-pHGFP reporter support the intriguing possibility that wound-induced recruitment of sarcolemma-derived dysferlin concentrates the lipid binding function of dysferlin at the wound, and creates an “active zone” of high calcium-dependent phospholipid binding activity specifically at the wound, in order to facilitate binding or fusion of intracellular vesicles (dysferlin-containing or otherwise) with the sarcolemma and reseal the sarcolemma following membrane injury in adult muscle

cells. In summary, using live-cell imaging to directly study dysferlin dynamics in adult muscle fibers, this study supports a new model that recruitment of sarcolemma-derived dysferlin to membrane lesions is required for membrane repair. The dysf-pHGFP reporter mouse described here will also be a valuable tool to directly visualize sarcolemma damage in real time, examine the localization and activation of the muscle membrane repair pathway in other physiological models of muscle injury and muscular dystrophy, and help understand the important role of membrane repair in these disorders.

## ACKNOWLEDGEMENTS

The pHluorin GFP construct was a kind gift from Dr. Gero Miesenböck and James Rothman. The MCK promoter was a kind gift from Dr. Jeffrey Chamberlain and Dr. Steve Hauschka. The University of Michigan Microscopy and Image Analysis Laboratory (MIL) provided expert support for confocal microscopy. We acknowledge Wanda Filipiak and Thom Saunders for preparation of transgenic mice and the Transgenic Animal Model Core of the University of Michigan's Biomedical Research Core Facilities. Core support was provided by the University of Michigan Cancer Center (P30 CA046592) and the University of Michigan Rheumatic Diseases Research Core Center (P30 AR048310). This work was supported by American Heart Association (12PRE12050130) and Systems and Integrative Biology Training Grant to J.M. and the NIH HL-080388, HL 895800, and the Muscular Dystrophy Association to D.M.




Scale bar in A=100 µm, C=200µm.

**Figure 3-1. Dysferlin-pHGFP transgenic reporter mouse. A)** Dysf-pHGFP transgene **B)** The dysf-pHGFP reporter places a pH-sensitive pHluorin within the acidic lumen of vesicles or the extracellular face of the sarcolemma or transverse tubules **C)** Dysferlin-pHGFP localizes to the lateral membrane of skeletal muscle fibers in dysf-pHGFP TG skeletal muscle (right-panel). No GFP signal from wt skeletal muscle (left-panel). **D)** Western blot reveals an upward shifted band specifically in dysf-pHGFP TG muscle using dysferlin antibody (top), and anti-GFP antibody (bottom). **E)** Dysferlin expression is increased between 2.5 and 5-fold in transgenic skeletal muscle. **F)** Muscles from dysf-pHGFP TG mice are comparable to wild-type muscle based on hematoxylin & eosin histological staining of gastrocnemius muscles from 14-wek old mice. **G)** Skeletal muscle from Dysf-pHGFP TG mice do not show an increase in centrally nucleated fibers (2888 fibers from 4 WT animals and 3058 fibers from 3 dysf-pHGFP TG animals).

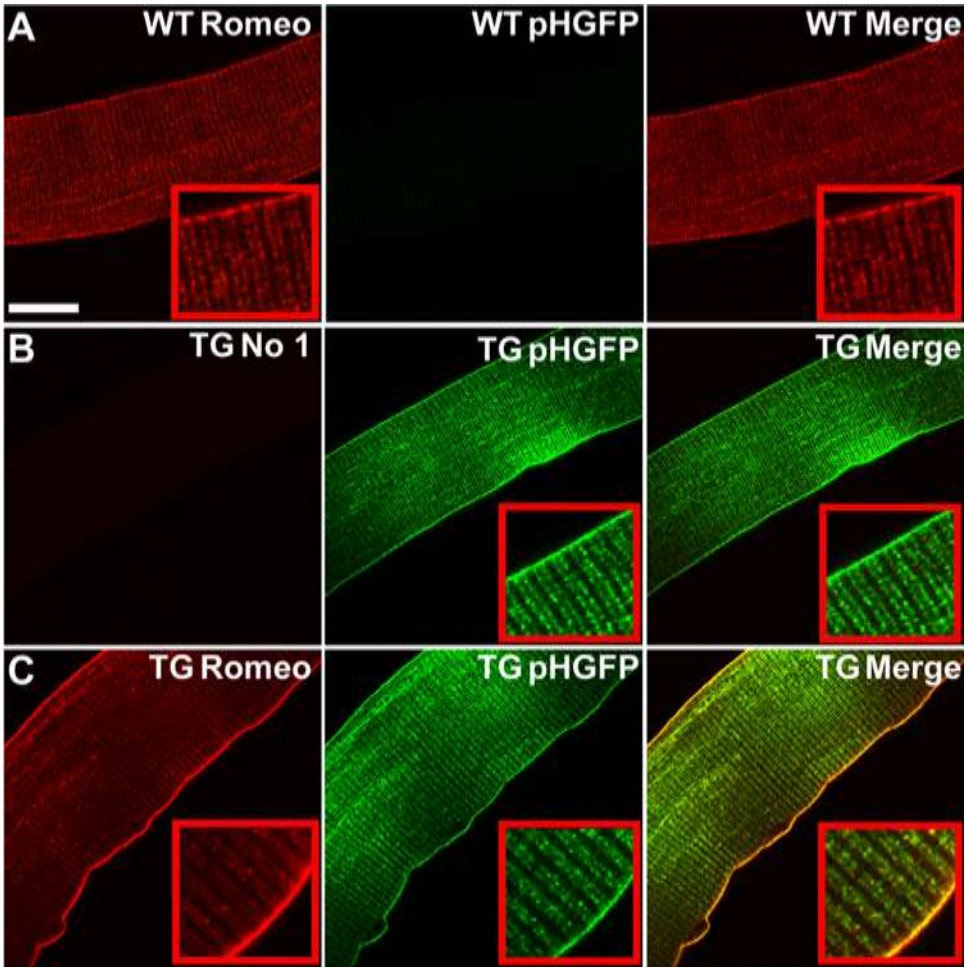



**Figure 3-2. Dysf-pHGFP is localized at the lateral sarcolemma and striated internal membranes in adult skeletal muscle fibers from dysf-pHGFP TG mice similar to endogenous dysferlin in wild-type mice.** **A)** Antibody labeling of endogenous dysferlin shows reveals that dysferlin localizes to the lateral sarcolemma and a striated internal membrane compartment in adult skeletal muscle fibers. Dysf-pHGFP localization in transgenic muscle fibers (**B**, middle) is similar to endogenous dysferlin (compare with **A**) and colocalizes with total dysferlin (Romeo antibody detection of dysf-pHGFP + endogenous dysferlin in transgenic fibers) at the lateral sarcolemma and striated internal compartment in adult skeletal muscle fibers **(C)**. Scale bar =20 µm.

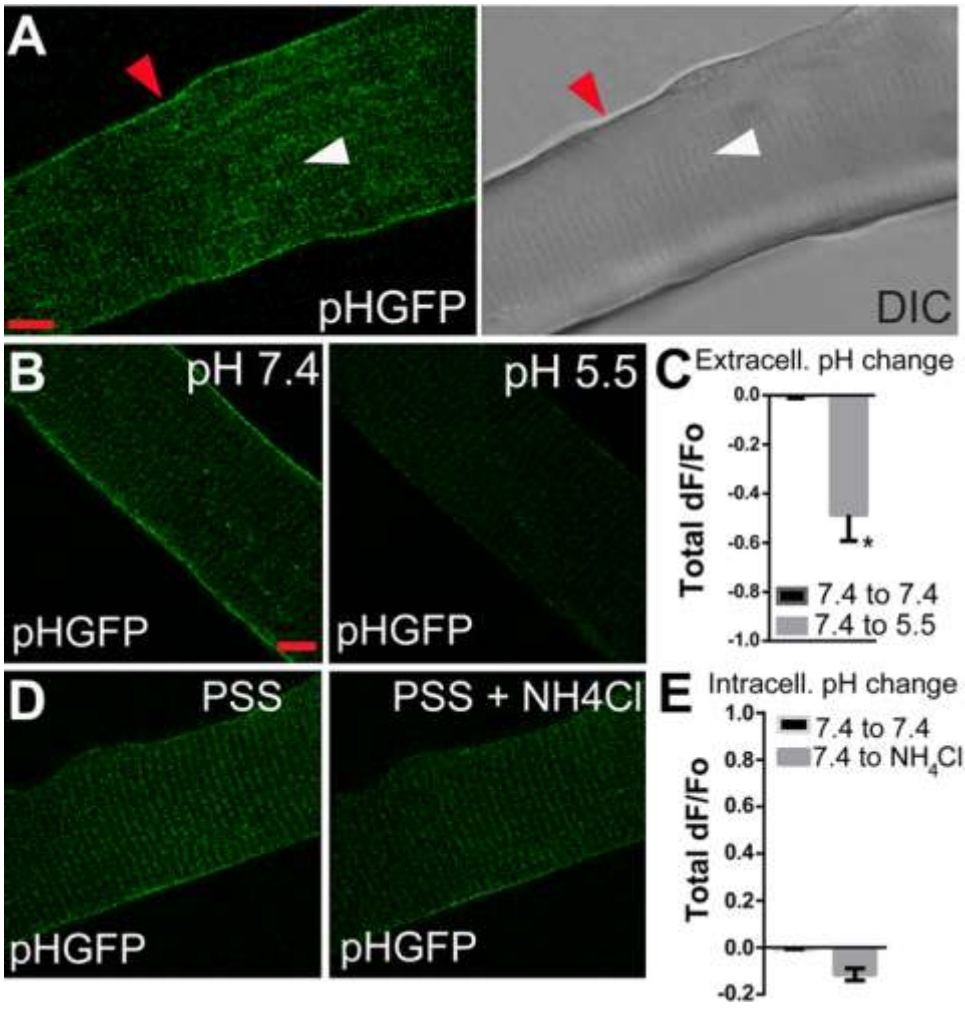



**Figure 3-3. Dysferlin is highly enriched in the plasma membrane and transverse tubules in adult skeletal muscle fibers.** **A)** Dysf-pHGFP localizes to the lateral membrane and internal membrane structures in live FDB fibers isolated from dysf-pHGFP TG mice. **B)** Dysf-pHGFP is visible at the lateral sarcolemma and internal membrane structures at extracellular pH 7.4 (left panel) and pHGFP signal is significantly reduced following reduction of extracellular pH from pH 7.4 to pH 5.5 (quantified in **C**). **D)** Dysf-pHGFP is visible at the lateral sarcolemma and internal membrane structures (left panel) and fluorescence intensity is not increased by $NH_4Cl$ treatment (quantified in **E**). (n=3 fibers in **B** and **C**, n= 6 fibers in **D** and **E**). Scale bar in A and B =10μm.

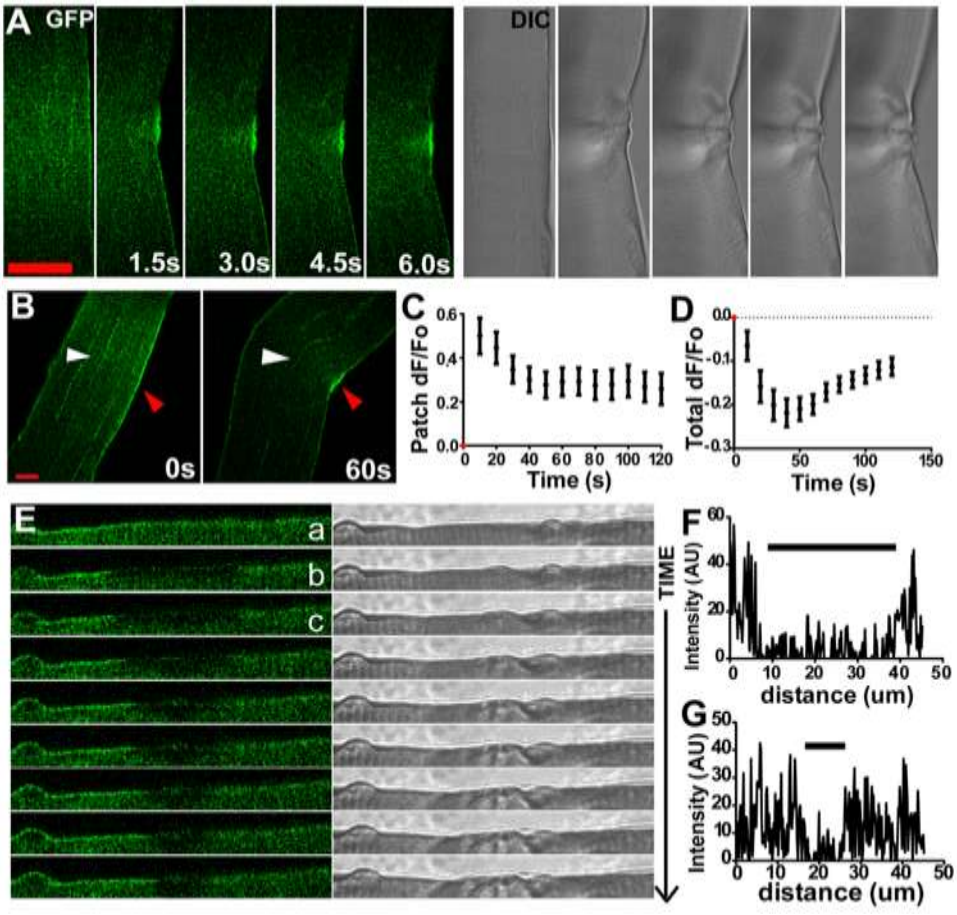



**Figure 3-4. Sarcolemma-derived dysferlin is recruited to membrane lesions following wounding in adult skeletal muscle fibers. A)** Dysferlin is rapidly recruited to membrane lesions following laser wounding in adult skeletal muscle fibers. **B)** Quantification of dysf-pHGFP fluorescence following wounding in adult skeletal muscle fibers. Dysf-pHGFP fluorescence is increased at membrane lesions (red arrowheads in **B**, quantification in **C**) but rapidly reduced from regions of sarcolemma and transverse tubules not adjacent to the wound (white arrows in **B**, quantification in **D**). **E)** Dysferlin used in membrane repair is derived from the adjacent sarcolemma in adult skeletal muscle fibers. Dysferlin is uniformly distributed within the sarcolemma and t-tubules of a resting fiber (**a**). A 30μm ROI containing sarcolemma was bleached (**b**), wounded in the center (**c**), and dysf-pHGFP from the lateral sarcolemma is pulled toward the wound and constricts the pre-bleached ROI (pre in **F**, and post in **G**) indicating that the lateral sarcolemma serves as a source of dysferlin in during membrane repair.

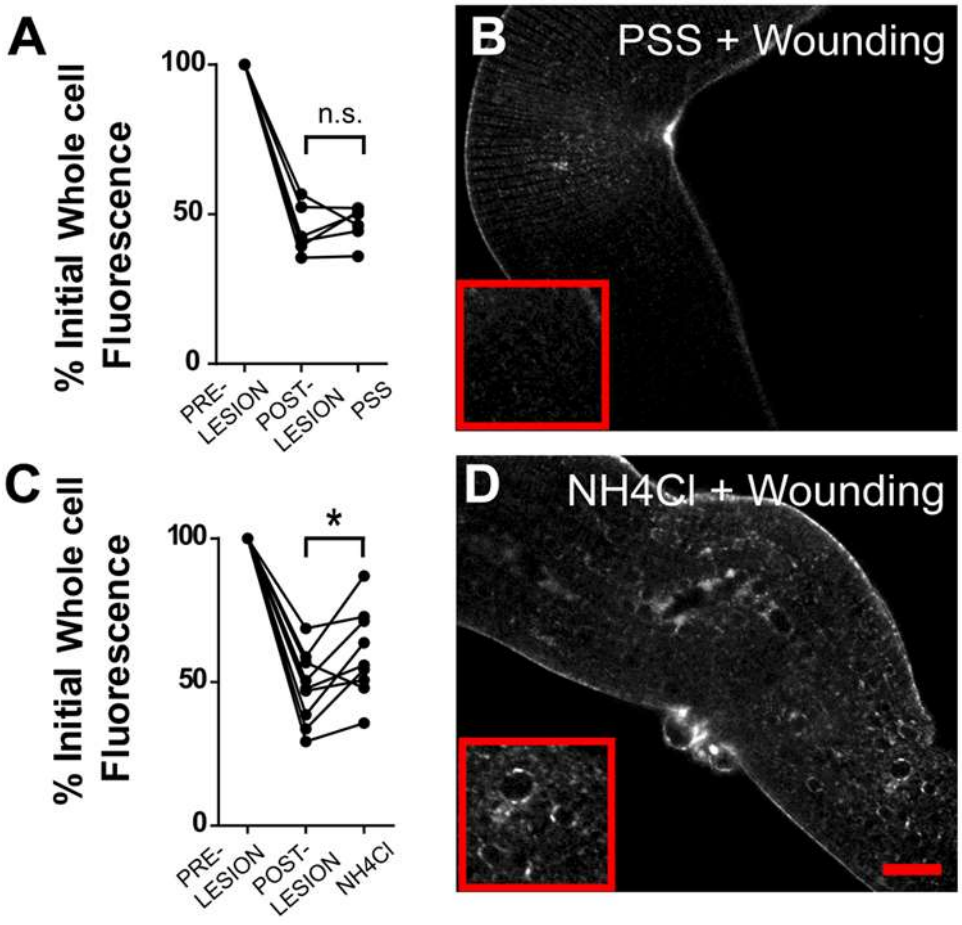



**Figure 3-5. Membrane damage induces formation of acidic dysferlin-containing vesicles in adult skeletal muscle fibers.** Fluorescence intensity was quantified prior to wounding (Pre-lesion), one minute following wounding (post-lesion) and following solution change to physiological saline (PSS, **A**), or physiological saline + 50mM $NH_4Cl$ **(C)** to alkalinize all cellular compartments. Treatment of wounded TG fibers with PSS alone had no effect on fluorescence intensity **(A)** and did not reveal dysferlin-containing vesicles in high resolution images **(B)**, while treatment of wounded fibers with 50mM $NH_4Cl$ led to a significant increase in fluorescence intensity back toward baseline **(C)** and revealed the presence of large intracellular dysferlin-containing vesicles **(D).** (n= 6 fibers for **A**, n= 9 fibers for **C**). Scale bar in D = 10μm.

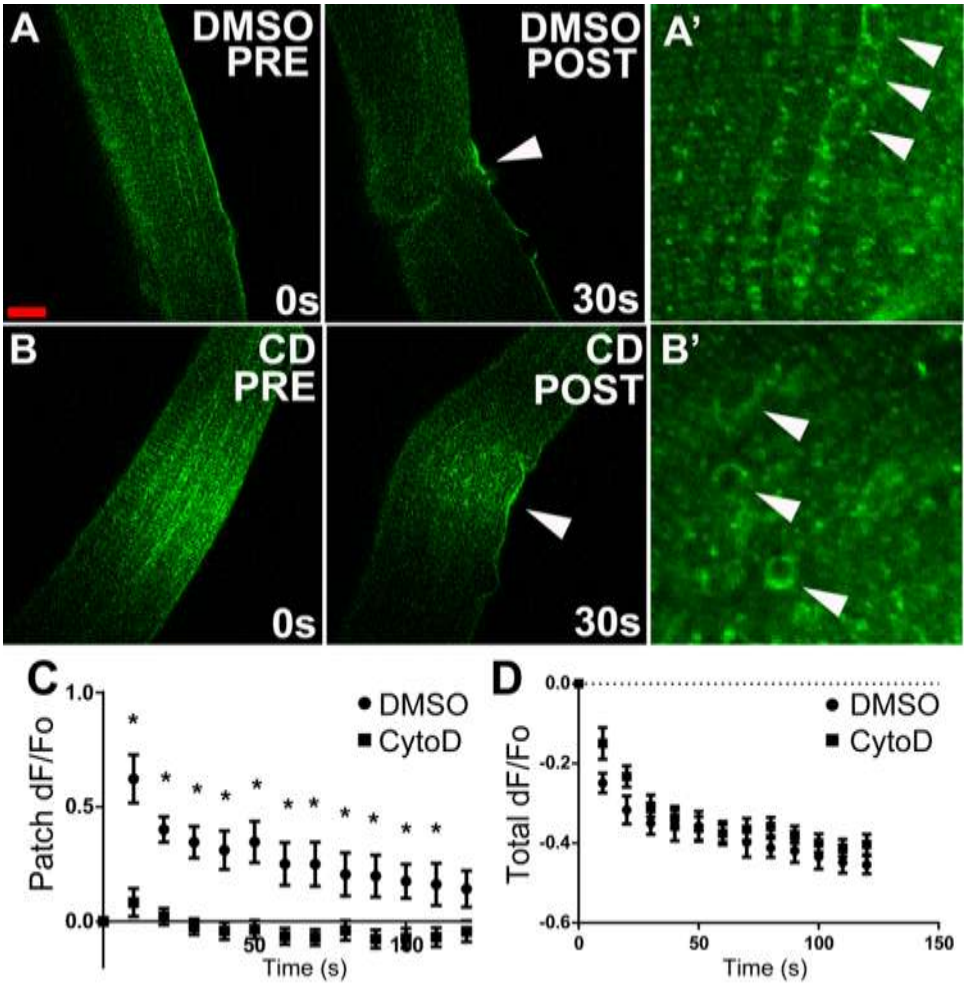



**Figure 3-6**. **Disruption of actin filaments impairs recruitment of sarcolemma-derived dysferlin without affecting damage-induced endocytosis of dysferlin.** TG skeletal muscle fibers were treated with DMSO +/- 50μM cytochalasin D for 1.5 hr and assayed for dysf-pHGFP fluorescence changes at the lesion **(A)** or endocytosis of dysferlin **(B)** as well as the presence of vesicles revealed by $NH_4Cl$ treatment (**A'** and **B'**). Recruitment of dysferlin-containing membrane is significantly reduced in cells treated with cytochalasin D (**A**, quantified in **C**), with no effect on endocytosis (**B**, quantified in D) (DMSO n= 6 fibers, CytoD n=12 fibers). Vesicles were detected following treatment with 50mM $NH_4Cl$ in both DMSO **(A')** and Cytochalasin D treated fibers following wounding **(B').** Scale bar in A = 10μm.

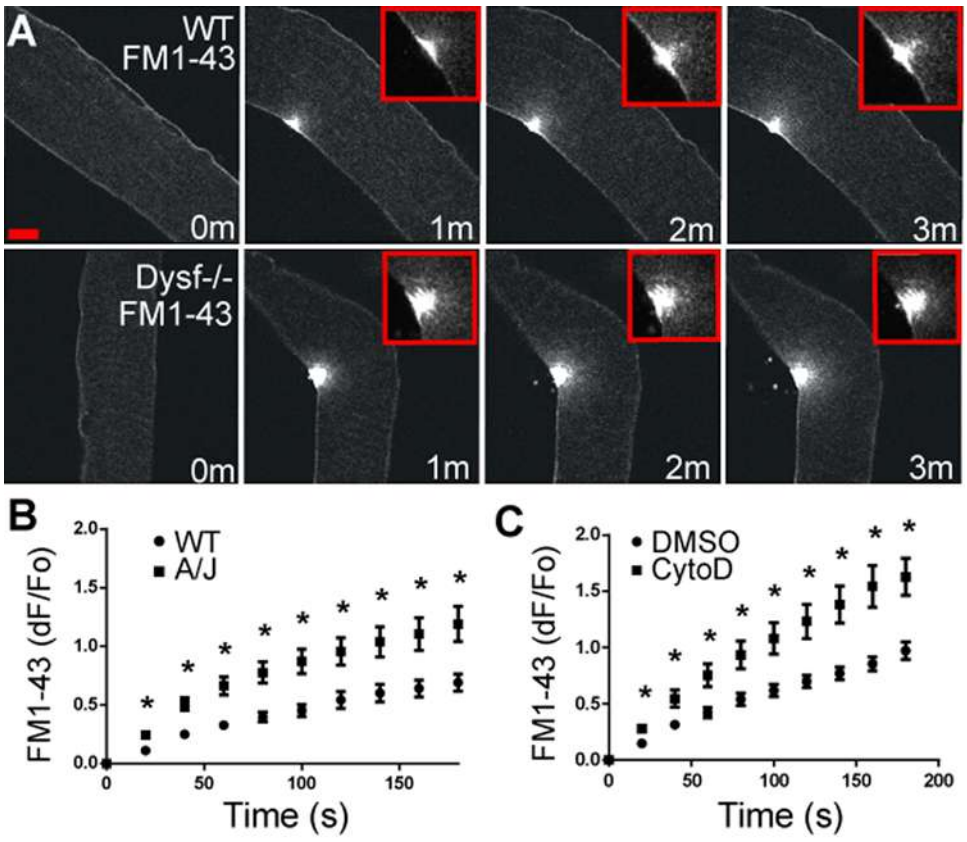



**Figure 3-7. Disruption of the actin cytoskeleton increases FM1-43 uptake following membrane wounding in adult wild-type skeletal muscle fibers. A)** Wounding induced uptake of FM1-43 dye is increased in dysferlin-deficient muscle fibers from A/J compared to wild type (Quantified in **B**, WT n=7 fibers, Dysferlin-null n=9 fibers). **C)** Laser-wounding induced uptake of FM1-43 is increased in wild-type fibers pre-treated for 1.5 hr with 50μM cytochalasin D compared to DMSO treated control fibers (DMSO n=8, CytoD n=10 fibers). Scale bar in A = 10μm.

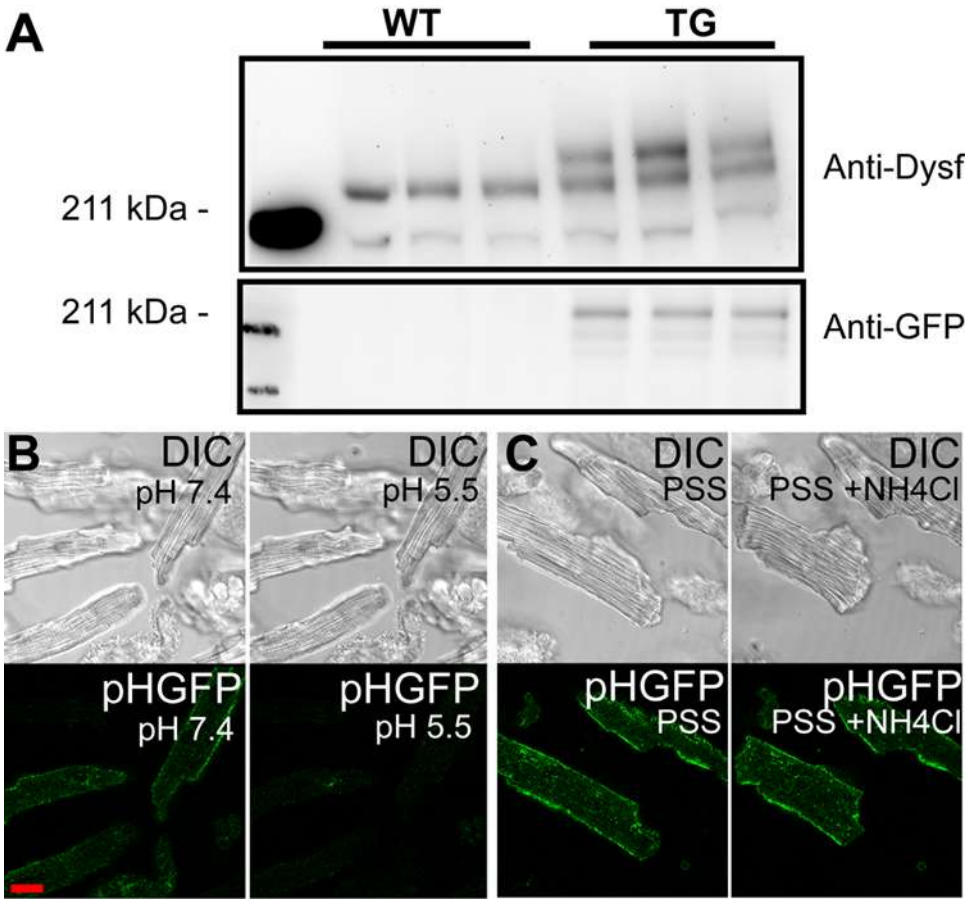



**Figure 3-8. Dysferlin localizes to the plasma membrane and transverse tubules in adult cardiac myocytes. A)** Dysf-pHGFP is detectable as an upward shifted band in western blots of KCl-washed microsomes isolated from wild-type or dysf-pHGFP TG adult hearts using an anti-dysferlin antibody (top), or anti-GFP antibody (bottom). **B)** Fluorescence intensity from dysf-pHGFP TG cardiac myocytes is dramatically reduced by switching cells from extracellular pH 7.4 (left panels) to extracellular pH 5.5 (right panels). **C)** Fluorescence intensity of dysf-pHGFP TG cardiac myocytes is not affected by treatment with 50mM $NH_4Cl$. Scale bar in B=10 µm.

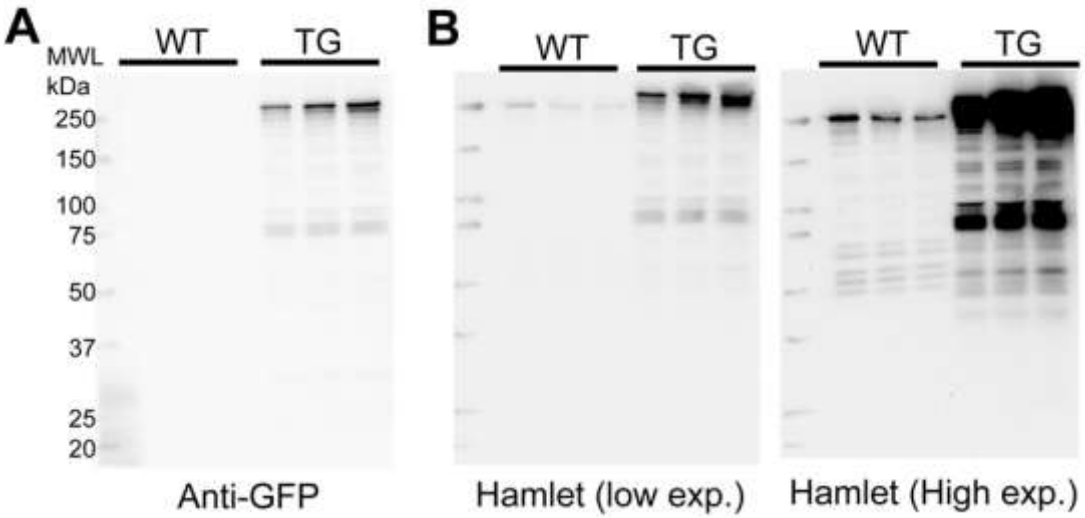



**Figure 3-9. Dysf-pHGFP expression in skeletal muscle from dysf-pHGFP TG mice.** **A)** Western blots using an anti-GFP show the presence of a dysf-pHGFP TG specific high molecular weight band corresponding to dysf-pHGFP as well as lower molecular weight putative C-terminal cleavage products in transgenic skeletal muscle microsomes. **B)** Western blots using NCL-hamlet antibody detect a prominent high molecular weight band in wild-type and transgenic skeletal muscle microsomes corresponding to endogenous dysferlin, as well as an upward shifted band specifically in dysf-pHGFP TG microsomes. Minor lower molecular weight bands corresponding to potential cleavage products are detectable in transgenic skeletal muscle microsomes (left panel). A similar pattern of low molecular weight endogenous C-terminal dysferlin isoforms are visible in wild-type microsomes at higher exposures (right panel).

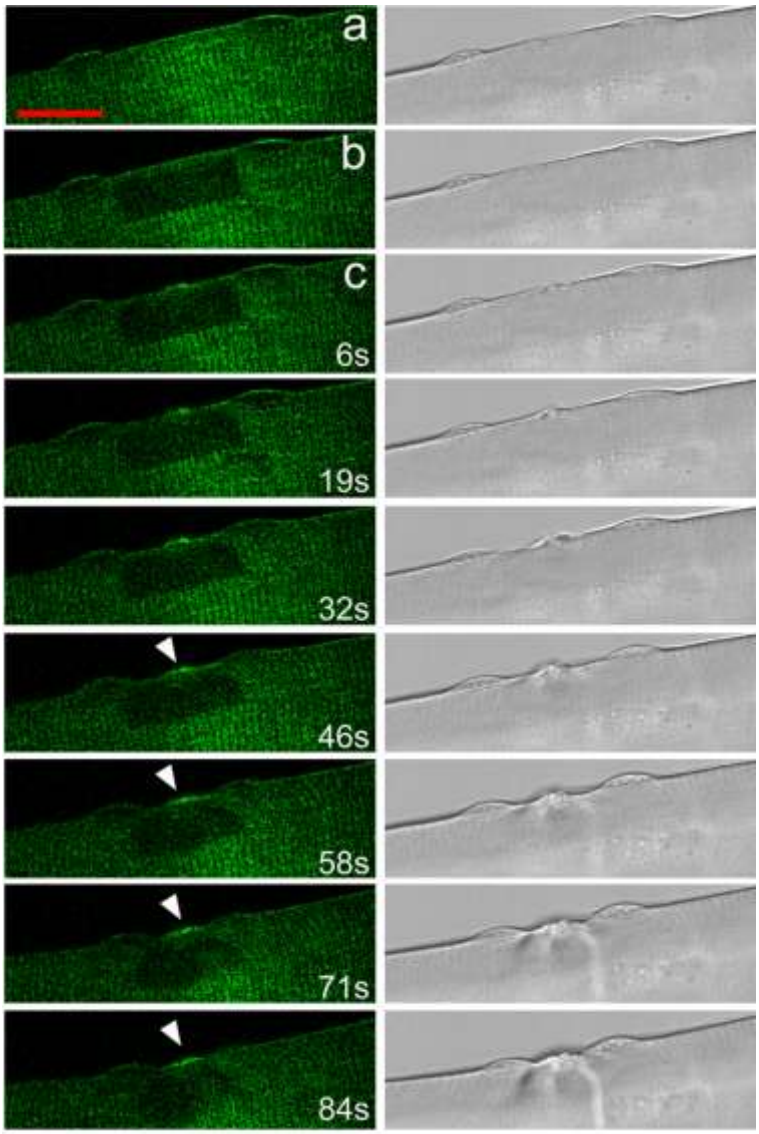



**Figure 3-10. Depletion of t-tubule GFP signal does not affect recruitment of dysf-pHGFP at membrane lesions in transgenic skeletal muscle fibers. a)** Dysf-pHGFP localizes to the plasma membrane and transverse tubules in adult resting skeletal muscle fibers. **b)** Dysf-pHGFP TG skeletal muscle fiber following t-tubule bleaching. **c)** Laser-wounding results in rapid and stable accumulation of dysf-pHGFP at membrane lesions. Scale bar=20 µm

# CHAPTER 4

# CONCLUSIONS AND FUTURE DIRECTIONS

## Summary of thesis work

Dysferlin-deficiency results in a complex set of muscular dystrophies, collectively termed dysferlinopathies [22]. The exact cellular deficits caused by dysferlin deficiency that lead to muscle disease are not clear, but dysferlin is required for efficient membrane resealing, a critical evolutionarily conserved process by which cells repair the plasma membrane following wounding [18, 61]. Bansal et al first demonstrated a role for dysferlin in membrane resealing by showing that damage induced uptake of membrane impermeable FM1-43 dye is increased in muscle fibers isolated from dysferlin-deficient mice compared with normal control muscle fibers. Furthermore, electron micrographs of dysferlin-deficient muscle reveal the presence of subsarcolemmal vesicles near PM disruptions, indicating that dysferlin-deficiency may impair fusion of intracellular repair vesicles with the PM following wounding [18]. Since the identification of dysferlin as a critical membrane repair protein in muscle cells, interactions between dysferlin and several additional proteins involved in membrane repair have been described including MG53 [51, 52], AHNAK [100], Annexin [53, 90] and caveolin-3 [52]. However, the mechanism by which dysferlin-containing membranes

contribute to membrane resealing in normal adult muscle is not clear. Furthermore, the cytoskeleton has been implicated in membrane resealing in non-muscle cells [82, 83], but whether dysferlin-containing membranes interact with the cytoskeleton to facilitate membrane repair in muscle is completely unknown. Therefore, the goal of this project was to examine the contribution of dysferlin-containing membranes and the cytoskeleton to membrane repair in skeletal muscle cells. To address this, live-cell imaging of fluorophore-labeled dysferlin in either developing (chapter 2) or adult muscle cells (chapter 3), in combination with either pharmacological or genetic disruption of the cytoskeleton, was used to examine the dynamic behavior of dysferlin-containing membranes prior to and following wounding and the requirement of the cytoskeleton for dysferlin-containing membrane function during membrane repair. Chapter 2 revealed that dysferlin-containing vesicles are capable of long-range movement along microtubules in differentiated L6 myotubes, and that mechanical wounding induces vesicle-vesicle fusion of dysferlin-containing vesicles, resulting in the formation of large "plug" forming cytoplasmic dysferlin-containing vesicles. Whether dysferlin is strictly required for vesicle-vesicle fusion following wounding is not clear, but this data, taken together with the proposed role for dysferlin in lipid binding *in-vitro* [42, 91], implicates dysferlin as a potential critical regulator of vesicle-vesicle fusion following wounding in muscle cells. Vesicle-vesicle fusion of dysferlin-containing vesicles was dependent on the presence of an in-tact microtubule cytoskeleton and functional KIF5B motors, which highlights a potential role for the microtubule cytoskeleton in membrane resealing in skeletal muscle myotubes. Chapter 3 expanded on the findings in chapter 2 by examining the behavior of dysferlin-containing membranes following laser-induced

membrane wounding in adult skeletal muscle fibers isolated from a novel dysferlin-pHGFP muscle-specific transgenic reporter mouse. The pH-sensitivity of this reporter was used to examine the localization of dysferlin in adult muscle cells prior to and following laser-induced wounding. Interestingly, our data indicate that dysferlin-expression is restricted to the sarcolemma and t-tubules in adult skeletal muscle fibers, with minimal dysferlin in intracellular vesicles in resting muscle fibers. Consistent with a role for sarcolemma derived dysferlin in membrane resealing, laser-induced wounding resulted in rapid and stable accumulation of adjacent dysferlin-containing sarcolemma at membrane lesions. Additionally, dysferlin from distant portions of the fiber is endocytosed into a heterogeneous population of intracellular vesicles. Pharmacological disruption of the actin-cytoskeleton impaired dysferlin recruitment at membrane lesions without affecting endocytosis, and impaired membrane resealing (based on FM1-43 uptake). These findings support the novel model that actin-dependent recruitment of dysferlin-containing sarcolemma at membrane lesions may facilitate membrane repair by creating a local "active-zone" of high lipid binding activity to enhance fusion of dysferlin-containing or non-dysferlin intracellular vesicles with the plasma membrane and facilitate resealing. The work presented in chapters 2 and 3, and summarized below expand the knowledge of dysferlin-mediated membrane resealing by directly demonstrating a role for dysferlin-containing membranes in membrane resealing and highlighting a critical role for the cytoskeleton in regulating dysferlin-mediated membrane resealing in skeletal muscle.

Chapter 2 showed that dysferlin localizes to non-secretory, non-lysosomal cytoplasmic vesicles within the cytoplasm of L6 myotubes. Live-cell imaging of dysferlin-

eGFP expressing myotubes revealed that the majority of dysferlin-containing vesicles undergo solely back and forth movements, with a small percentage undergoing long range movements. Long range movement of dysferlin-containing vesicles depends on intact microtubules, as pharmacological disruption of microtubules impairs dysferlin-vesicle movement. Furthermore, dysferlin-containing vesicles labeled with KIF5B, indicating that KIF5B may be the microtubule-based motor driving movement of dysferlin-containing vesicles in myotubes. In order to examine the contribution of dysferlin-containing vesicles to membrane repair, we employed a mechanical wounding technique to induce local membrane lesions. Interestingly, mechanical wounding commonly led to complete tearing of the muscle fiber, analogous to the formation of "hypercontraction-clots" formed in response to isometric tetanic contractions in mdx muscles *in-vitro* [14]. Strikingly, rather than accumulate specifically at membrane lesions, dysferlin-containing vesicles underwent rapid vesicle-vesicle fusion to form extremely large dysferlin-containing vesicles throughout the cytoplasm of L6 myotubes. The large damage-induced vesicles formed following wounding are remarkably stable, and although somewhat rare, are capable of collapsing on the wounded sarcolemma. Formation of large cytoplasmic vesicles was reminiscent of the "vesicular plug" model of membrane resealing, whereby membrane wounding induces the rapid formation of cytoplasmic vesicles that plug the membrane lesion [59]. Given that dysferlin-containing vesicles interact with microtubules and KIF5B in resting myotubes, we examined whether these interactions were critical for large vesicle formation in response to wounding. Vesicle-vesicle fusion of dysferlin-containing vesicles was impaired in cells pre-treated with microtubule disrupting reagent nocodazole, indicating that a functional

association between microtubules and dysferlin-containing vesicles is critical for vesicle-vesicle fusion following wounding. Expression of dnKIF5B significantly reduced formation of large dysferlin-containing vesicles following wounding without disrupting cytoskeletal architecture, Golgi formation or lysosomal organization, indicating that dnKIF5B impairs large vesicle formation by directly disrupting the function of dysferlin-containing vesicles following wounding. These data indicate that KIF5B based movement along microtubules is critical for large vesicle formation following wounding in differentiated L6 myotubes, and support the overall model that damage-induced formation of large dysferlin-containing vesicles contributes to membrane resealing by forming a vesicular plug to repair membrane lesions. Lysosomes are critical regulators of membrane resealing in non-muscle cells, but whether lysosomes contribute to membrane resealing muscle, and whether lysosomes interact with dysferlin-containing vesicles has not been explored. Dual labeling of lysosomes and dysferlin-containing vesicles in L6 myotubes revealed that dysferlin-containing vesicles do not localize to lysosomes prior to wounding, but undergo heterotypic fusion with lysosomes following wounding to form colabeled vesicles. Although lysosomes play a role in membrane resealing in non-muscle cell types [72], very few studies have addressed the specific role of lysosomes in membrane resealing in developing or fully differentiated muscle cells. Furthermore, whether lysosomes interact with dysferlin-containing membranes in adult muscle cells requires further examination. Taken together, these finding suggest that lysosomes may contribute to membrane repair in muscle by interacting with dysferlin-containing membranes following sarcolemma damage.

Chapter 3 expanded upon the findings in chapter 2 by utilizing a novel transgenic mouse (dysf-pHGFP TG) expressing dysferlin-pHGFP specifically in adult striated muscle cells. Our dysferlin-pHGFP construct places a pH-sensitive pHluorin molecule on the extracellular surface of muscle fibers or lumen of vesicles depending on the localization of dysferlin within the muscle cell. Dysferlin-pHGFP is expressed in skeletal muscle and heart and localizes to the lateral membrane of skeletal muscle fibers in dysf-pHGFP TG mice. Dysf-pHGFP TG mice are free of pathology throughout adulthood, indicating that dysf-pHGFP is functioning properly in-vivo. Treatment of dysf-pHGFP TG FDB muscle fibers with acidic media significantly reduced cellular fluorescence, indicating that dysferlin is enriched in the sarcolemma and t-tubules of skeletal muscle fibers prior to membrane disruption. Furthermore, treatment with $NH_4Cl$, which is commonly used to reveal pHGFP probes within acidic vesicles [118, 119], did not increase cellular fluorescence in resting adult muscle fibers. Taken together, these data support the novel hypothesis that dysferlin expression is restricted to the sarcolemma and t-tubules in adult muscle fibers with minimal dysferlin present in vesicles at rest. Membrane disruption induces rapid accumulation of local sarcolemma-derived dysferlin at membrane lesions, with minimal recruitment of dysferlin from intracellular stores, indicating that dysferlin-containing membranes involved in resealing may actually derive from the sarcolemma. Although dysf-pHGFP accumulates at membrane lesions, pHGFP signal from the surrounding regions of the sarcolemma and t-tubules is rapidly quenched, indicating that dysferlin may be endocytosed into acidic compartments following wounding. Consistent with this hypothesis, addition of $NH_4Cl$ to wounded fibers recovered pHGFP signal back toward baseline and revealed a

heterogeneous population of dysferlin-containing vesicles throughout the cytoplasm of wounded fibers. Interestingly, myoferlin, a dysferlin homolog, is critical for endocytosis potentially through interacting with caveolin-1, the muscle specific isoform of which (caveolin-3) also interacts with dysferlin, and myoferlin-mediated endocytosis may be critical for membrane resealing in endothelial cells [131]. Skeletal muscle from myoferlin knockout mice are myopathic and have altered t-tubule morphology, a phenotype that is also present in dysferlin-deficient muscle [50]. Therefore, it is interesting to speculate that myoferlin may also play a role in membrane resealing in skeletal muscle by regulating damage-induced endocytosis of dysferlin. Damage-induced vesicle formation in adult skeletal muscle fibers was reminiscent of vesicle-vesicle fusion of dysferlin-eGFP containing vesicles in developing myotubes described in chapter 2. It is therefore likely that these vesicles play a critical role in membrane repair, potentially by interacting with sarcolemmal-derived dysferlin at lesions to "plug" membrane lesions. However, our current model is limited in that vesicular dysferlin is not visible without harsh chemical treatments to alkinalize the cell. Therefore, the question of whether damage-induced vesicles contribute directly to membrane repair could be readily addressed using an analogous model utilizing a dysferlin reporter tagged with a non-pH sensitive reporters such as eGFP or mCherry. The rapid recruitment of dysferlin-containing sarcolemma and formation of stable dysferlin-rich structures at membrane lesions was suggestive of a role for the cortical actin cytoskeleton. Disruption of the actin-cytoskeleton with cytochalasin D impaired accumulation of dysferlin-containing sarcolemma without affecting endocytosis and formation of dysferlin-containing vesicles. Identical treatment of wild-type fibers with cytochalasin D increased uptake of FM1-43 dye following

wounding comparable to dysferlin-deficiency, indicating that actin-dependent recruitment of dysferlin-containing sarcolemma is a critical step in membrane resealing in skeletal muscle. These findings are consistent with recent data that the cortical actin cytoskeleton contributes to wound closure in drosophila larvae [84]. Although our pharmacological data strongly suggests that cortical actin-dependent recruitment of dysferlin-containing sarcolemma is critical for membrane resealing in skeletal muscle, examination of dysferlin-mediated membrane repair in genetic knockouts of specific actin isoforms would be useful in order to directly examine the role of the cortical actin cytoskeleton. The exact function of dysferlin downstream of actin-dependent recruitment to membrane lesions is not known, but *in-vitro* biochemical evidence indicates that dysferlin is involved in calcium-dependent lipid binding [42, 91] and potentially fusion of repair vesicles with the sarcolemma [18]. Therefore, these findings support the overall model that actin-dependent recruitment of dysferlin-containing sarcolemma at membrane lesions could facilitate membrane repair by generating an "active-zone" of high lipid-binding activity to enhance fusion of dysferlin-containing or non-dysferlin containing repair vesicles with the plasma membrane in adult skeletal muscle (summarized in Fig 4-1).

The work presented in chapters 2 and 3, and summarized above are consistent with a role for dysferlin-containing membranes and the cytoskeleton in membrane resealing in skeletal muscle. This work did, however, reveal specific differences in the mechanism by which dysferlin-containing membranes contribute to membrane resealing in myotubes versus adult terminally differentiated muscle fibers, specifically with respect to the localization of dysferlin in resting and wounded cells (intracellular vesicles in

myotubes versus sarcolemma and vesicles in adult muscle fibers). Dysferlin has been shown to localize to developing t-tubule system in developing C2C12 myotubes based on colocalization with t-tubule marker Bin-1 [49]. Given that fully developed transverse tubules are thought to develop from vesicular precursors in differentiating myotubes [132], it is possible that the dysferlin-containing vesicles in our myotube experiments could be t-tubule precursors, which would be consistent with the finding that dysferlin-pHGFP is present in t-tubules in adult skeletal muscle. The apparent lack of sarcolemmal dysferlin and lack of enrichment of sarcolemma-derived dysferlin in our myotube model may actually be due to technical limitations associated with epifluorescence imaging, given that a smaller signal (relative to vesicular dysferlin) may be difficult to detect using epifluorescence. Furthermore, there are likely important differences in the method of wounding in our mechanically wounded myotubes compared with laser-induced injury in adult myofibers. This is evident based on the cellular response to wounding, where myotubes undergo complete hyper-contraction, and adult myofibers are largely stable following wounding. This implies that the response (accumulation of sarcolemma-derived dysferlin and formation of damage-induced vesicles) is likely identical, but the "dose" of wounding may be much greater in mechanically wounded myotubes compared to laser-wounded adult myofibers. Regardless, the findings from myotubes and adult skeletal muscle are consistent in that both form large dysferlin-containing cytoplasmic vesicles following wounding. Therefore, these findings indicate that formation of damage-induced dysferlin containing vesicles and local enrichment of dysferlin-containing sarcolemma likely contribute to membrane resealing in skeletal muscle. The interaction between dysferlin-containing membranes

and the cytoskeleton is also consistent between developing myotubes and adult skeletal muscle. In differentiating myotubes, dysferlin-containing vesicles associated with microtubules, and this association was required for formation of damage-induced vesicles. While the requirement of microtubules for vesicle formation in adult skeletal muscle was not examined, it is plausible given that membrane wounding leads to formation of analogous dysferlin-containing vesicles in adult skeletal muscle fibers. Furthermore, disruption of cortical actin reduced accumulation of sarcolemma derived dysferlin at membrane lesions in adult skeletal muscle without affecting endocytosis and formation of dysferlin-containing vesicles. These findings are consistent with the observation that treatment with blebbistatin, a non-muscle myosin-II inhibitor, did not affect vesicle formation following wounding in differentiating myotubes. In conclusion, our data from differentiated myotubes and adult skeletal muscle fibers support the assertion that the cytoskeleton is a critical regulator of dysferlin-mediated membrane resealing in skeletal muscle.

## Implications and future directions

Mutations in dysferlin lead to progressive muscle disease, potentially through an impaired ability of dysferlin-containing membranes to repair membrane lesions [18]. Recent studies have begun to examine interactions between dysferlin and additional proteins involved in membrane repair [52, 90], however, the exact mechanism by which dysferlin-containing membranes contribute to membrane repair in normal muscle has not been fully explored. We used a novel live-cell imaging approach to examine the

behavior of dysferlin-containing membranes and vesicles following wounding in developing and adult skeletal muscle. Our data provide mechanistic evidence that interactions between the cytoskeleton and dysferlin-containing membranes may be critical for membrane repair in muscle cells. A deeper mechanistic understanding of how the cytoskeleton contributes to membrane resealing will provide valuable insight into the role of the cytoskeleton in the prevention of muscle disease and reveal potential targets for therapeutics aimed at enhancing membrane repair in muscle diseases.

The finding that enrichment of dysferlin-containing sarcolemma is critical for membrane repair in adult skeletal muscle fibers (chapter 3) is completely novel, and suggests that enrichment of sarcolemma-derived lipid-binding moieties (ie. C2A domain of dysferlin) at membrane lesions may be necessary for membrane resealing and implies that a major function of dysferlin may be to facilitate interactions between the plasma membrane and repair vesicles in skeletal muscle fibers. These findings have important therapeutic implications given that therapeutics aimed at repairing the sarcolemma (which could conceivably treat several muscular dystrophies independent of genetic origin) would not need to be transported into a specific sub-cellular compartment, rather a compound that remained extracellular but bound lipids may be sufficient restore sarcolemmal integrity. Furthermore, given that gene therapy for dysferlin-deficiency will likely be limited by the relatively large size of dysferlin, our findings indicate that a simplified molecule containing the calcium-dependent lipid binding C2A domain targeted to the plasma membrane may be sufficient to restore resealing capacity in dysferlin-deficient fibers. Interestingly, this is supported by a recent study that identified the C2A and transmembrane domain as critical functional domains

required for resealing, but whether this molecule ameliorates any of the pathology in dysferlin-deficient mice remains to be explored [29].

Although the pathogenesis of DGC-related muscular dystrophies is well described, the underlying cellular mechanisms by which mutations in DGC components lead to muscle disease are largely unknown. The current dogma is that loss of DGC components results in an "instability" of the muscle sarcolemma and susceptibility of muscle fibers to contraction-induced wounding. The field is limited, however, by a lack of reagents to study the development of membrane lesions in muscle fibers *in-vitro* and *in-vivo*. Our data from chapter 3 demonstrates clearly that dysferlin robustly and reproducibly labels membrane lesions in adult skeletal muscle fibers. Therefore, our novel dysferlin-pHGFP TG reporter mouse will serve as an important tool for studying disruption of the plasma membrane and activation of the repair pathway in various models of muscle disease, including the muscular dystrophies. Therefore, the future studies proposed below are aimed at both identifying critical molecules that regulate the membrane repair pathway, as well as using the dysferlin-pHGFP TG mouse to ask more fundamental questions about how mutations in various muscular dystrophy causing genes lead to the development of muscular dystrophy.

**Contribution of damage-induced dysferlin-containing vesicles to membrane repair in adult skeletal muscle cells:**

In chapter 3, the pH-sensitivity of dysferlin-pHGFP was used to explore the localization of dysferlin within adult skeletal muscle fibers at rest and following laser-induced wounding. Our data indicate that dysferlin expression is restricted to the

sarcolemma and t-tubules in resting adult muscle fibers, but following wounding, dysferlin is rapidly endocytosed and incorporated into large cytoplasmic vesicles, analagous to vesicle-vesicle fusion in myotubes (chapter 2), which may contribute to membrane repair by plugging membrane lesions. However, examination of vesicle behavior is limited in our transgenic model by the fact that pHGFP is not fluorescent at low pH (within the lumen of endocytic vesicles), and thus vesicles are not visible without treatment of the fibers ammonium chloride, which may have adverse effects on vesicle function. In order to circumvent this limitation, an MCK driven dysferlin-mCherry transgenic mouse could be generated, which will express constitutively fluorescent mCherry reporter gene to track the dynamic response of newly formed dysferlin-containing vesicles in live adult skeletal muscle cells following wounding (Fig 4-2A). Therefore, subjecting adult skeletal muscle fibers isolated from dysf-mCh TG mice to laser wounding and analyzing vesicle formation and accumulation at membrane lesions could be used to the hypothesis that damage-induced dysferlin-containing vesicles contribute to membrane resealing in adult skeletal muscle (Fig 4-2B).

Our data from dysf-pHGFP TG skeletal muscle fibers indicates that sarcolemma-derived dysferlin accumulates at membrane lesions and forms stable patches, but whether damage-induced dysferlin-containing vesicles accumulate with sarcolemma derived dysferlin at membrane lesions is not clear (as any endocytic dysferlin cannot be detected). Therefore, crossing the constitutively fluorescent dysf-mCh reporter mouse to the dysf-pHGFP TG mouse presented in Chapter 3 to generate a dysf-mCh/pHGFP double transgenic mouse will generate a muscle specific transgenic that expresses both a pH-sensitive GFP reporter as well as a constitutively fluorescent mCh reporter within

all muscle cells (Fig 4-2C). This model will allow for the simultaneous analysis of sarcolemma-localized dysferlin, (both green and red), and vesicular dysferlin (red but not green) at nascent lesions in adult skeletal muscle fibers. The ratio of green:red fluorescence (normalized for maximal intensity) at membrane lesions would hypothetically serve as an indicator of the relative percentage of vesicular dysferlin within nascent patches. Thus, a perfect 1:1 green:red intensity ratio within nascent patches would indicate that all dysferlin within the patch is within the sarcolemma, whereas values <1:1 would indicate that a portion of dysferlin within the nascent patch is derived from dysferlin present within endocytic vesicles (potential outcomes presented in Fig 4-2D). An additional dysf-eGFP/dysf-mCh transgenic mouse expressing constitutively fluorescent green and red probes could be generated, and skeletal muscle fibers isolated from this mouse could be used as a control that is constitutively 1:1 green to red. While the data presented in chapters 2 and 3 supports our current model (Fig 4-1), the proposed experiments would provide strong evidence that damage-induced dysferlin-containing vesicles contribute to membrane repair in adult skeletal muscle.

**Endocytosis, and myoferlin as a critical regulator of dysferlin-mediated membrane repair in adult skeletal muscle:**

The novel finding that membrane damage induces the formation of dysferlin-containing vesicles in skeletal muscle raises intriguing questions about the role of endocytosis in membrane resealing in adult skeletal muscle. Initial reports on membrane resealing in adult skeletal muscle overlooked endocytosis as a potential

regulator of membrane repair because of the apparent lack of caveolin-3 (a plasma membrane protein with a known role in endocytosis) at membrane lesions in mechanically wounded adult skeletal muscle fibers [18]. However, recent data suggests that caveolin-mediated endocytosis is activated in close proximity to membrane lesions leading to formation of caveolin-containing vesicles near membrane lesions, which implies that endocytic vesicles may contribute to membrane repair[89]. Furthermore, there are several lines of evidence that endocytosis contributes to membrane resealing in non-muscle cell types [56, 57, 89]. In neurons, axotomy induces rapid formation and accumulation of endocytic vesicles near the proximal cut end, and these endocytic vesicles form a semi-permeable barrier that rapidly excludes extracellular dyes [56]. In CHO cells, membrane lesions induced by SLO-toxin (which forms transmembrane pores within the plasma membrane) are rapidly endocytosed into caveolin-containing vesicles, a process that is required for resealing the plasma membrane [62]. Furthermore, enzyme release as a result of lysosomal exocytosis may facilitate endocytosis at membrane lesions, a process which contributes to membrane repair in NRK cells [78]. Despite growing evidence that endocytosis contributes to membrane repair in non-muscle cells, there have been few studies examining the role of endocytosis in membrane repair in adult skeletal muscle.

Myoferlin, a homolog of dysferlin, is highly expressed in developing cardiac and skeletal muscle and is expressed to somewhat lower levels in adult skeletal and cardiac muscle. Loss of myoferlin expression in mice results in an early-onset muscle myopathy with the most prominent phenotype being impaired cell-cell fusion during myogenesis, a phenotype which has also been reported for dysferlin-deficient muscle cells *in-vitro* and

*in-vivo*. Double knockout of myoferlin and dysferlin enhances the dysferlin-deficient phenotype, and deficiency of dysferlin, myoferlin, or both results in alterations in t-tubule morphology, suggesting that both myoferlin and dysferlin contribute to t-tubule stability or maintenance in skeletal muscle. Perhaps most intriguingly, membrane repair capacity is impaired in myoferlin-deficient endothelial cells [131] and over-expression of myoferlin may enhance membrane repair in dysferlin-deficient skeletal muscle [133]. Although the exact role for myoferlin in membrane resealing is not known, it is reasonable to suspect that myoferlin may contribute to membrane resealing by regulating damage-induced endocytosis. Surprisingly, there have been no studies addressing whether myoferlin is required for membrane resealing in adult skeletal muscle fibers, nor have there been any studies addressing the effect of myoferlin-deficiency on dysferlin function in membrane repair. Our data suggests that membrane damage induces rapid endocytosis of dysferlin into cytoplasmic vesicles, which may be critical for membrane resealing. Therefore, it is reasonable that myoferlin may contribute to membrane resealing in skeletal muscle by facilitating endocytosis of dysferlin into dysferlin-containing vesicles for subsequent use in membrane repair (Fig 4-3A).

Our data from chapter 3 suggests that skeletal muscle fibers from dysf-pHGFP TG mice can be used to track the endocytosis of dysferlin from the plasma membrane following wounding, providing a straightforward assay to assess endocytosis of dysferlin following wounding. Crossing the dysf-pH TG mouse described in chapter 3 to a myoferlin KO mouse to generate a mouse expressing dysf-pHGFP on a myoferlin-null background (myofKO pHGFP TG) mouse could be used to directly test the hypothesis that myoferlin is required for endocytosis of dysferlin following membrane damage in

adult skeletal muscle fibers. To test this hypothesis, muscle fibers could be isolated from myofKO TG mice and wild-type TG controls, subjected to our laser wounding assay, and analyzed for the pHGFP response to wounding (Fig 4-3C). If myoferlin is required for endocytosis of dysferlin following wounding, then the rapid decrease in "total" pHGFP fluorescence would be reduced in myofKO pHGFP TG skeletal muscle fibers following wounding. Crossing the aforementioned dysferlin-mCh transgenic mouse to a myofKO mouse to generate a myofKO mChTG mouse would avoid the limitations associated with pHGFP (outlined previously) and allow for direct examination of dysferlin vesicle formation, and behavior of damage-induced dysferlin-containing vesicles following wounding in myoferlin-null muscle fibers (Fig 4-3D). Finally, FM1-43 uptake following laser induced wounding in myofKO and wild-type adult muscle fibers could be examined to determine whether myoferlin is required for membrane resealing in muscle (Fig 4-3B).

Dysferlin and myoferlin share high structural homology (Fig 1-1), as well as lipid binding/fusion properties *in-vitro* [42]. Dysferlin has been shown to dimerize *in-vitro* and in live-cells [134], which raises the possibility that myoferlin-dysferlin heterodimerization could occur. Such an interaction could efficiently couple endocytosis and plug formation at membrane lesions. This hypothesis could be tested biochemically by expressing epitope tagged dysferlin and myoferlin constructs and using co-immunoprecipitation followed by western blotting. Additionally, the in-vivo relevance of these interactions could be directly examined using FRET-based imaging of acutely transfected myotubes expressing fluorophore labeled myoferlin and dysferlin constructs, as has been used previously to identify dimerization of dysferlin in live cells [134].

**Regulation of dysferlin-mediated membrane repair by cortical actin in adult skeletal muscle:**

In chapter 3, the contribution of sarcolemma-derived dysferlin to membrane repair in adult skeletal muscle was examined, which revealed that local dysferlin-containing sarcolemma is actively pulled into membrane lesions, resulting in the formation of a stable dysferlin-rich structure at membrane lesions. Based on the rapid and stable nature of nascent dysferlin-containing structures, it was proposed that the actin-cytoskeleton could play a critical role in recruitment of dysferlin-containing sarcolemma to membrane lesions. Pharmacological disruption of the actin-cytoskeleton significantly reduced dysf-pHGFP accumulation at membrane lesions, indicating that the actin cytoskeleton is critical for dysferlin-recruitment at membrane lesions and potentially resealing in adult skeletal muscle fibers. These findings are consistent with a growing body of evidence in single celled drosophila larvae and xenopus oocytes [84, 86], which indicates that the cortical actin cytoskeleton is a critical regulator of membrane repair. In the drosophila model, membrane damage induces the formation of a "contractile ring" composed of F-actin and non-muscle myosin II (nmII) which contracts to pull both the PM and intracellular vesicles into a repair plug [84]. Furthermore, it was recently shown that putative repair protein MG53 is actively transported to membrane lesions in an non-muscle myosin II (nmII) dependent manner, and pharmacological inhibition of nmII impaired membrane resealing in adult skeletal muscle [67]. Although our data supports a role for cortical actin in dysferlin-mediated

membrane resealing in skeletal muscle, our ability to selectively disrupt the cortical actin cytoskeleton is limited by the fact that cytochalasin D is not necessarily specific for cortical actin. Therefore, examination of genetic mutants for specific cortical actin isoforms could be useful in determining the precise molecular components of the cytoskeleton required for membrane repair in muscle.

Three major isoforms of actin are expressed in adult skeletal muscle, and which specific isoform is critical for membrane repair remains an open question [135]. The α-skeletal actin isoform is a major component of the thin filament of the contractile sarcomere, and regulates force production through direct interactions with the myosin containing thick filament [135]. β- and γ-actin are the major a components of the sub-sarcolemmal actin cortex and knockout of either gene results in progressive muscle myopathy [136, 137]. β-actin deletion results in decreased dystrophin expression and susceptibility to eccentric contraction, consistent with the primary deficits associated with impaired DGC function [136]. In contrast, γ-actin knockouts display a skeletal muscle myopathy independently of any effects on the DGC, and do not display susceptibility to eccentric contraction or impaired sarcolemmal integrity at rest, consistent with the effects of dysferlin-deficiency on muscle function [137]. Furthermore, γ-actin expression is increased in dystrophin-deficient muscle and may protect dystrophin-muscle from contraction induced damage [138, 139]. Our data indicates that disruption of either γ or β actin may result in impaired accumulation of dysferlin-containing membrane at membrane lesions, as well as membrane resealing in adult skeletal muscle fibers. Although γ-actin deficiency does not recapitulate the exact phenotype of dysferlin-null mice (ie. high CK), the development of myopathy in the

absence of DGC defects, relative insensitivity to lengthening contraction, and mild sarcolemmal permeability at rest suggest that the loss of γ-actin may result in muscle disease in part through impaired membrane repair [137]. Therefore, it is likely that γ-actin may be a critical regulator of dysferlin-mediated membrane resealing in adult skeletal muscle fibers.

The role of γ-actin in dysferlin-mediated membrane repair could be directly assessed by crossing our dysf-pHGFP TG mouse with muscle-specific γ-actin KO mice to generate a dysf-pHGFP TG mouse on a γ-actin deficient background (γ-KOpHGFP TG ) and subjecting dysf-pHGFP TG and γ-KOpHGFP TG muscle fibers to laser-wounding (Fig 4-4C). Based on our findings in chapter 3, it would be predicted that accumulation of sarcolemmal dysferlin would be markedly reduced in γ-KOpHGFP TG muscle fibers compared to wt-TG control. Examination of total cellular fluorescence following wounding in wt-TG and γ-KOpHGFP TG could be used to examine the effect of γ-actin deficiency on dysferlin endocytosis and formation of dysferlin-containing vesicles following wounding. Although our data indicates that endocytosis of dysferlin and formation of damage-induced dysferlin-containing vesicles is not affected by pharmacological disruption of the actin cytoskeleton, our data does not rule out whether cytoskeletal actin (γ-actin) regulates any downstream functions of dysferlin-containing vesicles such as accumulation of dysferlin-containing vesicles at membrane lesions in adult skeletal muscle fibers. Crossing γ-KO mice to the dysf-mCh transgenic described above would generate a constitutively fluorescent dysferlin reporter on a γ-actin deficient background (γ-KOmCh TG). This model could then be used to examine the behavior of damage-induced dysferlin-containing vesicles following membrane

disruption in live adult skeletal muscle fibers (Fig 4-4D). If γ-actin is required for accumulation of dysferlin-containing vesicles, one would expect that the γ-KOmCh TG fibers would show reduced vesicle accumulation at membrane lesions compared with the mChTG control. FM1-43 uptake following laser induced wounding in wt and γ-KO muscle fibers could be used to determine whether γ-actin and γ-actin-dependent recruitment of dysferlin-containing sarcolemma at membrane lesions is required for membrane resealing in adult skeletal muscle fibers (Fig 4-4B). Although the data presented in chapter 3 supports the overall model that the cortical actin cytoskeleton is critical for membrane resealing in adult skeletal muscle, the proposed experiments would provide strong evidence that the cortical actin cytoskeleton, and specifically γ-actin, is required for membrane resealing in adult skeletal muscle.

**Interaction of lysosomes and dysferlin-containing membranes and role of lysosomes in membrane repair in skeletal muscle:**

Although much of our work was devoted to investigating the behavior of dysferlin-containing membranes during membrane repair, the previously identified role for lysosomes in membrane resealing in non-muscle cell types led us to examine the interaction between dysferlin-containing vesicles and lysosomes in skeletal muscle cells [72]. This question was examined by simultaneously tracking the localization and behavior of dysferlin-containing vesicles (dysferlin-eGFP) and lysosomes (LGP120-mCh) in differentiated myotubes prior to and following mechanical wounding. Our data indicates that dysferlin-containing vesicles are distinct from lysosomes in resting cells, but undergo heterotypic fusion following membrane damage. These data indicate that

heterotypic fusion of dysferlin-containing vesicles with lysosomes maybe critical for membrane repair in skeletal muscle. Given the strong appreciation for lysosomal function in membrane resealing in non-muscle cells [72], the role of lysosomes membrane resealing and the relationship between lysosomes and dysferlin-containing membranes in adult muscle cells warrants further examination.

Our findings from chapter 2 support a potential role for lysosomes in membrane resealing in developing myotubes, but a role for lysosomes in membrane repair in adult muscle fibers remains to be demonstrated. Given that most proteins or organelles involved in membrane resealing accumulate at membrane lesions following wounding [18, 51, 90], an examination of lysosomal behavior following wounding in live adult skeletal muscle cells using commercially available dyes such as lysotracker, antibody labeling of endogenous proteins (Lamp-1) or expression of genetic labels (LGP120-mCh) in adult muscle fibers following laser-induced may be informative. FM1-43 uptake in wild-type adult skeletal muscle fibers treated with pharmacological inhibitors of lysosome function, such as GPN [140], could be used to explore the effect of pharmacological disruption of lysosomal function on membrane resealing in adult skeletal muscle fibers. Synaptotagmin-VII is a critical regulator of lysosomal exocytosis and function [141], and fibroblasts isolated from syt-VII knockout mice display impaired membrane resealing [71]. At the molecular level, dysferlin is highly homologous to syt-VII, but whether syt-VII is required for membrane resealing in muscle cells is not known. Examination of membrane resealing could be carried out in primary myotubes or adult skeletal muscle fibers isolated from sytVII-KO mice. If lysosomes are required for membrane resealing in adult skeletal muscle, then both pharmacological and genetic

inhibition of lysosomal function (syt-VII deficiency) should increase uptake of FM1-43 following wounding.

If syt-VII KO muscle fibers show a defect in membrane resealing, one potential explanation could be that loss of syt-VII on lysosomes impairs damage-induced fusion of lysosomes with dysferlin-containing vesicles following wounding. This could be tested by analyzing the behavior of dysferlin-containing vesicles and lysosomes following wounding in myoblasts derived from syt-VII knockout mice acutely transfected with dysferlin-eGFP and LGP120 (similar to chapter 2). If syt-VII on lysosomes is required for the interaction between dysferlin-containing vesicles and lysosomes, then formation of co-labeled vesicles following wounding would be reduced. If dysferlin-containing membranes and syt-VII containing lysosomes are required for membrane resealing in muscle cells, it would be interesting to examine whether there is functional redundancy between these compartments in membrane resealing in skeletal muscle. Our data from chapter 2 indicates that lysosomes may contribute to membrane resealing by fusing with dysferlin-containing vesicles, however, it is also possible that syt-VII containing lysosomes also serve dysferlin-independent role in membrane resealing. To address this, adult muscle fibers isolated from dysferlin-null mice could be treated with pharmacological inhibitors of lysosomal function, and assayed for FM1-43 uptake following wounding. If lysosomes contribute to membrane resealing independently of dysferlin, then pharmacological inhibition of lysosome function should impair membrane resealing to a greater degree than dysferlin-deficiency alone. Alternatively, if inhibition of lysosomal function in dysferlin-deficient muscle fibers does not further impair membrane resealing, it could be concluded that the function of lysosomes in membrane resealing is

entirely dependent on dysferlin in adult skeletal muscle. Furthermore, crossing dysferlin-deficient (A/J mice) to syt-VII KO mice to generate DKO mice would be especially useful, as the effect of combined loss of dysferlin and syt-VII could be examined in-vivo by analyzing H&E stain of skeletal muscle, plasma creatine kinase, central nucleated fibers, and Evan's blue dye uptake. If the muscle phenotype in DKO mice is worse than dysferlin-deficient mice, comparison of FM1-43 uptake in skeletal muscle fibers isolated from dysf-KO, syt-VII KO and DKO mice could be used to assess whether the effect of combined deficiency of dysferlin and syt-VII on the disease phenotype is due to further impaired resealing capacity when both dysferlin and syt-VII are absent.

**Plasma membrane instability and wounding as a common mechanism in the development of muscular dystrophy:**

The exact cellular mechanism by which loss of DGC or DGC-associated proteins result in muscular dystrophy is unclear and remains a critical question to be addressed in the field of muscular dystrophy. It is well known that skeletal muscle fibers from dystrophic human patients and mouse models of muscular dystrophy take up endogenous membrane impermeable molecules such as immunoglobulin (IgG, IgM) or exogenous membrane impermeant molecules such as Evan's blue dye from the blood stream [142, 143]. Further, dystrophic muscles release the cytosolic muscle enzyme creatine kinase (CK) into the blood stream, which is commonly used as an indication of dystrophic pathology [2]. These data indicate that reduced sarcolemma stability or increased permeability may be a common pathway by which mutations in the DGC or DGC-related proteins result in muscular dystrophy. Based on these findings, a model has been

proposed suggesting that the loss of DGC components renders the plasma membrane "unstable" and thus susceptible to contraction induced injury in the form of "microlesions" to the plasma membrane. Consistent with this hypothesis, sarcolemmal permeability to Evan's blue dye is elevated particularly following exercise in several mouse models of muscular dystrophy [13, 18]. However, there are several remaining questions as to the types of sarcolemmal lesions that occur in skeletal muscle as a result of contraction-induced injury in dystrophic skeletal muscles. Although all fibers from DGC-deficient muscles lack functional DGC, uptake of EBD is not uniform and typically occurs in discrete foci containing several muscle fibers, which seems to suggest that only a subset of muscle fibers show increased permeability as a result of membrane wounding [142, 143]. Furthermore, there is little direct evidence that small sarcolemmal lesions occur as a result of contraction in dystrophic skeletal muscle fibers. In fact, Brooks and Claflin 2008 showed that lesions generated in dystrophic muscle following tetanic isometric contraction consisted of calcium-dependent tearing of muscle fibers and formation of a "contraction clot" which suggests that wounds occuring as a result of DGC-deficiency may actually be more catastrophic than previously proposed [14]. Although this study implies that in dystrophic muscle, contraction leads to rupture of the sarcolemma or increased leakiness of the sarcolemma, over-activation of the local sarcomeres due to calcium influx, and subsequent failure of the muscle fiber, the development of sarcolemmal lesions as a result of contraction-induced injury in dystrophic muscle fibers remains to be demonstrated. It is interesting to note that some muscular dystrophy models develop a dystrophic phenotype independent of changes in skeletal muscle membrane permeability. For example, laminin (the extracellular

receptor for dystroglycan within the basal lamina), considered a critical component of the DGC, results in a muscular dystrophy phenotype without an increase in skeletal muscle EBD uptake [143]. Furthermore, dysferlin-deficient mice display a myopathic phenotype with robust CK elevation, even in the absence of considerable EBD uptake (compared to the mdx mouse) [18]. More recently, several laboratories have questioned whether sarcolemmal wounds contribute to development of muscular dystrophy entirely. In this alternative model, stretch-induced calcium channels present on the muscle sarcolemma are increased in mdx skeletal muscle and enhanced stretch-induced calcium influx activates secondary calcium-dependent degredative pathways that contribute to disease progression [144]. This model is supported by findings that pharmacological inhibition of these channels with streptomycin, a stretch-induced calcium channel blocker, partially protects mdx muscles from eccentric contractions in-vitro and following eccentric exercise in-vivo and reduces exercise-induced dye uptake into mdx skeletal muscle fibers following eccentric exercise in-vivo [144]. Furthermore, long-term in-vivo administration of streptomycin into mdx mice reduced dystrophic pathology and decreased EBD uptake into mdx tibialis anterior muscles, indicating that calcium-influx through stretch activated calcium channels may have deleterious effects on mdx skeletal muscle [145]. Therefore, a closer examination of sarcolemma wounding in dystrophic skeletal muscle following contraction in-vitro and following exercise or contraction in-vivo is critical for understanding the effects of DGC mutations on plasma membrane homeostasis and understanding the contribution of sarcolemmal wounding to the development of muscular dystrophy.

Our dysf-pHGFP TG mouse is ideally suited to address this question, as we have shown (chapter 3) that dysf-pHGFP robustly and reproducibly identifies lesions in the plasma membrane in adult skeletal muscle fibers following wounding. Therefore, crossing the dysf-pHGFP TG (heretofore referred to as simply WT-TG) mouse to various models of muscular dystrophy including the mdx (dystrophin-deficient) and dy/dy (laminin-deficient) [146] and alpha-sarcoglycan [147] deficient mice (heretofore referred to as simply "dystrophic" or "DGC-deficient") would generate useful (mdx-TG, dy-TG, aSG-TG, heretofore referred to as "DGC-deficient TG") reporter mice to examine whether sarcolemmal lesions develop as a result of contraction in skeletal muscles from several independent DGC-deficient mouse models and facilitate direct comparison of membrane defects in each model. Adult skeletal muscle fibers could be subjected to contraction in-vitro and simultaneously analyzed for the formation of sarcolemmal lesions (similar to those shown in chapter 3) using live-cell microscopy. Given that dysf-pHGFP is an indirect measure of sarcolemmal permeability, simultaneous analysis of contraction-induced uptake of extracellular dyes or intracellular calcium using a calcium reporter dye could be useful. While isometric contraction is capable of generating muscle damage in dystrophic muscle [14], dystrophic muscle also shows a marked susceptibility to "lengthening", or "eccentric" contractions [116]. Therefore, direct analysis of contraction-induced sarcolemmal lesions developed in response to isometric and eccentric contractions could be useful to explore whether more injurious eccentric contractions are associated with increased incidence of sarcolemmal wounds. Based on the model from Brooks and Claflin 2008, it would be predicted that small sarcolemmal lesions developed following contraction contribute to

elevations in intracellular calcium, "over-activation" and failure of the local cytoskeleton, and fiber "hyper-contraction" [14]. Thus, it would be predicted that the formation of sarcolemmal lesions would also precede fiber hypercontraction, as well as any decrements in force production following eccentric contraction in DGC-deficient skeletal muscle fibers. This could be directly tested in this model by examining the formation of dysf-pHGFP patches following each contraction, and measuring maximal force produced by the muscle fiber following each successive contraction. Furthermore, treatment of WT-TG and mdx-TG muscle fibers (which have been suggested to have increased levels of strecth-activated channels) with streptomycin and subsequent analysis of lesion formation following contraction could be used to analyze the contribution of stretch-activated channel-dependent calcium influx to the development of membrane lesions. If the prevalence of membrane lesions increases with contraction (based on dysf-pHGFP accumulation and calcium indicator fluorescence increases), and the development of lesions is not affected by the presence of streptomycin, then it can concluded that contraction leads to sarcolemmal disruption in dystrophic models likely due to impaired sarcolemmal stability, and not as a secondary consequence of increased stretch-induced calcium channel activation.

In addition to in-vitro analysis of sarcolemmal wounding, these models would also serve as useful tools to explore the development of sarcolemmal lesions in dystrophic muscle in-vivo. It has been assumed that prevalence of sarcolemmal rupture increases in dystrophic muscle following exercise due to the finding that CK is elevated in dystrophic muscle and EBD uptake into skeletal muscle from dystrophic mice increases following exercise [13], but direct examination of membrane lesions in

skeletal muscle following exercise in-vivo has not been definitively shown. Therefore, the presence of sarcolemmal lesions (dysf-pHGFP accumulation) could be examined in longitudinal sections of skeletal muscle from WT-TG mice compared with DGC-deficient TG mice with or without treadmill exercise. Alternatively, development of sarcolemmal lesions could be analyzed in skeletal muscles subjected to in-situ lengthening contraction protocols which would allow examination of "injured" versus "non-injured" muscles from individual mice within each genotype. If the number of sarcolemmal lesions increases in DGC-deficient muscle fibers following exercise or lengthening contraction to a greater extent than in normal skeletal muscle, it could be concluded that development of sarcolemmal wounds following contraction may be a common mechanism by which disruption of the DGC leads to the development of muscular dystrophy. It is unclear whether muscle fibers in dystrophic muscle take up EBD as a result of small sarcolemmal lesions or whether EBD labels a more catastrophic injury such as muscle fibers undergoing a "hyper-contraction clot". Further, whether EBD positive fibers are the only fibers that have undergone sarcolemmal lesions, or whether formation of small sarcolemmal injuries precedes EBD uptake has not been explored. Therefore, analysis of exercise-induced sarcolemmal lesions in WT-TG and DGC-deficient TG mice injected with EBD could be used to examine the relationship between the development of sarcolemmal lesions and the uptake of EBD in-vivo. These findings will have important implications given that uptake of EBD does not appear to be uniform throughout dystrophic muscles [142], and does not occur in skeletal muscle from all models of muscular dystrophy [18, 143]. Thus, an understanding of whether sarcolemmal lesions occur in the absence of EBD uptake may shed light on the

relationship between sarcolemmal wounds and EBD uptake and whether EBD uptake should be used as the sole indicator to identify membrane instability in novel mouse models with a suspected muscular dystrophy phenotype.

A deeper understanding of how sarcolemmal lesions develop in-vivo across a variety of muscular dystrophies is a critical question, as the development of sarcolemmal wounds has been proposed as a unifying mechanism for the development of muscular dystrophy, but has not been directly examined. If development of sarcolemmal lesions is a critical pathway by which genetic mutations lead to muscular dystrophy, then development of therapeutics aimed at protecting the sarcolemma from injury or restoring plasma membrane integrity following wounding could be an effective therapeutic approach. The studies outlined above would directly examine for the first time whether the development of sarcolemmal lesions following contraction-induced injury is a common occurrence in dystrophic muscle, and would lead to a deeper understanding of plasma membrane defects associated with DGC-deficiency in skeletal muscle.

## Conclusions

Membrane resealing is an evolutionarily conserved process by which cells repair even the most severe wounds in the plasma membrane. Impaired membrane resealing results in muscle disease [18, 52], which makes enhancement of membrane resealing an attractive therapeutic target [95]. However, the mechanism by which the sarcolemma is resealed in normal muscle is largely unknown. The goal of our study was to examine the behavior of dysferlin-containing membranes following wounding in skeletal muscle,

and determine the contribution of the cytoskeleton to dysferlin-mediated membrane repair in skeletal muscle. Our data support the novel model that recruitment of sarcolemma-derived dysferlin and formation of damage-induced vesicles contribute contribute to membrane repair in skeletal muscle, and demonstrate for the first time, that dynamic interactions between dysferlin-containing membranes and the cytoskeleton are critical for membrane repair in adult skeletal muscle. In addition to the mechanistic analysis of membrane repair in skeletal muscle, our novel dysf-pHGFP TG mouse model will serve as a useful tool to examine membrane wounding and activation of membrane repair in diseases of skeletal muscle, including the muscular dystrophies. Our data provide important mechanistic evidence that will serve as the basis for understanding dysferlin-mediated membrane resealing, and the development of novel therapeutics for enhancing membrane repair to treat muscle diseases.

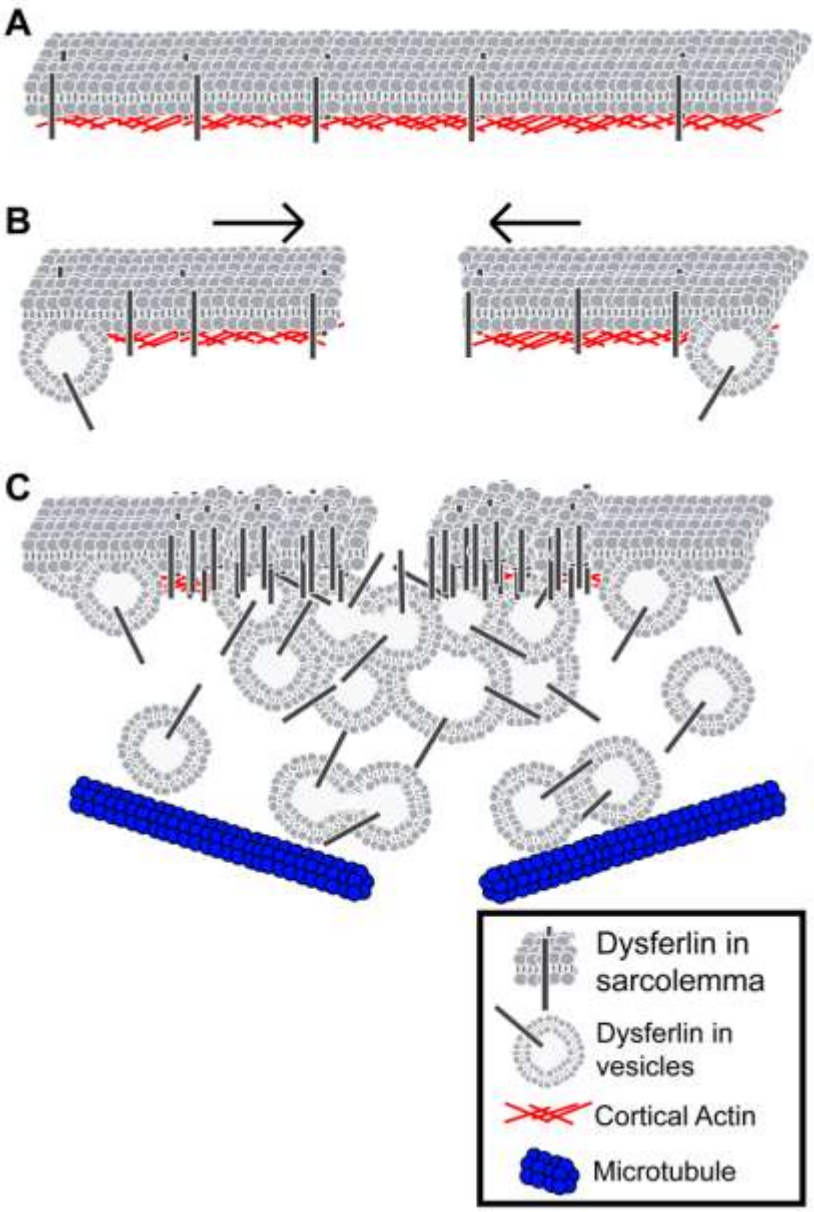



**Figure 4-1. Proposed model for membrane resealing in skeletal muscle cells.**
**A)** Dysferlin localizes to the plasma membrane and transverse tubules in resting striated muscle cells. **B)** After wounding, cortical actin (red) pulls adjacent dysferlin containing sarcolemma toward membrane lesions (arrows), constricting the size of the wound. Simultaneously, dysferlin from distal portions of the fiber is endocytosed into dysferlin-containing vesicles. **C)** Dysferlin-containing vesicles undergo vesicle-vesicle fusion on microtubules (blue) to form large dysferlin-containing vesicles that "plug" the lesion via interactions with the stable dysferlin-rich "active-zone".

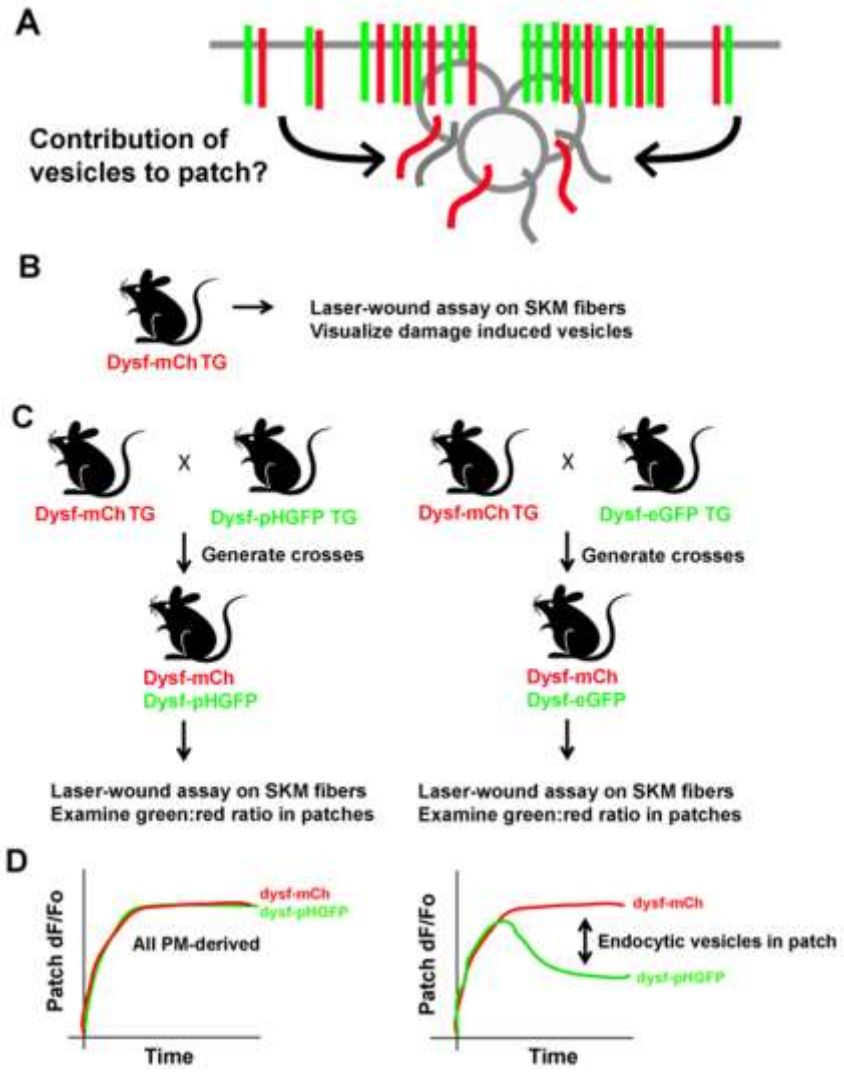



**Figure 4-2. Contribution of dysferlin-containing vesicles to membrane resealing in adult skeletal muscle. A)** Proposed model to examine the contribution of damage-induced dysferlin-containing vesicles to membrane repair in adult skeletal muscle. Dysf-mCherry is constitutively fluorescent, and will reveal all dysferlin populations at nascent patches. Dysf-pHGFP is not fluorescent within acidic vesicles. The combination of both labels will provide relative estimate of dysferlin at the PM (red and green) and dysferlin only in vesicles (red alone). **B)** Laser wounding of dysf-mCH TG fibers will be used to examine vesicle behavior. **C)** Crosses between Dysf-mCh and Dysf-pHGFP (left) and Dysf-mCh and Dysf-eGFP (right). **D)** Potential outcomes of laser wounding experiments outlined in **C**. If dysferlin-containing vesicles contribute to patch formation, relative red:green intensity within the patch will be shifted toward red>green due to pHGFP quenching.

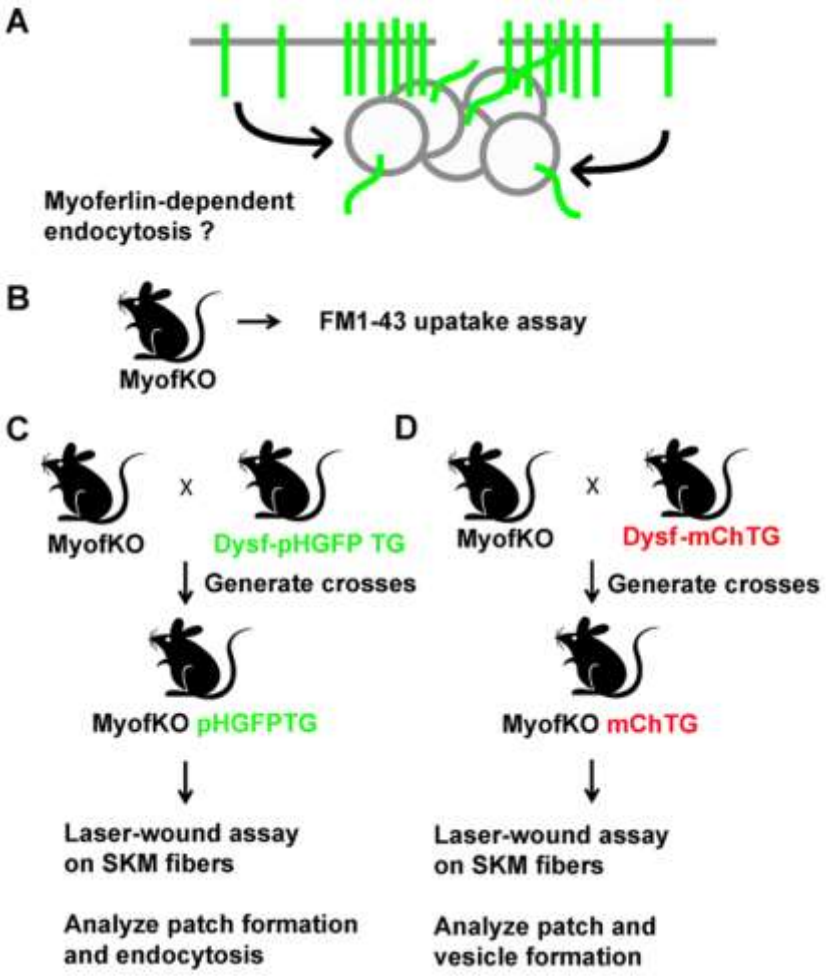



**Figure 4-3. Potential role for myoferlin in damage-induced endocytosis of dysferlin and membrane repair in adult skeletal muscle cells. A)** Simplified schematic of the role for myoferlin in damage-induced endocytosis of dysferlin following wounding in adult skeletal muscle fibers. **B)** FM1-43 dye uptake assay could be used to assess membrane resealing in isolated myof-KO skeletal muscle fibers. **C)** Genetic cross of myof-KO and dysf-pHGFP mice to examine endocytosis of dysferlin in myoferlin-deficient muscle. **D)** Genetic cross of myof-KO and dysf-mCh to examine the formation of dysferlin-containing vesicles following membrane wounding in myoferlin-deficient muscle.

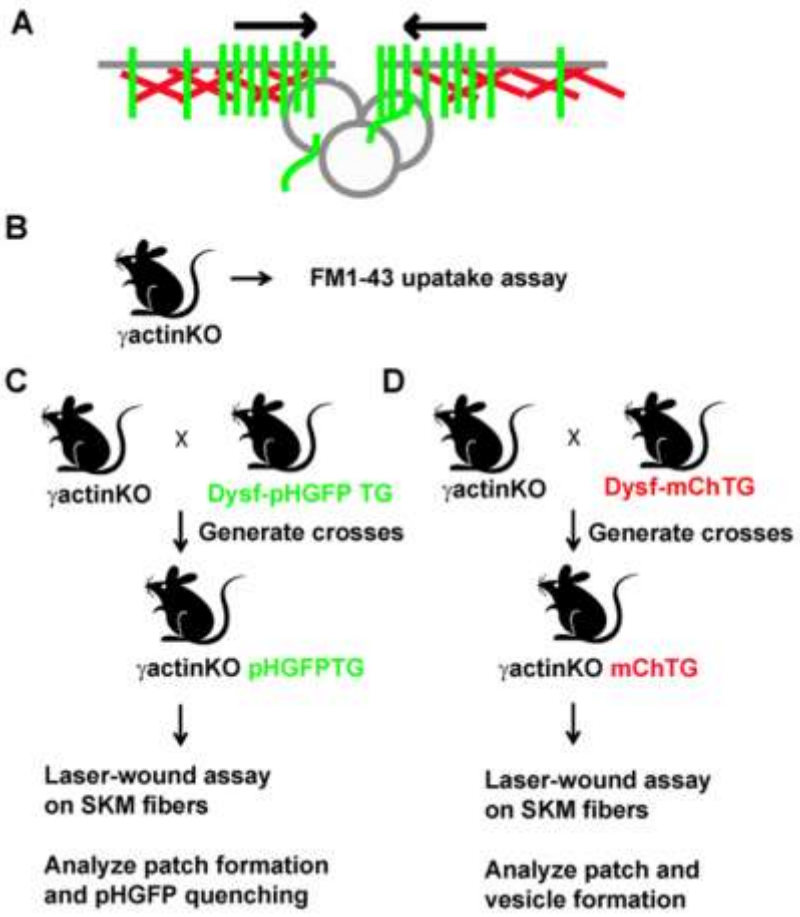



**Figure 4-4. Proposed role for gamma actin in damage-induced recruitment of sarcolemmal dysferlin and membrane resealing adult skeletal muscle fibers. A)** Simplified schematic of the role for gamma actin in damage-induced patch-formation following wounding in adult skeletal muscle fibers. **B)** FM1-43 dye uptake assay could be used to assess membrane resealing in isolated gamma actin deficient skeletal muscle fibers. **C)** Genetic cross of gamma actin-KO and dysf-pHGFP mice to examine endocytosis of dysferlin in gamma actin-deficient muscle. **D)** Genetic cross of gamma actin-KO and dysf-mCh to examine the formation of dysferlin-containing vesicles following membrane wounding in gamma actin-deficient muscle.

## REFERENCES


1. Mercuri, E. and F. Muntoni, *Muscular dystrophies.* The Lancet. **381**(9869): p. 845-860.
2. McNally, E.M. and P. Pytel, *Muscle Diseases: The Muscular Dystrophies.* Annual Review of Pathology: Mechanisms of Disease, 2007. **2**(1): p. 87-109.
3. Mayer, U., et al., *Absence of integrin alpha-7 causes a novel form of muscular dystrophy.* Nature Genetics, 1997. **17**(3): p. 318-2323.
4. Michele, D.E., et al., *Post-translational disruption of dystroglycan-ligand interactions in congenital muscular dystrophies.* Nature, 2002. **418**(6896): p. 417-421.
5. Bonnemann, C.G., et al., *Beta-sarcoglycan (A3b) mutations cause autosomal recessive muscular dystrophy with loss of the sarcoglycan complex.* Nature Genetics, 1995. **11**(3): p. 266-273.
6. Muñoz-Mármol, A.M., et al., *A dysfunctional desmin mutation in a patient with severe generalized myopathy.* Proceedings of the National Academy of Sciences, 1998. **95**(19): p. 11312-11317.
7. Udd, B., et al., *Titinopathies and extension of the M-line mutation phenotype beyond distal myopathy and LGMD2J.* Neurology, 2005. **64**(4): p. 636-642.
8. McNally, E.M., et al., *Caveolin-3 in Muscular Dystrophy.* Human Molecular Genetics, 1998. **7**(5): p. 871-877.
9. Gumerson, J.D. and D.E. Michele, *The Dystrophin-Glycoprotein Complex in the Prevention of Muscle Damage.* Journal of Biomedicine and Biotechnology, 2011. **2011**: p. 13.
10. Koenig, M., A.P. Monaco, and L.M. Kunkel, *The complete sequence of dystrophin predicts a rod-shaped cytoskeletal protein.* Cell, 1988. **53**(2): p. 219-228.
11. Ibraghimov-Beskrovnaya, O., et al., *Primary structure of dystrophin-associated glycoproteins linking dystrophin to the extracellular matrix.* Nature, 1992. **355**(6362): p. 696-702.

12. Hamer, P.W., et al., *Evans Blue Dye as an in vivo marker of myofibre damage: optimising parameters for detecting initial myofibre membrane permeability.* Journal of Anatomy, 2002. **200**(1): p. 69-79.
13. Wooddell, C.I., et al., *Use of evans blue dye to compare limb muscles in exercised young and old mdx mice.* Muscle & Nerve, 2010. **41**(4): p. 487-499.
14. Claflin, D.R. and S.V. Brooks, *Direct observation of failing fibers in muscles of dystrophic mice provides mechanistic insight into muscular dystrophy.* American Journal of Physiology - Cell Physiology, 2008. **294**(2): p. C651-C658.
15. Petrof, B.J., et al., *Dystrophin protects the sarcolemma from stresses developed during muscle contraction.* Proceedings of the National Academy of Sciences, 1993. **90**(8): p. 3710-3714.
16. Anderson, L.V.B., et al., *Dysferlin is a Plasma Membrane Protein and is Expressed Early in Human Development.* Human Molecular Genetics, 1999. **8**(5): p. 855-861.
17. Liu, J., et al., *Dysferlin, a novel skeletal muscle gene, is mutated in Miyoshi myopathy and limb girdle muscular dystrophy.* Nat Genet, 1998. **20**(1): p. 31-36.
18. Bansal, D., et al., *Defective membrane repair in dysferlin-deficient muscular dystrophy.* Nature, 2003. **423**(6936): p. 168-172.
19. Weiler, T., et al., *Identical Mutation in Patients with Limb Girdle Muscular Dystrophy Type 2B Or Miyoshi Myopathy Suggests a Role for Modifier Gene(s).* Human Molecular Genetics, 1999. **8**(5): p. 871-877.
20. Piccolo, F., et al., *Intracellular accumulation and reduced sarcolemmal expression of dysferlin in limb–girdle muscular dystrophies.* Annals of Neurology, 2000. **48**(6): p. 902-912.
21. Mafalda Cacciottolo, G.N., Stefania Aurino, Imma Rosaria Caserta, Marina Fanin, Luisa Politano, Carlo Minetti, Enzo Ricci, Giulio Piluso, Corrado Angelini, and Vincenzo Nigro, *Muscular dystrophy with marked Dysferlin deficiency is consistently caused by primary dysferlin gene mutations.* European journal of human genetics, 2011. **19**: p. 974-980.

22. Nguyen, K., et al., *Phenotypic study in 40 patients with dysferlin gene mutations: High frequency of atypical phenotypes.* Archives of Neurology, 2007. **64**(8): p. 1176-1182.
23. Illarioshkin, S.N., et al., *Identical dysferlin mutation in limb-girdle muscular dystrophy type 2B and distal myopathy.* Neurology, 2000. **55**(12): p. 1931-1933.
24. Rosales, X.Q., et al., *Cardiovascular magnetic resonance of cardiomyopathy in limb girdle muscular dystrophy 2B and 2I.* Journal of cardiovascular magnetic resonance : official journal of the Society for Cardiovascular Magnetic Resonance, 2011. **13**: p. 39.
25. Angelini, C., W. Grisold, and V. Nigro, *Diagnosis by protein analysis of dysferlinopathy in two patients mistaken as polymyositis.* Acta Myologica, 2011. **30**(3): p. 185-187.
26. Angelini C, P.E., Gaiani A, Bortolussi L, Borsato C., *Dysferlinopathy course and sportive activity: clues for possible treatment.* Acta Myologica, 2011. **2**: p. 127-132.
27. Biggar, W.D., et al., *Deflazacort treatment of Duchenne muscular dystrophy.* The Journal of Pediatrics, 2001. **138**(1): p. 45-50.
28. Walter, M., et al., *Treatment of dysferlinopathy with deflazacort: a double-blind, placebo-controlled clinical trial.* Orphanet Journal of Rare Diseases, 2013. **8**(1): p. 26.
29. Azakir, B.A., et al., *Modular Dispensability of Dysferlin C2 Domains Reveals Rational Design for Mini-dysferlin Molecules.* Journal of Biological Chemistry, 2012. **287**(33): p. 27629-27636.
30. Hornsey, M.A., et al., *Muscular dystrophy in dysferlin-deficient mouse models.* Neuromuscular Disorders, 2013. **23**(5): p. 377-387.
31. Ho, M., et al., *Disruption of muscle membrane and phenotype divergence in two novel mouse models of dysferlin deficiency.* Human Molecular Genetics, 2004. **13**(18): p. 1999-2010.
32. Bittner, R.E., et al., *Dysferlin deletion in SJL mice (SJL-Dysf) defines a natural model for limb girdle muscular dystrophy 2B.* Nat Genet, 1999. **23**(2): p. 141-142.

33. Chiu, Y.-H., et al., *Attenuated muscle regeneration is a key factor in dysferlin-deficient muscular dystrophy.* Human Molecular Genetics, 2009. **18**(11): p. 1976-1989.
34. Han, R., et al., *Dystrophin deficiency exacerbates skeletal muscle pathology in dysferlin-null mice.* Skeletal Muscle, 2011. **1**(1): p. 35.
35. Dellorusso, C., et al., *Tibialis anterior muscles in <i>mdx</i> mice are highly susceptible to contraction-induced injury.* Journal of Muscle Research and Cell Motility, 2001. **22**(5): p. 467-475.
36. Roche, J.A., et al., *Extensive mononuclear infiltration and myogenesis characterize recovery of dysferlin-null skeletal muscle from contraction-induced injuries.* American Journal of Physiology - Cell Physiology, 2010. **298**(2): p. C298-C312.
37. Bashir, R., et al., *A gene related to Caenorhabditis elegans spermatogenesis factor fer-1 is mutated in limb-girdle muscular dystrophy type 2B.* Nat Genet, 1998. **20**(1): p. 37-42.
38. Lek, A., et al., *Ferlins: Regulators of Vesicle Fusion for Auditory Neurotransmission, Receptor Trafficking and Membrane Repair.* Traffic, 2012. **13**(2): p. 185-194.
39. Lek, A., et al., *Phylogenetic analysis of ferlin genes reveals ancient eukaryotic origins.* BMC Evolutionary Biology, 2010. **10**(1): p. 231.
40. Washington, N.L. and S. Ward, *FER-1 regulates Ca2+-mediated membrane fusion during C. elegans spermatogenesis.* Journal of Cell Science, 2006. **119**(12): p. 2552-2562.
41. Duncker, S.V., et al., *Otoferlin Couples to Clathrin-Mediated Endocytosis in Mature Cochlear Inner Hair Cells.* The Journal of Neuroscience, 2013. **33**(22): p. 9508-9519.
42. Davis, D.B., et al., *Calcium-sensitive Phospholipid Binding Properties of Normal and Mutant Ferlin C2 Domains.* Journal of Biological Chemistry, 2002. **277**(25): p. 22883-22888.
43. Marty, N.J., et al., *The C2 Domains of Otoferlin, Dysferlin, and Myoferlin Alter the Packing of Lipid Bilayers.* Biochemistry, 2013. **52**(33): p. 5585-5592.

44. de Morree, A., et al., *Dysferlin regulates cell adhesion in human monocytes.* Journal of Biological Chemistry, 2013.
45. Sharma, A., et al., *A New Role for the Muscle Repair Protein Dysferlin in Endothelial Cell Adhesion and Angiogenesis.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2010. **30**(11): p. 2196-2204.
46. Klinge, L., et al., *Dysferlin associates with the developing T-tubule system in rodent and human skeletal muscle.* Muscle and Nerve, 2010. **41**(2): p. 166-173.
47. Belanto, J.J., et al., *Dexamethasone induces dysferlin in myoblasts and enhances their myogenic differentiation.* Neuromuscular disorders : NMD, 2010. **20**(2): p. 111-121.
48. Demonbreun, A.R., et al., *Impaired muscle growth and response to insulin-like growth factor 1 in dysferlin-mediated muscular dystrophy.* Human Molecular Genetics, 2011. **20**(4): p. 779-789.
49. Klinge, L., et al., *From T-tubule to sarcolemma: damage-induced dysferlin translocation in early myogenesis.* The FASEB Journal, 2007. **21**(8): p. 1768-1776.
50. Demonbreun, A.R., et al., *Dysferlin and Myoferlin Regulate Transverse Tubule Formation and Glycerol Sensitivity.* The American Journal of Pathology, 2013.
51. Cai, C., et al., *MG53 nucleates assembly of cell membrane repair machinery.* Nat Cell Biol, 2009. **11**(1): p. 56-64.
52. Cai, C., et al., *Membrane Repair Defects in Muscular Dystrophy Are Linked to Altered Interaction between MG53, Caveolin-3, and Dysferlin.* Journal of Biological Chemistry, 2009. **284**(23): p. 15894-15902.
53. Lennon, N.J., et al., *Dysferlin Interacts with Annexins A1 and A2 and Mediates Sarcolemmal Wound-healing.* Journal of Biological Chemistry, 2003. **278**(50): p. 50466-50473.
54. Tamada, M., et al., *Two distinct modes of myosin assembly and dynamics during epithelial wound closure.* The Journal of Cell Biology, 2007. **176**(1): p. 27-33.
55. McNeil, P.L. and S. Ito, *Gastrointestinal cell plasma membrane wounding and resealing in vivo.* Gastroenterology, 1989. **96**(5): p. 1238-1248.

56. Eddleman, C.S., et al., *Endocytotic Formation of Vesicles and Other Membranous Structures Induced by Ca2+ and Axolemmal Injury.* The Journal of Neuroscience, 1998. **18**(11): p. 4029-4041.
57. Eddleman, C.S., et al., *Repair of plasmalemmal lesions by vesicles.* Proceedings of the National Academy of Sciences of the United States of America, 1997. **94**(9): p. 4745-4750.
58. Fishman, H.M. and G.D. Bittner, *Vesicle-Mediated Restoration of a Plasmalemmal Barrier in Severed Axons.* Physiology, 2003. **18**(3): p. 115-118.
59. Spira, M.E., D. Benbassat, and A. Dormann, *Resealing of the proximal and distal cut ends of transected axons: Electrophysiological and ultrastructural analysis.* Journal of Neurobiology, 1993. **24**(3): p. 300-316.
60. Yawo, H. and M. Kuno, *Calcium dependence of membrane sealing at the cut end of the cockroach giant axon.* The Journal of Neuroscience, 1985. **5**(6): p. 1626-1632.
61. Han, R., et al., *Dysferlin-mediated membrane repair protects the heart from stress-induced left ventricular injury.* The Journal of Clinical Investigation, 2007. **117**(7): p. 1805-1813.
62. Idone, V., et al., *Repair of injured plasma membrane by rapid Ca2+-dependent endocytosis.* The Journal of Cell Biology, 2008. **180**(5): p. 905-914.
63. Huynh, C., et al., *Defective lysosomal exocytosis and plasma membrane repair in Chediak–Higashi/beige cells.* Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(48): p. 16795-16800.
64. Bi, G.Q., J.M. Alderton, and R.A. Steinhardt, *Calcium-regulated exocytosis is required for cell membrane resealing.* The Journal of Cell Biology, 1995. **131**(6): p. 1747-1758.
65. Togo, T., T.B. Krasieva, and R.A. Steinhardt, *A Decrease in Membrane Tension Precedes Successful Cell-Membrane Repair.* Molecular Biology of the Cell, 2000. **11**(12): p. 4339-4346.
66. Han, R., et al., *Basal lamina strengthens cell membrane integrity via the laminin G domain-binding motif of α-dystroglycan.* Proceedings of the National Academy of Sciences, 2009. **106**(31): p. 12573-12579.

67. Lin, P., et al., *Nonmuscle myosin IIA facilitates vesicle trafficking for MG53-mediated cell membrane repair.* The FASEB Journal, 2012. **26**(5): p. 1875-1883.
68. McNeil, P.L., K. Miyake, and S.S. Vogel, *The endomembrane requirement for cell surface repair.* Proceedings of the National Academy of Sciences, 2003. **100**(8): p. 4592-4597.
69. McNeil, P.L., et al., *Patching plasma membrane disruptions with cytoplasmic membrane.* Journal of Cell Science, 2000. **113**(11): p. 1891-1902.
70. Terasaki, M., K. Miyake, and P.L. McNeil, *Large Plasma Membrane Disruptions Are Rapidly Resealed by Ca2+-dependent Vesicle–Vesicle Fusion Events.* The Journal of Cell Biology, 1997. **139**(1): p. 63-74.
71. Chakrabarti, S., et al., *Impaired membrane resealing and autoimmune myositis in synaptotagmin VII–deficient mice.* The Journal of Cell Biology, 2003. **162**(4): p. 543-549.
72. Reddy, A., E.V. Caler, and N.W. Andrews, *Plasma Membrane Repair Is Mediated by Ca2+-Regulated Exocytosis of Lysosomes.* Cell, 2001. **106**(2): p. 157-169.
73. Shen, S.S., et al., *Molecular Regulation of Membrane Resealing in 3T3 Fibroblasts.* Journal of Biological Chemistry, 2005. **280**(2): p. 1652-1660.
74. Lee, B., K. McKenna, and J. Bramhall, *Kinetic studies of human erythrocyte membrane resealing.* Biochimica et Biophysica Acta (BBA) - Biomembranes, 1985. **815**(1): p. 128-134.
75. McNeil, P.L. and M.M. Baker, *Cell surface events during resealing visualized by scanning-electron microscopy.* Cell and Tissue Research, 2001. **304**(1): p. 141-146.
76. Steinhardt, R., G. Bi, and J. Alderton, *Cell membrane resealing by a vesicular mechanism similar to neurotransmitter release.* Science, 1994. **263**(5145): p. 390-393.
77. Bi, G.-Q., et al., *Kinesin- and Myosin-driven Steps of Vesicle Recruitment for Ca2+-regulated Exocytosis.* The Journal of Cell Biology, 1997. **138**(5): p. 999-1008.

78. Tam, C., et al., *Exocytosis of acid sphingomyelinase by wounded cells promotes endocytosis and plasma membrane repair.* The Journal of Cell Biology, 2010. **189**(6): p. 1027-1038.
79. Eddleman, C.S., et al., *Repair of plasmalemmal lesions by vesicles.* Proceedings of the National Academy of Sciences, 1997. **94**(9): p. 4745-4750.
80. Erez, H., et al., *Formation of microtubule-based traps controls the sorting and concentration of vesicles to restricted sites of regenerating neurons after axotomy.* The Journal of Cell Biology, 2007. **176**(4): p. 497-507.
81. Fletcher, D.A. and R.D. Mullins, *Cell mechanics and the cytoskeleton.* Nature, 2010. **463**(7280): p. 485-492.
82. Togo, T., *Disruption of the plasma membrane stimulates rearrangement of microtubules and lipid traffic toward the wound site.* J Cell Sci, 2006. **119**(13): p. 2780-2786.
83. Togo, T. and R.A. Steinhardt, *Nonmuscle Myosin IIA and IIB Have Distinct Functions in the Exocytosis-dependent Process of Cell Membrane Repair.* Mol. Biol. Cell, 2004. **15**(2): p. 688-695.
84. Abreu-Blanco, M.T., J.M. Verboon, and S.M. Parkhurst, *Cell wound repair in Drosophila occurs through three distinct phases of membrane and cytoskeletal remodeling.* The Journal of Cell Biology, 2011. **193**(3): p. 455-464.
85. Bement, W.M., C.A. Mandato, and M.N. Kirsch, *Wound-induced assembly and closure of an actomyosin purse string in Xenopus oocytes.* Current biology : CB, 1999. **9**(11): p. 579-587.
86. Abreu-Blanco, Maria T., Jeffrey M. Verboon, and Susan M. Parkhurst, *Coordination of Rho Family GTPase Activities to Orchestrate Cytoskeleton Responses during Cell Wound Repair.* Current biology : CB, 2014.
87. Marg, A., et al., *Sarcolemmal Repair Is a Slow Process and Includes EHD2.* Traffic, 2012. **13**(9): p. 1286-1294.
88. Parton, R.G. and M.A. del Pozo, *Caveolae as plasma membrane sensors, protectors and organizers.* Nature Reviews Molecular Cell Biology, 2013. **14**(2): p. 98-112.

89. Corrotte, M., et al., *Caveolae internalization repairs wounded cells and muscle fibers.* eLife, 2013. **2**.
90. Roostalu, U. and U. Strähle, *In Vivo Imaging of Molecular Interactions at Damaged Sarcolemma.* Developmental cell, 2012. **22**(3): p. 515-529.
91. Fuson, K., et al., *Alternate Splicing of Dysferlin C2A Confers Ca2+-Dependent and Ca2+-Independent Binding for Membrane Repair.* Structure, (0).
92. Matsuda C, et al., *The C2A domain in dysferlin is important for association with MG53 (TRIM72).* PLoS Currents, 2012.
93. Hernández-Deviez, D.J., et al., *Aberrant dysferlin trafficking in cells lacking caveolin or expressing dystrophy mutants of caveolin-3.* Human Molecular Genetics, 2006. **15**(1): p. 129-142.
94. Azakir, B.A., et al., *Dysferlin Interacts with Tubulin and Microtubules in Mouse Skeletal Muscle.* PLoS ONE, 2010. **5**(4): p. e10122.
95. Weisleder, N., et al., *Recombinant MG53 Protein Modulates Therapeutic Cell Membrane Repair in Treatment of Muscular Dystrophy.* Science Translational Medicine, 2012. **4**(139): p. 139ra85.
96. Michele, D.E. and K.P. Campbell, *Dystrophin-Glycoprotein Complex: Post-translational Processing and Dystroglycan Function.* Journal of Biological Chemistry, 2003. **278**(18): p. 15457-15460.
97. Jaiswal, J.K., et al., *Patients with a Non-dysferlin Miyoshi Myopathy have a Novel Membrane Repair Defect.* Traffic, 2007. **8**(1): p. 77-88.
98. Demonbreun, A.R., et al., *Impaired muscle growth and response to insulin-like growth factor 1 in dysferlin-mediated muscular dystrophy.* Human Molecular Genetics, 2010.
99. Togo, T., *Disruption of the plasma membrane stimulates rearrangement of microtubules and lipid traffic toward the wound site.* Journal of Cell Science, 2006. **119**(13): p. 2780-2786.
100. Huang, Y., et al., *AHNAK, a novel component of the dysferlin protein complex, redistributes to the cytoplasm with dysferlin during skeletal muscle regeneration.* The FASEB Journal, 2007. **21**(3): p. 732-742.

101. Gelfand, V., et al., *A Dominant Negative Approach for Functional Studies of the Kinesin II Complex*, in *Kinesin Protocols*, I. Vernos, Editor. 2001, Humana Press. p. 191-204.
102. Tanaka, Y., et al., *Targeted Disruption of Mouse Conventional Kinesin Heavy Chain kif5B, Results in Abnormal Perinuclear Clustering of Mitochondria.* Cell, 1998. **93**(7): p. 1147-1158.
103. Wang, Z., et al., *Kif5b controls the localization of myofibril components for their assembly and linkage to the myotendinous junctions.* Development, 2013. **140**(3): p. 617-626.
104. Wenzel, K., et al., *Dysfunction of dysferlin-deficient hearts.* Journal of Molecular Medicine, 2007. **85**(11): p. 1203-1214.
105. de Morrée, A., et al., *Proteomic Analysis of the Dysferlin Protein Complex Unveils Its Importance for Sarcolemmal Maintenance and Integrity.* PLoS ONE, 2010. **5**(11): p. e13854.
106. Cai, Q., C. Gerwin, and Z.-H. Sheng, *Syntabulin-mediated anterograde transport of mitochondria along neuronal processes.* The Journal of Cell Biology, 2005. **170**(6): p. 959-969.
107. Cai, D., K.J. Verhey, and E. Meyhöfer, *Tracking Single Kinesin Molecules in the Cytoplasm of Mammalian Cells.* Biophysical Journal, 2007. **92**(12): p. 4137-4144.
108. Kawano, T., et al., *A Small Peptide Sequence is Sufficient for Initiating Kinesin-1 Activation Through Part of TPR Region of KLC1.* Traffic, 2012. **13**(6): p. 834-848.
109. Farina, F., et al., *Kinesin KIFC1 actively transports bare double-stranded DNA.* Nucleic Acids Research, 2013. **41**(9): p. 4926-4937.
110. Han, R., et al., *Genetic ablation of complement C3 attenuates muscle pathology in dysferlin-deficient mice.* The Journal of Clinical Investigation, 2010. **120**(12): p. 4366-4374.
111. Han, W.-Q., et al., *Lysosome fusion to the cell membrane is mediated by the dysferlin C2A domain in coronary arterial endothelial cells.* Journal of Cell Science, 2012. **125**(5): p. 1225-1234.

112. Lapidos, K.A., R. Kakkar, and E.M. McNally, *The Dystrophin Glycoprotein Complex: Signaling Strength and Integrity for the Sarcolemma.* Circ Res, 2004. **94**(8): p. 1023-1031.
113. Miesenbock, G., D.A. De Angelis, and J.E. Rothman, *Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins.* Nature Protocols, 1998. **394**(6689): p. 192-195.
114. Hauser, M., et al., *Analysis of Muscle Creatine Kinase REgulatory Elements in Recombinant Adenoviral Vectors.* Molecular Therapy, 2000. **2**(1): p. 16-25.
115. Becker, K. and B. Jerchow, *Generation of Transgenic Mice by Pronuclear Microinjection*, in *Advanced Protocols for Animal Transgenesis*, S. Pease and T.L. Saunders, Editors. 2011, Springer Berlin Heidelberg. p. 99-115.
116. Gumerson, J.D., et al., *Soleus muscle in glycosylation-deficient muscular dystrophy is protected from contraction-induced injury.* American Journal of Physiology - Cell Physiology, 2010. **299**(6): p. C1430-C1440.
117. Shefer G and Y.-R. Z, *Isolation and culture of skeletal muscle myofibers as a means to analyze satellite cells.* Methods in Molecular Biology, 2005. **290**: p. 281-304.
118. Burrone, J., Z. Li, and V.N. Murthy, *Studying vesicle cycling in presynaptic terminals using the genetically encoded probe synaptopHluorin.* Nature Protocols, 2007. **1**(6): p. 2970-2978.
119. Sankaranarayanan, S., et al., *The Use of pHluorins for Optical Measurements of Presynaptic Activity.* Biophysical Journal, 2000. **79**(4): p. 2199-2208.
120. McDade, J.R. and D.E. Michele, *Membrane damage-induced vesicle–vesicle fusion of dysferlin-containing vesicles in muscle cells requires microtubules and kinesin.* Human Molecular Genetics, 2013.
121. Matsuda, C., et al., *The sarcolemmal proteins dysferlin and caveolin-3 interact in skeletal muscle.* Human Molecular Genetics, 2001. **10**(17): p. 1761-1766.
122. Roche, J.A., et al., *Unmasking Potential Intracellular Roles For Dysferlin through Improved Immunolabeling Methods.* Journal of Histochemistry & Cytochemistry, 2011. **59**(11): p. 964-975.

123. Demonbreun, A.R., et al., *Dysferlin and Myoferlin Regulate Transverse Tubule Formation and Glycerol Sensitivity.* The American Journal of Pathology, 2014. **184**(1): p. 248-259.
124. Kerr, J.P., et al., *Dysferlin stabilizes stress-induced Ca2+ signaling in the transverse tubule membrane.* Proceedings of the National Academy of Sciences, 2013. **110**(51): p. 20831-20836.
125. Lek, A., et al., *Calpains, Cleaved Mini-DysferlinC72, and L-Type Channels Underpin Calcium-Dependent Muscle Membrane Repair.* The Journal of Neuroscience, 2013. **33**(12): p. 5085-5094.
126. Glover, L.E., et al., *Dysferlin overexpression in skeletal muscle produces a progressive myopathy.* Annals of Neurology, 2010. **67**(3): p. 384-393.
127. Mandato, C.A. and W.M. Bement, *Contraction and polymerization cooperate to assemble and close actomyosin rings around Xenopus oocyte wounds.* The Journal of Cell Biology, 2001. **154**(4): p. 785-798.
128. Tian, Q., et al., *Functional and morphological preservation of adult ventricular myocytes in culture by sub-micromolar cytochalasin D supplement.* Journal of Molecular and Cellular Cardiology, 2012. **52**(1): p. 113-124.
129. Moench, I., et al., *Resolution of hyposmotic stress in isolated mouse ventricular myocytes causes sealing of t-tubules.* Experimental Physiology, 2013. **98**(7): p. 1164-1177.
130. Waddell, L.B., et al., *Dysferlin, Annexin A1, and Mitsugumin 53 Are Upregulated in Muscular Dystrophy and Localize to Longitudinal Tubules of the T-System With Stretch.* Journal of Neuropathology & Experimental Neurology, 2011. **70**(4): p. 302-313 10.1097/NEN.0b013e31821350b0.
131. Bernatchez, P.N., et al., *Myoferlin is critical for endocytosis in endothelial cells.* American Journal of Physiology - Cell Physiology, 2009. **297**(3): p. C484-C492.
132. Yuan, S.H., W. Arnold, and A.O. Jorgensen, *Biogenesis of transverse tubules and triads: immunolocalization of the 1,4-dihydropyridine receptor, TS28, and the ryanodine receptor in rabbit skeletal muscle developing in situ.* The Journal of Cell Biology, 1991. **112**(2): p. 289-301.

133. Lostal, W., et al., *Lack of Correlation between Outcomes of Membrane Repair Assay and Correction of Dystrophic Changes in Experimental Therapeutic Strategy in Dysferlinopathy.* PLoS ONE, 2012. **7**(5): p. e38036.
134. Xu, L., et al., *Dysferlin Forms a Dimer Mediated by the C2 Domains and the Transmembrane Domain In Vitro and in Living Cells.* PLoS ONE, 2011. **6**(11): p. e27884.
135. Kee, A., P. Gunning, and E. Hardeman, *Diverse roles of the actin cytoskeleton in striated muscle.* Journal of Muscle Research and Cell Motility, 2009. **30**(5-6): p. 187-197.
136. Prins, K.W., et al., *Quadriceps myopathy caused by skeletal muscle-specific ablation of βcyto-actin.* Journal of Cell Science, 2011. **124**(6): p. 951-957.
137. Sonnemann, K.J., et al., *Cytoplasmic γ-Actin Is Not Required for Skeletal Muscle Development but Its Absence Leads to a Progressive Myopathy.* Developmental Cell, 2006. **11**(3): p. 387-397.
138. Hanft, L.M., et al., *Cytoplasmic γ-actin contributes to a compensatory remodeling response in dystrophin-deficient muscle.* Proceedings of the National Academy of Sciences, 2006. **103**(14): p. 5385-5390.
139. Baltgalvis, K., et al., *Transgenic overexpression of gamma-cytoplasmic actin protects against eccentric contraction-induced force loss in mdx mice.* Skeletal Muscle, 2011. **1**(1): p. 32.
140. Liu, Y., Y. Zhou, and K. Zhu, *Inhibition of Glioma Cell Lysosome Exocytosis Inhibits Glioma Invasion.* PLoS ONE, 2012. **7**(9): p. e45910.
141. Martinez, I., et al., *Synaptotagmin VII Regulates Ca2+-Dependent Exocytosis of Lysosomes in Fibroblasts.* The Journal of Cell Biology, 2000. **148**(6): p. 1141-1150.
142. Matsuda, R., A. Nishikawa, and H. Tanaka, *Visualization of Dystrophic Muscle Fibers in Mdx Mouse by Vital Staining with Evans Blue: Evidence of Apoptosis in Dystrophin-Deficient Muscle.* Journal of Biochemistry, 1995. **118**(5): p. 959-963.
143. Straub, V., et al., *Animal Models for Muscular Dystrophy Show Different Patterns of Sarcolemmal Disruption.* The Journal of Cell Biology, 1997. **139**(2): p. 375-385.

144. Whitehead, N.P., et al., *Streptomycin reduces stretch-induced membrane permeability in muscles from mdx mice.* Neuromuscular Disorders, 2006. **16**(12): p. 845-854.
145. Jørgensen, L.H., et al., *Long-Term Blocking of Calcium Channels in mdx Mice Results in Differential Effects on Heart and Skeletal Muscle.* The American Journal of Pathology, 2011. **178**(1): p. 273-283.
146. Xu, H., et al., *Defective muscle basement membrane and lack of M-laminin in the dystrophic dy/dy mouse.* Proceedings of the National Academy of Sciences, 1994. **91**(12): p. 5572-5576.
147. Duclos, F., et al., *Progressive Muscular Dystrophy in α-Sarcoglycan–deficient Mice.* The Journal of Cell Biology, 1998. **142**(6): p. 1461-1471.