Mechanisms of Plasma Membrane Repair in Striated Muscle

by

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ABSTRACT

Sarcolemma instability is a hallmark of multiple types of muscular dystrophy and is typically caused by two major pathological mechanisms: increased susceptibility of the sarcolemma to contraction-induced damage or decreased capacity to repair the sarcolemma after contraction-induced damage. The former mechanism results from mutations in critical transmembrane and cytoskeletal proteins of the dystrophin-glycoprotein complex (DGC), a key component of the membrane-stabilizing costameres. The latter mechanism is due to loss of function mutations in key membrane repair proteins, such as dysferlin, that lead to aberrant membrane resealing after membrane injury. In both cases, the respective vulnerabilities of the plasma membrane result in muscle fiber death and progressive muscle disease. Modulating membrane repair could represent a potential therapeutic target to improve sarcolemma integrity in either type of muscular dystrophy. However, while many putative membrane repair proteins have been identified, the mechanisms of membrane repair in striated muscle remain unclear.

The primary model of membrane repair in muscle posits that membrane repair proteins form a complex at membrane wounds to facilitate the fusion of cytoplasmic vesicles to repair the membrane barrier. Therefore, the goal of this thesis was to investigate the factors that regulate trafficking of the membrane repair protein dysferlin after injury – both to the wound and into cytoplasmic vesicles. These studies investigate

dynamin-dependent endocytosis and cytoskeletal rearrangement after injury and their roles in dysferlin trafficking and ultimately membrane resealing.

Dynamin-dependent endocytosis was interrogated prior to and after injury in isolated adult mouse skeletal muscle fibers using live cell imaging after *in vitro* laser injury in the presence of an endocytic marker, FM1-43, and pharmacological inhibitors of dynamin. We show that dynamin-dependent endocytosis is highly active at rest. We also show that injury results in a massive endocytic response, measured by FM1-43, and dynamin inhibition severely blunts FM1-43 uptake after injury in wildtype and dysferlin-deficient cells. For that reason, we measured calcium flux as a dynamin-independent measure of membrane repair and show that dysferlin-deficient cells have increased calcium uptake after injury, consistent with defective membrane repair. Interestingly, dynamin inhibition had no effect on dysferlin recruitment to membrane wounds or dysferlin endocytosis after injury, suggesting the two pathways are independent and the mechanisms for dysferlin endocytosis are still unclear.

The contribution of subsarcolemmal cytoskeleton remodeling to membrane repair was investigated using live cell imaging approaches to monitor dynamic actin after membrane injury in conjunction with a muscle-specific knockout model of one of the major subsarcolemmal isoforms of actin, γ-actin. We show that calcium drives actin polymerization at the wound, forming a stable structure that remains localized at the wound for minutes after injury. We also show that muscle-specific knockout of γ-actin results in a membrane repair defect. However, defective membrane repair observed in this model is independent of dysferlin, as dysferlin trafficking to membrane wounds and dysferlin endocytosis are unaffected by genetic loss of γ-actin. It remains possible that

alternate forms of subsarcolemmal actin partially compensate or contribute to dysferlin trafficking to the wound, or alternatively, actin may play additional roles in stabilizing the membrane repair complex during or after resealing.

These studies demonstrate that injury-induced dysferlin trafficking is independent of both dynamin-dependent endocytosis and γ-actin. However, our data emphasize that both dynamin-dependent endocytosis and the subsarcolemma cytoskeleton are activated after muscle membrane injury and are required for efficient membrane resealing.

CHAPTER 1

Introduction

Membrane Stability during Muscle Contraction

Striated muscle has evolved as a highly specialized tissue with a unique role in generating and transmitting biomechanical forces responsible for coordinated movement and daily activity. Within muscle, the highly organized structure of individual muscle fibers, including the sarcomeres and membrane compartments that control the rise and fall of intracellular calcium, is critical to producing and releasing tension on a cellular level that when coordinated among the fibers within a myofibril bundle results in muscle contraction and relaxation. Because muscle fibers are terminally differentiated cells, muscle fibers must achieve high levels of efficient and repetitive contractile function while protecting themselves against mechanical, metabolic, and other injuries.

A considerable body of work has shown that muscle contraction can result in significant damage to myofibrils. Studies in healthy human subjects showed that muscle contraction during exercise, particularly lengthening contractions, resulted in histological signs of damage in muscle biopsy and leakage of cytoplasmic contents, like creatine kinase (CK), into patient serum [1-4]. These studies were followed up by controlled studies in rodent models that confirmed

muscle contraction resulted in disruptions in myofibrillar ultrastructure of both the T-Tubules and the sarcomere, as well as decreased efficiency of excitation-contraction coupling, both of which could potentially explain the observation that lengthening contraction results in transient decreased force production in subsequent lengthening contractions [5-7].

Most important to the studies herein, lengthening contraction has also been shown to disrupt cellular integrity. Muscle sections taken from rabbits after repetitive lengthening contractions showed intracellular staining of plasma fibronectin and hypercontraction of muscle fibers [8]. Additional work showed that histological samples of exercised rats show an increase in mononuclear cells as well as an increase concentration of CK in plasma, but again, muscles were most severely affected by lengthening contraction during downhill running [9]. McNeil et al. went on to show that rat muscle fibers after downhill exercise had taken up extracellular albumin, which presumably gained entry through plasma membrane wounds. Albumin staining and serum CK was highest immediately following exercise but both parameters decreased by 24hrs post exercise, suggesting acute injury can be repaired [10].

To protect the membrane from contraction-induced damage, specific cytoskeletal components, namely the costameres, align perpendicular to the thin and thick filaments of the sarcomere forming a strong mechanical linkage between the Z-disk at the ends of the sarcomere and the plasma membrane [11]. Not only do costameres serve to transmit force laterally along the fiber and aid in coordinated muscle contraction along myofibrillar bundles, but they also ensure that the plasma membrane travels in unison with shortening sarcomeres [12]. By distributing force on the sarcolemma along its

whole length, costameres prevent uneven mechanical forces from disturbing the lipid bilayer, which is well-exemplified by electron micrographs of contracted muscle fibers showing parallel costameres with membrane bulging in between, termed festoons [13, 14]. Lastly, the strong mechanical linkage provided by costameres prevents undue stretch of the plasma membrane during lengthening contraction. It is when the connection is disrupted that the membrane is permitted to stretch unabated with the potential to inflict membrane injury [14-16].

Additional evidence for the importance of costameres in muscle contraction and membrane stability is provided by studies using mouse models of muscular dystrophy, the most prominent of which is the *mdx* mouse model of Duchenne Muscular Dystrophy (DMD). DMD is an X-linked disorder caused by loss of function mutations in dystrophin, a 427kDa protein located at the sarcolemma that is associated with the dystrophin-glycoprotein complex (DGC) [17]. Dystrophin forms a strong mechanical linkage between the DGC and the sarcomere through C-terminal binding of β-dystroglycan and N-terminal binding of the γ_{cyto}-actin filaments at costameres [18, 19]. Pathological mutations in dystrophin result in disorganization of the costameres, which increases membrane susceptibility to contraction-induced injury [20-23]. Both DMD patients and the *mdx* mouse model show dramatically increased serum CK, an indicator of plasma membrane breach. In addition, characterization of the *mdx* mouse shows that at rest, *mdx* myofibrils have an increased uptake of the cell-impermeant Evan's blue dye, and dye uptake is exacerbated by exercise [24]. In addition, DMD patients and *mdx* mice have an increased number of muscle fibers with a branched morphology, and these branch points are particularly susceptible to injury, which is evidenced by an increased

intracellular Evan's blue dye specifically at branch-points [25]. These data together suggest a critical role for costameres, and particularly the DGC, in maintaining membrane stability. Moreover, these data highlight membrane instability associated with disorganization of the DGC or costameres as a pathologic contributor to several muscular dystrophies.

While the membrane's susceptibility to damage has been studied extensively in the context of DGC dysfunction, it has become clear more recently that increased contraction-induced injury is not the only source for increased membrane permeability. While many disease-causing mutations had been identified to be associated with the DGC and an increased susceptibility to contraction-induced damage due to instability within the complex, dysferlin mutations were identified in patients with Limb-Girdle Muscular Dystrophy Type 2B and Miyoshi Myopathy, that presented with increased membrane permeability and evidence of membrane lesions measured by increased serum CK [26-30]. However, the mechanism was found to be separate from DGC dysfunction (**Figure 1.1**) [31].

Bansal et. al. showed that dysferlin-null mice present with a progressive muscular dystrophy associated with an increased number of centrally nucleated fibers and an increased number of smaller diameter fibers, both parameters indicative of an increase in regeneration typically associated with muscular dystrophy [28]. However, in dysferlin-null mice, all components of the DGC were expressed at similar levels to WT, and the DGC was properly localized to the plasma membrane. Even though the DGC was observed to be intact, dysferlin-null mice showed increased intracellular staining of cell-impermeable Evan's Blue dye at rest; however, the uptake of dye was not

increased with exercise, which is contrary to muscular dystrophies associated with DGC-dysfunction like the *mdx* mouse model [24, 28]. Based on the observation that the absence of dysferlin leads to accumulation of subsarcolemma vesicles and due to the structural similarity of dysferlin to synaptotagmin (described later in this chapter), at the time, dysferlin was thought to be a membrane vesicle protein [28]. These data taken together resulted in the hypothesis that the increased membrane permeability in dysferlin-null mice was a result of an inability to repair contraction-induced damage possibly through a vesicle fusion mediated process. This hypothesis was supported by an assay of membrane damage and repair that has since become the “gold-standard” for identifying putative membrane repair proteins. The authors isolated muscle fibers from dysferlin-null mice and, using a high-powered microscope laser, ablated a small area of plasma membrane, after which the uptake of the cell-impermeable dye, FM1-43, was monitored. Genetic loss of dysferlin resulted in an increased uptake of FM1-43 after injury providing the first experimental evidence that dysferlin is a critical membrane repair protein and that membrane instability caused by defects in membrane repair may be a pathologic mechanism for a subset of muscular dystrophies [28, 32]. Therefore, the two mechanisms of maintaining membrane integrity and the ability of muscle to repair the sarcolemma represent two distinct but convergent mechanisms resulting in sarcolemma disruption, muscle cell degeneration, and muscular dystrophy (**Figure 1.2**).

Muscle contraction can significantly damage the plasma membrane of individual muscle fibers. Structures like the costameres are critically important for maintaining cellular integrity by providing stability to the sarcolemma and acting as a shock-absorber for mechanical forces. Furthermore, a concerted membrane repair response is required

to restore cellular integrity if sarcolemma damage does occur. Studies have shown that the loss of membrane stability through either mechanism – increased susceptibility to damage or defects in membrane repair - contributes to the pathology of several muscle diseases. Therefore, understanding the contributing factors that influence either membrane damage or repair are of particular interest to the pathology and treatment of muscular dystrophy. The goal of this thesis was to contribute to the basic understanding of plasma membrane repair, though the studies herein could have implications for both mechanisms of membrane instability.

Mechanisms of Plasma Membrane Repair

The "Patch" Hypothesis

Extensive research using tractable, non-mammalian systems to investigate the mechanisms of plasma membrane repair has resulted in several proposed models of membrane repair. One prevailing model is the "patch hypothesis" which proposes that plasma membrane repair occurs through the addition of endomembrane to restore the lipid barrier (reviewed in [33]). Early work in Sea urchin embryos showed calcium influx after injury stimulated the formation of large vesicles beneath membrane disruptions, which were later suggested to be the result of homotypic fusion of secretory vesicles docked near the plasma membrane, known as cortical granules [34-36]. Further studies showed that inhibiting exocytosis with inhibitors of various SNARE proteins or manipulating vesicle availability prevented efficient resealing [35-38]. Taken together, these data suggest that exocytosis of fusion-capable cortical vesicles participates in membrane repair in Sea urchin embryos, which led to the hypothesis that reserve

vesicles may serve as a membrane patch to restore barrier function to the plasma membrane.

These studies, while compelling, do not necessarily reflect plasma membrane repair in mammalian cell types, nor in muscle fibers. Very few mammalian cells exist in a state with vesicles poised for quick release, though axons with synaptic vesicles and other types of secretory cells are notable exceptions [39, 40]. However, additional work in a variety of cell types has shown that some of the tenets of the patch hypothesis may hold true in mammalian cell types. For example, exocytosis of the lysosome has been implicated as a potential intramembranous source for patch repair in mammalian fibroblasts, particularly repair after injury with pore-forming toxins (PFTs) (reviewed in [41]). Successful lysosomal exocytosis was described after injury by the presence of lumenal markers of the lysosome, such as Lamp-1, at the plasma membrane and active proteolytic enzymes in the extracellular space [42]. Inhibition of lysosome exocytosis prevented membrane repair, suggesting lysosome exocytosis is a critical step to the repair process [42, 43].

The role of lysosome exocytosis in membrane repair has been linked to regulating wound-induced endocytosis through the release of the enzyme acid-sphingomyelinase (ASM). ASM was found to be secreted after injury where it works to generate membrane-bending ceramide, thereby promoting endocytosis after injury. Inhibiting lysosomal exocytosis or inhibiting ASM negatively affected both wound-induced endocytosis and membrane repair, though inhibiting ASM alone did not affect lysosomal exocytosis [44]. Additional studies have used extracellular tracers to identify endosomes, which have been observed to increase in number and size, suggesting

highly active wound-induced endocytosis that culminates in homotypic fusion of intracellular vesicles [45]. Additionally, endocytic machinery has been suggested to play a role in membrane repair, including but not limited to dynamin-2, caveolin1 and caveolin3 [45, 46]. How endocytosis contributes to membrane resealing is still not fully understood and whether submembrane vesicles merely plug the hole temporarily or participate in fusion to restore membrane continuity is not known. The evidence suggests that both lysosomal exocytosis and wound-induced endocytosis are possibly important for membrane repair, at least in some mammalian cell types, and may potentially participate in the formation of a membranous patch for either plugging or repairing a plasma membrane breach.

Purse-String Model

Additional studies in *Xenopus* oocytes and *Drosophila* embryos have suggested a model of membrane repair that relies heavily on the formation of an actin-myosin based contractile ring, similar to that formed during cytokinesis, that encircles and closes the wound (reviewed in [47]). While the ring components differ slightly between the two animal models, both models agree that actin and myosin are recruited to membrane wounds, which is required for membrane repair. In both model organisms, inhibiting either actin or myosin genetically or with specific inhibitors prevented both ring formation, ring closure, and thus membrane repair [48, 49].

Additional studies suggest that microtubules are also recruited to membrane wounds to focus actin filaments into a specific wound-perimeter zone; treating *Xenopus* oocytes with nocodazole affected the recruitment of the actin nucleator Arp2/3 complex and myosin II, and also resulted in a diffuse and destabilized actin ring at the wound

[50]. In addition, microtubules are involved in the formation of distinct zones of several Rho GTPases, which are required for membrane repair [51]. Active RhoA, Rac1, and Cdc42 have all been shown to localize to distinct concentric zones surrounding the contractile ring independently of actin polymerization. Inhibiting any of these Rho GTPases disrupted ring formation and delayed wound closure [51, 52]. In *Drosophila* embryos, inhibiting Rho1 prevented ring assembly completely and was shown to be required for myosin II activation, and inhibiting Cdc42 and Rac dysregulated actin recruitment to membrane wounds [52]. Studies using photoactivatable GTPase activity probes show that the activities of RhoA and Cdc42 are modulated to maintain distinct zones encircling the wound that were important for maintaining localized activity of actin and myosin within the ring [53]. These data together implicate the Rho GTPases as upstream effectors responsible for targeting actin and myosin to membrane wounds to facilitate ring contraction and wound closure in non-mammalian model systems.

Dysferlin-Mediated Membrane Repair in Striated Muscle

A Role for Dysferlin in Membrane Repair

While several models of membrane repair have been proposed in a variety of cell types, it remains unclear if some or all of the mechanisms of plasma membrane repair are conserved in mammalian cells, let alone skeletal muscle. A main tenet of the patch hypothesis requires significant contributions from endomembranes that are released rapidly after injury, but skeletal muscle is not a secretory tissue and therefore does not have vesicles poised for release. In addition, the formation of a contractile ring has yet to be identified in mammalian cell types or skeletal muscle, which argues against the

purse-string model being strictly true in skeletal muscle. Therefore, investigating plasma membrane repair in skeletal muscle, a highly dynamic and vulnerable tissue, becomes critically important.

Skeletal muscle Dysferlin was originally hypothesized to promote membrane stability through an interaction with the DGC based on the findings that causative mutations within dysferlin were identified in a subset of Limb-Gridle Muscular Dystrophies that shared phenotypic similarities (i.e. increased centrally nucleated fibers and high patient serum CK) with dystrophies caused by DGC dysfunction [26, 27]. Furthermore, dysferlin was found to be mislocalized from the sarcolemma in several muscular dystrophies caused by disruption to DGC components [31]. However, upon biochemical analysis, no such interaction between the DGC components and dysferlin was identified (**Figure 1.1B**).Electron micrographs of human dysferlin-deficient muscle showed gaps and microvilli-like projections on the sarcolemma, an accumulation of intracellular vesicles, and a multi-layered basal lamina [31, 54]. Increased muscle fiber damage along with an accumulation of intracellular membranes combined with dysferlin's homology to a vesicle fusion protein involved in spermatogenesis in C. elegans, led to the initial hypothesis that dysferlin facilitates vesicle fusion to create a membrane patch during membrane repair [27, 31, 54].

Analysis of the domain structure of dysferlin also pointed to a role for dysferlin in vesicle fusion events (**Figure 1.1A**). Dysferlin, a type 2 transmembrane protein that localizes to the t-tubules and lateral sarcolemma of the muscle fiber, contains seven C2 domains (C2A-G) and shares sequence homology with the vesicle-fusion family, the ferlins, and synaptotagmin [27]. The C2 domain structure typically contains a compact

beta sandwich at its core with three loops at the apex of the domain that contain the “C2 motif” of five negatively charged aspartate residues to coordinate the binding of calcium ions [55]. Calcium-binding stabilizes the protein and increases the surface charge thereby increasing affinity for negatively charged phospholipids [55, 56].

Whether all seven C2 domains of dysferlin are functionally important is still unclear. The most well-characterized C2 domain of dysferlin, the C2A domain, contains multiple calcium binding sites, shares the C2 domain fold, and maintains three of the five conserved aspartate residues in loops 1 and 3 [57, 58]. The other C2 domains have the conserved aspartate residues in varying degrees with C2B and C2D being the least conserved and C2E, F, and G being the most conserved [57]. For this reason, studies were devoted to characterizing the calcium-sensitivity, the phospholipid-binding, and the membrane repair capacity of each of the dysferlin C2 domains.

Testing a construct of three C2 domains – C2A, B, and C - Marty et. al. showed that in response to calcium these three domains were sufficient to increase the order of membrane lipids *in vitro*. Subsequent truncations of each domain decreased lipid ordering activity, suggesting the domains possibly act synergistically to increase lipid ordering [59]. These data are consistent with findings of another study that noted all dysferlin C2 domains have some degree of lipid-binding activity, though the lipid-binding activity of some domains was more calcium-sensitive than others [60]. Additional studies used truncation mutants of each C2 domain separately in isothermal chemistry experiments to show that the lipid-binding activity of all seven C2 domains is calcium sensitive, with C2A being the most calcium sensitive and C2D and E being the least

sensitive, which is consistent with other reports that C2A is the most calcium-sensitive C2 domain [60, 61].

Further mutation analyses indicate that C2A, C2F, C2G, and to some degree C2D are required for functional dysferlin. Immunocytochemistry and western blotting shows that the C2A, F, and G domains are required for dysferlin localization to the plasma membrane, and C2A, F, G and to some degree C2D are required for proper membrane resealing after laser injury [62]. These findings suggested that proper localization of dysferlin to the plasma membrane is required for proper membrane repair, but also indicate that some C2 domains may be dispensable for dysferlin function. The C2 domains of dysferlin have also been shown to facilitate the formation of a dysferlin homodimer, which is thought to coordinate the lipid-binding activity of the most calcium-sensitive domain, C2A [63]. However, it is still unclear whether this dimer is formed in a transient manner or if it is functionally important for membrane repair.

These data led to the several unanswered questions regarding dysferlin function during membrane repair. The calcium-dependent lipid binding activity of the dysferlin C2 domains suggests a potential role in the insertion of dysferlin into endosomes, or in the fusion of dysferlin-containing vesicles to each other, or in the fusion of vesicles to the plasma-membrane wound. Another interesting aspect of the dysferlin C2 domains is the ability to act as a homodimer, which could also implicate dysferlin as a scaffolding protein for other membrane repair machinery.

To elucidate the function of dysferlin in membrane repair, additional studies focused on dysferlin trafficking after membrane injury. Immunohistochemistry of injured muscle fibers showed that dysferlin is present at membrane wounds, which was later

shown to be the result of active transport of dysferlin from the lateral sarcolemma [28, 64]. Inhibiting actin polymerization prevented dysferlin recruitment to the injury site and increased the uptake of cell-impermeable dye after injury, suggesting dysferlin translocation to membrane wounds is both dependent upon the actin cytoskeleton and critical to membrane repair [64]. In addition, dysferlin was shown in both adult isolated muscle fibers and L6 myotubes to be rapidly endocytosed after injury. In L6 myotubes, these endosomes were shown to be trafficked along microtubules by the kinesin motor KIF5B [64, 65]. However, these studies also highlight that the majority of dysferlin is localized to the plasma membrane, with very little dysferlin present in intracellular vesicles at rest [64]. These data taken together suggest dysferlin is critical to membrane repair but also present the possibility that dysferlin may regulate, either as a scaffold by facilitating membrane fusion, vesicle-vesicle and/or vesicle-plasma membrane fusion events after injury. However, the function of dysferlin in repair still remains unclear.

The Muscle Membrane Repair Complex

After the discovery of dysferlin as a membrane repair protein, many studies have used similar approaches to try to identify other proteins that might participate in muscle membrane repair [28]. The fundamental approach to identifying putative membrane repair proteins was to identify candidate proteins through genetic screens in dysferlin-deficient mice or immunoproteomic analysis of dysferlin interacting partners [66-69]. Then, researchers would test whether the candidate proteins rapidly translocated to wounds after muscle membrane injury and test whether their loss of function altered FM1-43 uptake. Collectively, this work has gathered evidence for a rapidly assembling

membrane repair complex with many components that may contribute to distinct processes during sarcolemma repair. Here we describe the evidence for additional membrane repair proteins and discuss what is known about the membrane repair response in skeletal muscle.

Annexins in Dysferlin-Mediated Plasma Membrane Repair in Muscle

The annexins were first investigated in plasma membrane repair due to their calcium-sensitive, lipid-binding properties [70]. Furthermore, it was identified that expression levels of Annexin1 and 2 were significantly elevated in Limb-Girdle Type 2B patient muscle, which was correlated with clinical severity of muscle disease, suggesting Annexin1 and 2 may modify dysferlinopathies through participation in membrane repair [71]. Co-immunoprecipitation and fluorescence-lifetime imaging microscopy (FLIM) after injury identified a calcium-dependent interaction between Annexin1 and Annexin2 with dysferlin at membrane wounds [72]. Additional studies in zebrafish muscle showed that Annexin1, Annexin2, AnnexinV and Annexin6 all localize at the site of membrane injury, observations which were recently replicated in isolated adult mouse muscle fibers [73, 74]. However, while Annexin1 was shown to be recruited to membrane wounds in muscle, a knockout mouse model of Annexin1$^{-/-}$ showed neither a dystrophic muscle phenotype nor any deficit in membrane repair despite evidence in other cell types suggesting Annexin1 is required for membrane repair, [73, 75, 76]. In contrast, Annexin2$^{-/-}$ myofibers showed decreased capacity for membrane repair [77]. Lastly, mutations in AnnexinV and Annexin6 were shown to increase extracellular dye uptake after membrane injury, and Annexin6 was shown to require actin polymerization for efficient recruitment to membrane wounds [74, 78, 79].

Additional studies in other cell types have shown that AnnexinV oligomers form a lattice structure, hypothesized to stabilize the wound and prevent pore expansion [80, 81]. Several annexins have also been shown to interact with both the actin cytoskeleton and phospholipids, potentially suggesting a role for the annexins as a scaffold to mediate plasma membrane repair complex formation [82-84]. These data taken together from plasma membrane repair studies specifically in muscle suggest that multiple of the annexins, including Annexin1, 2, 3, 5, and 6 are recruited to membrane wounds, in part by the actin cytoskeleton, to facilitate dysferlin-mediated membrane repair, though the individual contribution of each annexin to membrane repair and dysferlin function is less clear.

Membrane Trafficking during Dysferlin-Mediated Membrane Repair in Muscle

The contribution of lysosome exocytosis to membrane repair in other cell types was discussed, but there is additional evidence that dysferlin may play a role in lysosome exocytosis in sarcolemma repair. Multiple types of injury in cultured myotubes showed the lysosome lumenal protein, LAMP-1 accumulated at the cell surface in a calcium-dependent manner [42, 72]. Dysferlin-null myofibers also presented with defects in fusion of the lysosome at the plasma membrane after injury, resulting in accumulation of lysosomes at the surface membrane and reduced release of the lysosomal enzyme acid sphingomyelinase, both steps which have been shown to be critical to membrane repair in other cell types [85]. Lastly, dysferlin colocalized and co-immunoprecipitated with SNAREs previously identified as critical to lysosome exocytosis - syntaxin4 and SNAP23 – and dysferlin accelerated lipid mixing in the presence of SNARE proteins *in vitro* [86]. Lysosomes have been implicated as the

major source of donor membrane for a membrane patch in mammalian cells, and these data suggest that dysferlin may be involved, but it is likely more complicated.

Other endomembrane sources have also been implicated in the membrane repair response; for example, the enlargeosome, a calcium-dependent exocytosis vesicle. A marker for the enlargeosome, AHNAK, has been shown in epithelial cells to translocate to the plasma membrane in response to calcium where it interacts with Annexin2 and the cortical actin cytoskeleton [87, 88]. Furthermore, through mass-spectrometry and co-immunoprecipitation, AHNAK, has been identified as a dysferlin binding partner in muscle [89]. These data support the idea that AHNAK may play a role in membrane repair, though more evidence is required to implicate either AHNAK or the enlargeosome in the formation of a membrane patch.

While these data suggest a potential role for dysferlin in the exocytic response to injury, more evidence implicates dysferlin in the endocytic response to injury. Dysferlin has been shown to be endocytosed following injury and a number of dysferlin-containing vesicles can be seen accumulating in the cytosol of injured myofibers [64, 65]. It has also been shown that the muscle-specific TRIM family protein, MG53, likely plays a role in dysferlin-vesicle trafficking, which is critical to membrane repair. MG53 knockout resulted in a progressive myopathy characterized by increased membrane permeability after exercise and increased uptake of extracellular FM1-43 after injury in isolated muscle fibers. Furthermore, electron micrograph images of MG53 knockout muscle show a decrease in intracellular vesicle accumulation, suggesting that MG53 is upstream of dysferlin and required for injury-induced vesicle formation. MG53 also accumulated at membrane wounds where it interacted with negatively charged

phospholipids and colocalized with AnnexinV [68]. Lastly, MG53 was shown to co-immunoprecipitate with dysferlin, suggesting an interaction that was later pinpointed to dysferlin C2A domain [90, 91]. These data highlight the importance of the endocytic response to membrane repair and demonstrate a role for dysferlin in that response. However, the exact mechanism of dysferlin function in the endocytic or the exocytic response to membrane repair remains unclear.

Based on these and other studies, at the time I Began this dissertation research, a fairly well-accepted model of membrane repair had been developed (**Figure 1.3**). Plasma membrane breach permits extracellular calcium influx into the cell that activates membrane repair proteins, dysferlin and annexin, stimulating their translocation to membrane wounds *via* the actin cytoskeleton. Several of the annexins, namely annexin 1, 2, 5, and 6, form a membrane repair cap that is flanked by dysferlin and actin. In addition, membrane wounding stimulates dysferlin accumulation into intracellular vesicles, likely facilitated by a direct interaction with MG53. Dysferlin-containing vesicles then undergo homotypic fusion and are trafficked back to the site of a plasma membrane breach. Here these vesicles plug the wound and, through an unknown mechanism, the plasma membrane repair protein complex at the wound repairs the injury by facilitating vesicle docking and fusion and restoration of phospholipid bilayer integrity.

Rationale and Approach

Muscle membrane repair has been identified as a crucial pathway to preserve membrane integrity and promote membrane stability. Primary defects in muscle

membrane repair, most commonly caused by loss of function mutations in dysferlin, result in dystrophic muscle pathology and progressive muscle disease [26, 27]. While several models of membrane repair have been proposed, it remains unclear if one mechanism serves for every cell type or for every type of membrane disruption. Since skeletal muscles are highly dynamic tissues with a propensity for membrane damage, it is important that the studies herein consider the mechanisms of membrane repair specifically in muscle. Several putative membrane repair proteins have been identified, and several cellular pathways have been implicated, but the distinct mechanism of dysferlin function and membrane repair remains unclear. While enhancing membrane repair may be a viable option for improving patient outcomes, only through a better understanding of sarcolemma repair can novel therapeutic strategies be identified [92].

Therefore, the goals of this dissertation were to contribute to the understanding of membrane repair in skeletal muscle by clarifying the molecular pathways involved in the injury-induced trafficking of the critical membrane repair protein dysferlin. We test the overall hypothesis that both dynamin-dependent endocytosis and the subsarcolemma cytoskeleton are critical for dysferlin trafficking, both to the wound and into cytoplasmic vesicles. In Chapters 2 and 3, I will address this hypothesis with the following two aims, and in Chapter 4, I will summarize the collective contributions of these studies and future directions.

Specific Aim 1: Demonstrate the contribution of wound-induced endocytosis in membrane repair in striated muscle.

Previous data have shown that dysferlin is rapidly endocytosed following injury; however, both the regulators of dysferlin endocytosis and the contribution of

endocytosis to membrane repair in skeletal muscle remain unclear. We tested the working hypothesis that dynamin-dependent endocytosis of dysferlin-containing vesicles is critical for membrane repair following acute wounding in adult skeletal muscle fibers. To address this, live cell imaging approaches monitoring endocytosis were applied during injury, and the role of endocytosis was interrogated using pharmacological inhibitors of dynamin. Isolated muscle fibers were injured in the presence of the endocytic marker, FM1-43, in the presence or absence of a dynamin inhibitor, dynasore. Surprisingly, inhibition of dynamin markedly reduced uptake of FM1-43 by endocytosis at rest and after injury. These data suggested that FM1-43 uptake is highly dependent upon dynamin-dependent endocytosis and further called for an investigation into alternate methods of monitoring membrane repair. To that end, calcium flux after injury was measured as a supplemental, dynamin-independent approach to the canonical assay of FM1-43 uptake after injury. These studies indicated that calcium flux reports a much faster membrane repair response than what is traditionally indicated by FM1-43 uptake.

Specific Aim 2: Define the role of the subsarcolemma cytoskeleton and γ-actin in plasma membrane repair in striated muscle.

Actin has been shown to be a critical regulator of both dysferlin and annexin recruitment to membrane wounds and has also been shown to be required for efficient membrane repair [64, 74]. However, the actin isoform responsible for dysferlin recruitment remains unknown. Cytoplasmic γ-actin localizes to the costameres of the muscle fiber and is one of the major actin isoforms that forms the subsarcolemmal actin cytoskeleton. Genetic disruption of γ-actin in mouse muscle causes a mild degenerative

myopathy with some shared, mildly progressive phenotypes as dysferlin deficiency. Therefore, in the current study, we studied mice with genetic loss of γ-actin in differentiated muscle to investigate whether γ-actin may be a critical actin isoform in dysferlin recruitment and thus efficient membrane repair.

We tested the working hypothesis that the subsarcolemmal cytoskeleton, and in particular, γ-actin, may regulate dysferlin function after membrane injury and its genetic loss would disrupt dysferlin trafficking and result in a membrane repair defect. Novel live cell imaging approaches were used to monitor dynamic actin after membrane repair in conjunction with a muscle-specific knockout model of a critical subsarcolemmal isoform of actin, γ-actin, to assess the role of the subsarcolemmal cytoskeleton in membrane repair. The data show that a subsarcolemmal actin structure polymerizes at the site of a membrane injury in a calcium-dependent manner. In addition, the subsarcolemmal-specific isoform of actin, γ-actin, plays a role in membrane repair, as its loss of function in adult muscle fibers leads to a membrane repair defect. The mechanism by which γ-actin mediates membrane repair is independent of dysferlin recruitment to membrane wounds and therefore still an area of active investigation.

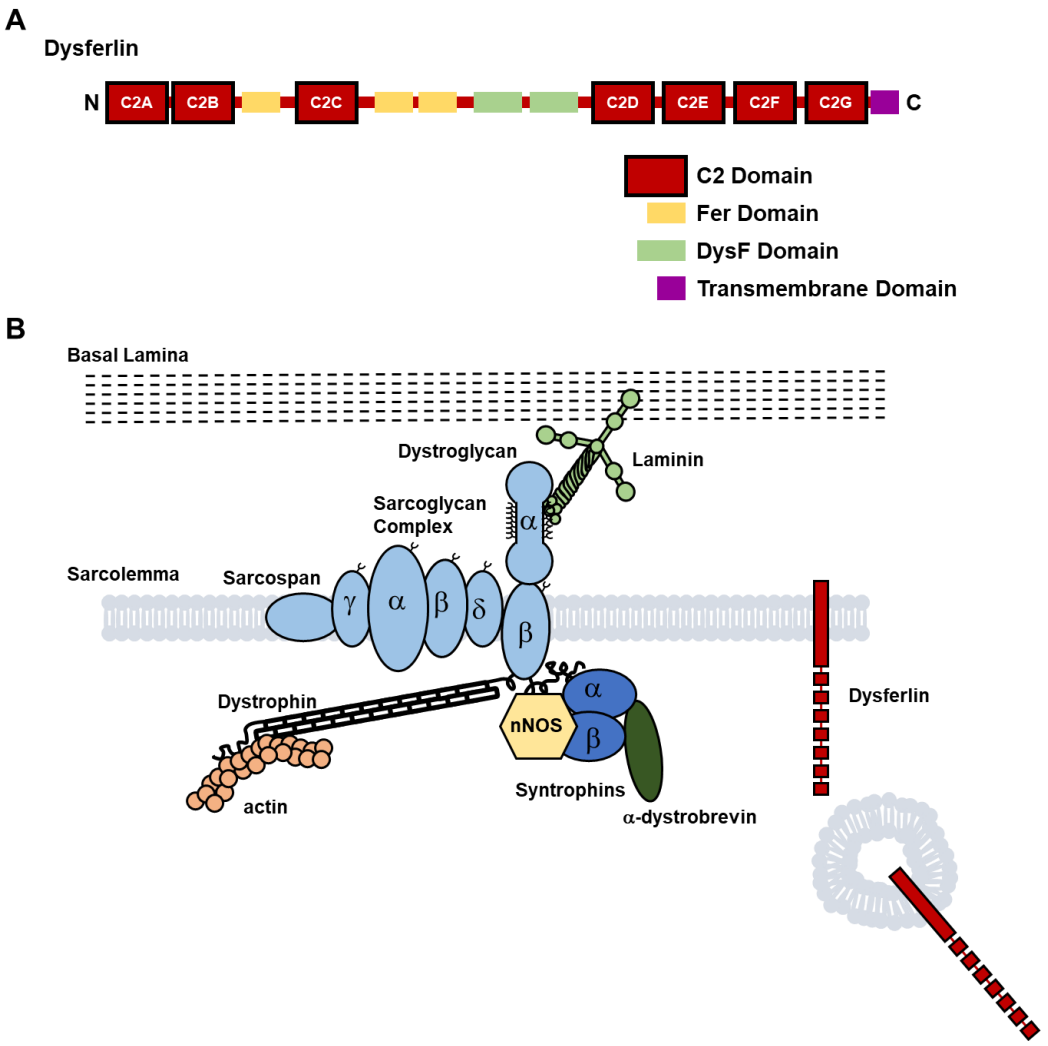

Figure 1.1 Dysferlin is not an integral component of the Dystrophin Glycoprotein Complex. (**A**) Dysferlin is a ~230kDa protein composed of seven C2 domains (C2A-G), three Fer domains and two DysF domains of unknown function, and a C-terminal transmembrane domain. (**B**) The dystrophin glycoprotein complex (DGC) serves as the mechanical linkage between the actin cytoskeleton and the extracellular matrix. The transmembrane complex associates with cytoplasmic dystrophin, which forms a physical bond with the actin cytoskeleton. Extracellularly, alpha dystroglycan binds to the matrix protein laminin. Loss of function mutations in several of the DGC components destabilize the complex and disrupt these stabilizing interactions, which is thought to result in increased susceptibility to contraction-induced membrane damage. Dysferlin is a transmembrane protein that primarily resides in the plasma membrane but can also be endocytosed into intracellular vesicles; however, it is mechanistically distinct from the DGC and is primarily involved in repairing contraction-induced membrane damage.

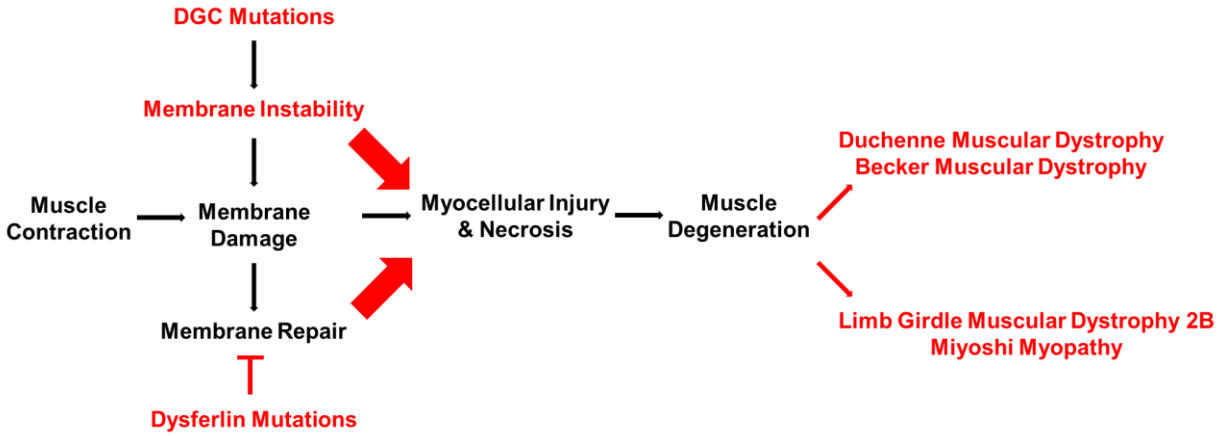

Figure 1.2 Membrane Instability is a pathological mechanism for multiple types of Muscular Dystrophy. Membrane instability occurs *via* two main mechanisms: increased membrane damage or decreased membrane repair. Increased susceptibility to membrane damage usually results from mutations in components of the dystrophin glycoprotein complex (DGC) and is most commonly associated with Duchenne Muscular Dystrophy or Becker Muscular Dystrophy. Decreased membrane repair is a result of loss of function mutations in critical membrane repair proteins, such as dysferlin, which is most usually associated with Limb Girdle Muscular Dystrophy Type 2B or Miyoshi Myopathy.

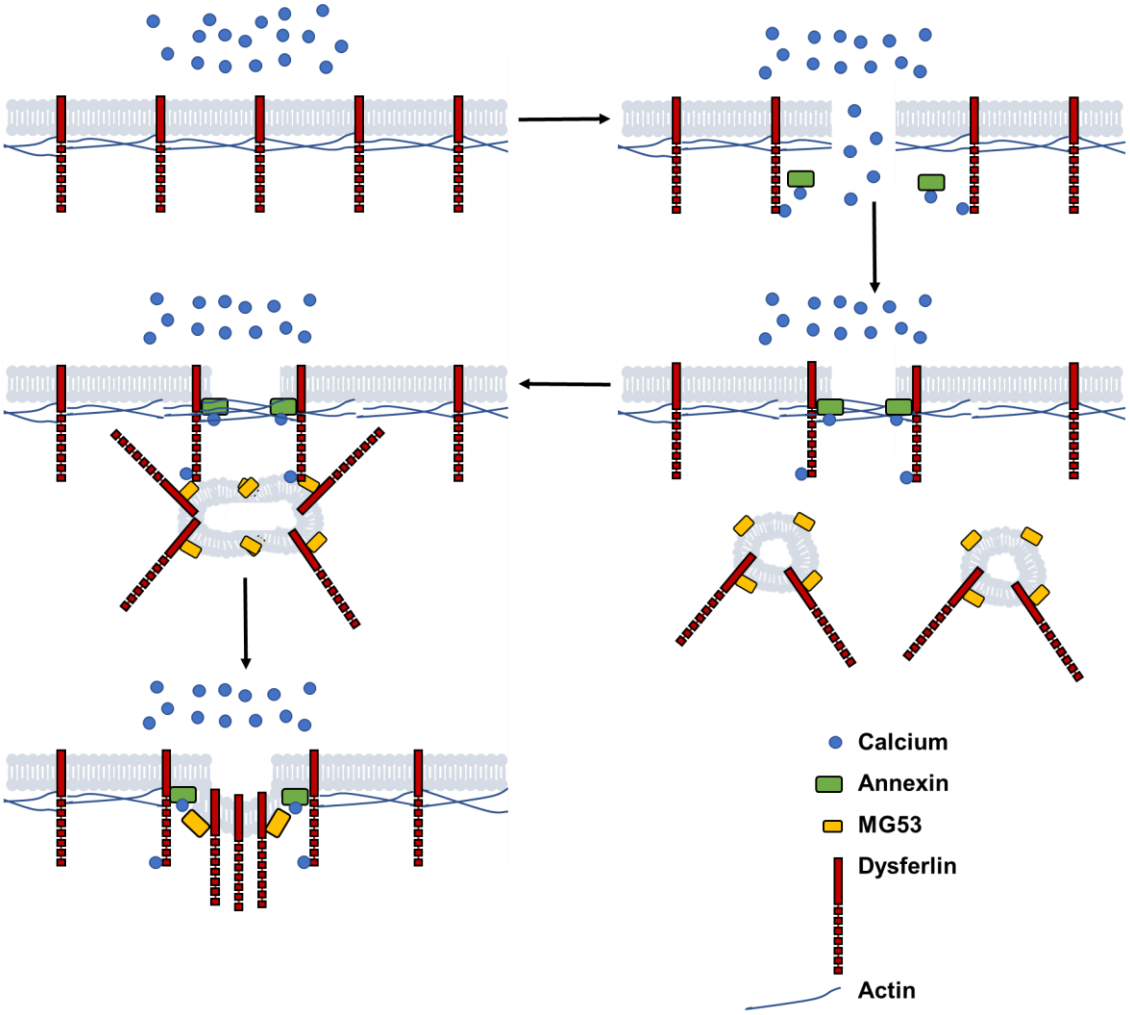

Figure 1.3. Previously accepted model of plasma membrane repair. Plasma membrane injury results in influx of extracellular calcium that binds to and activates membrane repair proteins and promotes their translocation to membrane wounds. MG53 facilitates dysferlin recruitment into intracellular vesicles that accumulate, fuse, and traffic back to membrane wounds. Through an unknown mechanism, membrane repair proteins facilitate homotypic vesicle fusion and vesicle fusion to the plasma, membrane restoring barrier function.

CHAPTER 2

Sarcolemma Wounding Activates Dynamin Dependent Endocytosis in Striated Muscle

ABSTRACT

Plasma membrane repair is an evolutionarily conserved mechanism by which cells can seal breaches in the plasma membrane. Mutations in several proteins with putative roles in sarcolemma integrity, membrane repair, and membrane transport result in several forms of muscle disease, however the mechanisms activated and responsible for sarcolemma resealing are not well understood. Using the standard assays for membrane repair, which track the uptake of FM 1-43 dye into adult skeletal muscle fibers following laser-induced sarcolemma disruption, we show that labeling of resting fibers by FM1-43 prior to membrane wounding and the induced FM1-43 dye uptake after sarcolemma wounding occurs *via* dynamin-dependent endocytosis. Dysferlin-deficient muscle fibers show elevated dye uptake following wounding, which is the basis for the assertion that membrane repair is defective in this model. Our data show that dynamin inhibition mitigates the differences in FM1-43 dye uptake between dysferlin-null and wild-type muscle fibers, suggesting that elevated wound-induced FM1-43 uptake in dysferlin-deficient muscle may actually be due to enhanced dynamin-

dependent endocytosis following wounding, though dynamin inhibition had no effect on dysferlin trafficking after wounding. By monitoring calcium flux after membrane wounding we show that reversal of calcium precedes the sustained, slower increase of dynamin-dependent FM1-43 uptake in WT fibers, and that dysferlin-deficient muscle fibers have persistently increased calcium after wounding, consistent with its proposed role in resealing. These data highlight a previously unappreciated role for dynamin-dependent endocytosis in wounded skeletal muscle fibers and identify overactive dynamin-dependent endocytosis following sarcolemma wounding as a potential mechanism or consequence of dysferlin deficiency.

INTRODUCTION

Proper maintenance of the plasma membrane is critical in cardiac and skeletal muscle as mutations that render the sarcolemma susceptible to membrane injury or disrupt membrane repair result in muscle disease [27, 93, 94]. Mutations in several putative membrane transport proteins including dysferlin [95], annexin [67], MG53 [96], synaptotagmin-VII [97] and PTRF/Cavin-1 [98] have been shown in mouse models to result in muscle disease, potentially through reduced capacity to reseal the plasma membrane following wounding [99]. This hypothesis is based largely on *in-vitro* laser-wounding experiments, which demonstrate that mutant skeletal muscle cells take up excess extracellular FM-dyes compared to normal cells following wounding [95, 96, 98]. One important caveat of these studies is that FM-dyes can also be taken up by endocytosis [100], a possibility which has not been investigated in adult skeletal muscle fibers neither at rest nor after injury. Early studies from non-muscle cells indicate that delivery and fusion of intracellular vesicles with the plasma membrane is required for

efficient wound repair [37, 101, 102]. Based on these findings, most research on muscle membrane repair to date has focused on wound-induced exocytosis as a means to reseal the sarcolemma. However, we have recently demonstrated that membrane wounding induces endocytosis of at least one putative membrane repair protein, dysferlin, resulting in the formation of large dysferlin-containing cytoplasmic vesicles [65], raising the intriguing possibility that endocytosis may contribute to efficient membrane repair in adult skeletal muscle. In spite of this, there is very little direct evidence that membrane wounding activates endocytic pathways in adult skeletal muscle, and whether endocytosis contributes to membrane repair is not clear.

Dynamin is a large GTPase that facilitates endocytosis by forming oligomerized rings around nascent vesicles leading to vesicle release. Mutations or deficiency of dynamin-2, a dynamin isoform highly expressed in adult skeletal muscle, results in a centronuclear myopathy phenotype characterized by internalized nuclei and t-tubule membrane and cytoskeletal disorganization by a largely unknown mechanism [103, 104]. Dysferlin is mislocalized away from the sarcolemma in muscle from dynamin-2 heterozygous mutant mice, indicating that dynamin may regulate some aspect of dysferlin function [104], but the relevance of this interaction to membrane repair has not been explored. Interestingly, dynamin-dependent endocytosis contributes to membrane repair in NRK cells following perforin-induced injury by removing toxin pores that assemble in the plasma membrane [105]. These data suggest that dynamin and dynamin-dependent endocytosis may play a role in membrane repair, but whether dynamin-dependent endocytosis is activated following sarcolemma wounding, and

whether dysferlin and dynamin are components of the same membrane repair pathway has not been explored.

We tested the overall hypothesis that dynamin-dependent endocytosis of dysferlin-containing vesicles is critical for membrane repair following acute wounding in adult skeletal muscle fibers. We tested this hypothesis by examining FM1-43 uptake into adult skeletal muscle fibers at rest and following laser-induced wounding with or without pharmacological inhibition of dynamin-dependent endocytosis. Surprisingly, basal and wound-induced FM1-43 uptake is severely reduced in skeletal muscle fibers treated with a dynamin-inhibitor, indicating that wounding stimulates a large endocytic response that is measured by FM1-43. We also use calcium flux following membrane wounding as an alternative approach to show that the reversal of calcium precedes the slower and continued increase of FM1-43 uptake after wounding, which suggests resealing and wound-induced increases in endocytosis may be distinct temporal events in the membrane repair process. Together, these data have important implications for future studies of membrane repair, give mechanistic insight into membrane repair in muscle, and highlight the modulation of dynamin-activity as a potential therapeutic approach for muscle disease.

MATERIALS AND METHODS

Animals: Wild-type (C57BL/6 or A/WySnJ) and dysferlin deficient (A/J) mice were purchased from Jackson Laboratories, Bar Harbor, ME. Dysferlin-deficient BLA/J mice harbor the same mutation as A/J mice on a C57BL/6 background and were a gift from The Jain Foundation Inc. [106]. Dysferlin-pHluorin (Dysf-pHGFP) transgenic mice were

generated as previously described [107]. All procedures with animals were approved by the Institutional Animal Care and Use Committee at the University of Michigan.

Muscle fiber isolation and imaging setup: Muscle fibers were isolated and imaged as previously described [11]. Briefly, flexor digitorum brevis muscles were isolated from the hind-paws of anaesthetized (i.p. injection of 15µl/gram of 2.5% Avertin solution) wild-type, dysferlin-deficient or Dysf-pHGFP transgenic mice and incubated for 4 hours at 37°C in an MEM solution containing 0.2% collagenase. Muscle fibers were triturated with glass pipettes of decreasing radius to liberate individual muscle fibers. Muscle fibers were plated on 35-mm glass bottom dishes and incubated at 37°C in MEM + 10% FBS until used. All live cell imaging was carried out on a Leica SP8 confocal microscope equipped with a temperature-controlled chamber. Cells were imaged through a 63x oil objective using an argon laser (excitation wavelength of 488nm) and an HyD detector set from either 498-525nm (GFP, Fluo-4), or 580-620nm (FM1-43). Wounding was carried out by imaging a 2x2µm ROI at the sarcolemma using an MP laser at ~70% power. In general, images were acquired every 1.3 seconds for ~3min. Photobleach controls were carried out when necessary to ensure minimal dye uptake or GFP bleaching as a result of imaging.

FM1-43 uptake assay: For analysis of FM1-43 uptake in resting muscle fibers, cells were imaged in residual physiological saline solution (PSS: 15mM Hepes, 145mM NaCl, 5.6mM KCl, 2.2mM $CaCl_2$, 0.5mM $MgCl_2$, and 5.6mM dextrose) to obtain a "baseline"

recording of fluorescence intensity. Media was then changed to PSS containing 2.5μM FM1-43 (Invitrogen: T3163) and cells were imaged using the FM1-43 imaging set-up described above at a frame rate of 1/30 sec for a total of 15 min. To examine the effect of dynasore on resting FM1-43 uptake, muscle cells were first incubated in PSS containing DMSO ±80μM dynasore (no FM1-43) for 5-40 min and subsequently switched to the appropriate dye containing solution (±dynasore). Laser-wounding assays were performed similar to previously published protocols [95, 107]. Briefly, cells were pre-incubated for 10min in physiological saline containing 2.5μM FM1-43 to ensure complete labeling of all exposed membrane compartments. Fibers were then subjected to laser-induced wounding as described above. The multiphoton laser was calibrated prior to every experiment and was used at a fixed intensity for the duration of each experiment to ensure production of equivalent wounds across all cells. To examine the effect of dynasore on wound-induced FM1-43 uptake, cells were ”loaded” for 10min with FM1-43 to ensure equal labeling prior to wounding, incubated for 5 min in PSS + FM1-43 ±dynasore, and subjected to laser-induced wounding.

Calcium influx assays: Isolated fibers were incubated with 3μM Fluo-4-AM (Invitrogen: F-14201) diluted in MEM+10%FBS for 60min at 37°C [108]. Cells were washed once with MEM+10%FBS and incubated for 10min at 37°C to allow cleavage by esterases and reduce dye leakage. Prior to imaging, media was changed to PSS and fibers subjected to laser wounding as described above. Fluorescence intensity at the wound was quantified by a 10x10μm ROI centered at the wound and distal fluorescence intensity was quantified using a 10x10μm ROI placed on the membrane opposite the

wound. Fluorescence intensity is plotted as $\Delta F/F_0$ ($(F_t-F_0)/F_0$) and the time to reversal in Fluo-4 experiments was estimated as the inflection point on the first derivative graph of this time course.

RESULTS

FM1-43 uptake in resting adult skeletal muscle fibers requires dynamin activity. Multiple membrane transport proteins have been linked to muscle disease, including dynamin-2, and in some cell types, endocytosis has been shown to play a critical role in plasma membrane repair [103, 105]. FM1-43 dye uptake after laser wounding is a standard assay for membrane repair, but FM1-43 also has been used in other cell types to monitor cellular endocytosis [100]. Little is known about the resting endocytic activity of adult skeletal muscle fibers. The standard assay for membrane repair utilizes a preincubation of fibers in media containing FM1-43 dye prior to laser-induced membrane wounding. Wild-type skeletal muscle fibers from adult C57BL/6 mice were isolated and incubated with a solution containing 2.5μM FM1-43, which led to a rapid increase in cellular fluorescence that reached maximal intensity at ~10 min post FM1-43 addition (**Fig 2.1A**). To determine whether the prolonged increase in FM1-43 labeling was due to dye uptake *via* endocytosis, resting adult skeletal muscle fibers were incubated with FM1-43 in the presence of DMSO ± dynasore, a potent inhibitor of dynamin-dependent endocytosis (outlined in **Fig 2.1B**). FM1-43 labeling in resting adult skeletal muscle fibers was not affected by DMSO-treatment, (**Fig 2.1B** top, quantified **2.1C**) but is almost completely abolished in the presence of dynasore (**Fig 2.1B** middle, quantified in **2.1C**). The inhibitory effect of dynasore on FM1-43 labeling is reversible, as FM1-43 uptake commences upon removal of dynasore from the extracellular solution

(**Fig 2.1B** bottom, quantified **2.1D**). These data indicate that FM1-43 dye uptake *via* dynamin-dependent endocytosis is the major mechanism by which adult skeletal muscle fibers become labeled with FM1-43 at rest.

Wound-induced FM1-43 uptake is dynamin-dependent in adult skeletal muscle fibers. Elevated wound-induced FM1-43 fluorescence after membrane wounding has been assumed to occur by dye entry from the extracellular buffer through nascent lesions and binding to intracellular lipids [109]. The accumulation of dye fluorescence is commonly used as a gold standard assay to study membrane resealing and quantify defective membrane repair [95, 96, 110]. However, previous studies have not definitively examined the mechanism of how wound-induced dye uptake occurs, and whether this might be mediated by endocytosis. Given that FM1-43 uptake in resting adult skeletal muscle fibers is highly dependent on dynamin activity, we posited that wound-induced FM1-43 uptake may also be dynamin-dependent. To test this, muscle fibers were isolated from adult C57BL/6 mice and "loaded" with FM1-43 for 10 minutes to ensure equal and complete FM1-43 labeling prior to wounding. Once loaded, fibers were switched to solution containing FM1-43 ± dynasore and subjected to laser-induced wounding (outlined in **Fig 2.2A**). Consistent with our hypothesis, acute (~5 min) treatment with 80μM dynasore markedly reduced FM1-43 uptake following wounding compared to DMSO treated control cells, and this effect was reversible following removal of dynasore from the extracellular solution (**Fig 2.2B**, quantified **2.2C**). We independently confirm these results using an even more potent inhibitor of dynamin, Dyngo4a, and show that Dyngo4a also significantly decreases the uptake of FM1-43

after wounding (**Fig 2.2C**) [111]. These findings suggest that membrane wounding elicits a considerable dynamin-dependent endocytic response that contributes to FM1-43 uptake after wounding. We show that wound-induced FM1-43 uptake does not occur solely through membrane lesions, but in fact the majority of wound-induced dye uptake occurs *via* dynamin-dependent endocytosis in adult skeletal muscle fibers.

FM1-43 uptake after membrane wounding is dependent upon extracellular dye. To further exemplify that the increase in FM1-43 fluorescence after wounding is caused by uptake of the extracellular dye in the media, the plasma membrane of skeletal muscle fibers isolated from adult C57BL/6 mice was labeled with FM1-43 for ~10min, and then all extracellular FM1-43 was removed, and the fibers were wounded in the presence or absence of 80μM dynasore, (outlined in **Fig 2.3A)**. We note that the washout of extracellular FM1-43 removes a majority of the FM1-43 fluorescence that appears at the wound, and again, most of the FM1-43 uptake in the presence of extracellular dye is inhibited by dynasore (**Fig 2.3B**, quantified **2.3C**). These data suggest that FM1-43 is required in the extracellular media to produce wound-induced dye uptake both by endocytic mechanisms (inhibited by dynasore) and through the membrane wound itself (not inhibited by dynasore). Interestingly, however, there is a small area of FM1-43 fluorescence directly at the wound that appears rapidly after wounding in the absence of extracellular FM1-43. This suggest that lipids labeled by FM1-43 prior to wounding rapidly occupy the wounded area and can contribute to FM1-43 fluorescence following a membrane wound, albeit to a smaller extent than dye uptake through endocytosis and membrane lesions. Together this data suggests that dynamic movement of lipids through endocytosis and other mechanisms, are significant contributors to wound

induced FM1-43 uptake and these mechanisms must be considered to fully appreciate the utility of FM1-43 for identifying contributing mechanisms to membrane repair.

Dynamin inhibition reduces wound-induced uptake of FM1-43 into wild-type and dysferlin-deficient muscle fibers. Dysferlin-deficiency has been shown to result in elevated uptake of FM1-43 after membrane wounding, which is the basis for the assertion that dysferlin mutations result in defective membrane repair [95]. Given that wound-induced FM1-43 uptake is dependent upon dynamin-activity in wild-type muscle fibers, we posited that the elevated FM1-43 uptake observed in dysferlin-null skeletal muscle fibers is due to over-activation of dynamin-dependent endocytosis following wounding. Neither maximum FM1-43 labeling nor the rate of uptake were different between resting wild-type (A/WySnJ) and dysferlin-null (A/J) muscle fibers, indicating that dysferlin-deficiency does not affect resting dynamin-dependent FM1-43 uptake (**Fig 2.4A**). Consistent with previous reports, DMSO treated, wounded A/J muscle fibers show increased uptake of FM1-43 after wounding. However, dynasore treatment significantly reduced wound-induced FM1-43 uptake in both wild-type and dysferlin-null muscle fibers and obscured any differences in FM1-43 uptake between the two genotypes (**Fig 2.4B**, quantified **2.4C**). Consistent with our hypothesis, these data suggest that elevated wound-induced uptake of FM1-43 in dysferlin-deficient muscle fibers may be actually due to elevated dynamin activity following wounding in adult muscle fibers.

Dynamin inhibition does not affect endocytosis of dysferlin following wounding in adult skeletal muscle cells. In order to determine whether dynamin and dysferlin are within the same membrane repair pathway and if dynamin may regulate dysferlin function in

some way, we tracked dysferlin trafficking following wounding in the presence or absence of dynamin inhibition. We previously developed a dysferlin-pHluorin muscle specific transgenic mouse (dysf-pHGFP) which allows for real-time tracking of dysferlin endocytosis based on varying fluorescence intensity depending on the specific subcellular compartment (outlined in **Fig 2.4D**) [107]. Consistent with our previous report, surface localized dysferlin-pHGFP molecules adjacent to the lesion are rapidly recruited to the membrane wound (white arrow **Fig 2.4E**, quantified **2.4F**), whereas remaining dysferlin-pHGFP molecules are rapidly quenched in response to wounding (red arrow **Fig 2.4E**, quantified **2.4G**)). Given the fact that dynamin-mediated endocytosis is activated in response to wounding, we sought to examine whether dysferlin endocytosis is a dynamin-dependent pathway in adult skeletal muscle fibers. To address this, real-time changes in dysferlin-pHGFP fluorescence intensity were analyzed following wounding in adult dysf-pHGFP skeletal muscle fibers following treatment with DMSO ± 80μM dynasore. Our data indicate that treatment with dynasore does not affect recruitment of dysf-pHGFP to membrane lesions (**Fig 2.4E**), or endocytosis following wounding (**Fig 2.4F**). Taken together, these data indicate that while dynamin-dependent endocytosis is activated in response to wounding, dysferlin trafficking to membrane wounds and dysferlin endocytosis after wounding is dynamin-independent.

Calcium flux following membrane wounding indicates a rapid repair response as compared to FM1-43 uptake. Since we show that FM1-43 uptake after wounding is largely dependent upon wound-induced endocytosis, we used calcium flux as an endocytosis-independent measure of membrane repair. Calcium influx through

membrane lesions is considered the proximal event following membrane disruption and is thought to be required for activation of the membrane repair machinery [101, 112, 113]. Adult skeletal muscle fibers isolated from adult C57BL/6 mice were incubated with 3μM Fluo-4, a fluorescent calcium indicator, and intracellular calcium levels were measured following laser wounding. Membrane wounding leads to a rapid increase in intracellular calcium concentration near the wound that peaks on average around 40 sec post-wounding and decreases down to a steady state calcium concentration that is higher than the pre-wounded level (**Fig 2.5A, B**). Shortly after wounding, calcium diffuses, increasing the calcium concentration at sites distal to the wound that peaks and reverses near 60s post wound (**Fig 2.5C, D**). Fluo-4 fluorescence shows a clear peak and reversal which seems to suggest that shortly after wounding, influx of extracellular calcium ions is restricted, and calcium begins to be actively removed from the cytoplasm.

We also show that stimulating calcium release from the sarcoplasmic reticulum by treating wounded myofibers with a bolus of caffeine (PSS+10mM Caffeine) increases cytoplasmic calcium above calcium levels measured immediately after membrane wounding (**Fig. 2.5E)**. These data indicate that the reversal in Fluo-4 fluorescence is a direct result of decreased cytoplasmic calcium concentrations and not artifact caused by dye leakage through the plasma membrane breach. These data suggest that Fluo-4 under these conditions can capture the peak post-wound cytoplasmic calcium without saturating the microscope detectors or binding capacity of the Fluo-4 indicator. We go on to show that cytoplasmic calcium increases after wounding are dependent upon extracellular calcium flowing down its electrochemical gradient as when extracellular

calcium is removed and any trace amounts of calcium chelated with 1mM EGTA, minimal changes in intracellular calcium concentrations are observed (**Fig 2.5F**). Importantly, the kinetics of calcium reversal are rapid and in stark contrast to FM1-43 dye uptake, which shows a bi-phasic response of an initial rapid increase followed by a gradual persistent increase in fluorescence intensity over the course of several minutes post sarcolemma wounding (**Fig 2.5G**). Taken together, these data confirm that measuring calcium concentrations provide a sensitive, robust assay for the accurate indication of the point at which calcium influx slows and efflux mechanisms begin to dominate, and can provide an important readout for when barrier function to the plasma membrane is likely restored.

Calcium influx after membrane wounding is largely dynamin-independent. We show that FM1-43 uptake after wounding is dependent upon dynamin activity and FM1-43 uptake shows markedly slow kinetics compared to the estimates of wound-induced resealing as measured by calcium influx. To confirm that calcium flux after wounding is not dependent upon dynamin activity, Fluo-4 loaded muscle fibers were treated with 80µM dynasore or vehicle control (DMSO) and wounded. Following treatment and wounding, muscle fibers show a rapid increase in calcium similar to vehicle control treated cells (**Fig 2.6A,** quantified **2.6B**). There is a modest effect of dynasore treatment on the reversal of Fluo-4 fluorescence that results in a slightly faster reversal in calcium toward baseline compared to vehicle control treated cells (**Fig 5C**). These modest effects on cytoplasmic calcium after wounding suggest inhibition of dynamin by dynasore has some effects on compartmentalization of calcium after wounding, but importantly, the calcium influx immediately after membrane wounding is unaffected by dynasore

treatment. These data further support the conclusions that calcium influx and reversal is largely dynamin-independent, and FM 1-43 uptake is largely dynamin-dependent, and these two assays provide complimentary insight into the distinct kinetic steps and mechanisms of membrane repair.

Dysferlin-Deficient muscle fibers have increased calcium influx after wounding. We showed that dynasore treatment severely blunts the uptake of FM1-43 into wounded dysferlin-deficient muscle fibers. We also show that dynasore treatment effectively removes any difference in FM1-43 uptake between wild-type and dysferlin-deficient fibers. Therefore, we investigated whether calcium influx in dysferlin-deficient muscle was increased compared to wild-type. Isolated muscle fibers from dysferlin-deficient BLA/J mice and wild-type (C57BL/6) littermate controls were loaded with 3μM Fluo-4 and laser-wounded. Immediately after wounding, calcium influx into dysferlin-deficient muscle is increased compared to wild-type (**Fig 2.7A-D**). However, while the peak amount of calcium is increased in BLA/J mice, calcium reverses similarly in both genotypes (solid line **Fig 2.7B, 2.7E**). Interestingly, while we noted previously that calcium after wounding does not return to pre-wound levels in wild-type cells, this persistent increase in calcium is exacerbated in dysferlin-deficient muscle fibers (**Fig 2.7F**).

DISCUSSION

Elevated uptake of extracellular dyes following laser-wounding has been noted in multiple models of muscle disease; a phenotype that is commonly attributed to defective membrane repair following membrane wounding. In fact, almost all experiments investigating the role of a given protein in membrane repair have relied on measuring

uptake of extracellular dyes following wounding. Importantly, FM1-43 uptake was traditionally used to measure endocytosis, but whether endocytosis is activated following wounding in adult muscle fibers is unknown. In this study, we show that FM1-43 labeling in resting muscle fibers is completely reduced in the presence of a dynamin inhibitor. Furthermore, wound-induced FM1-43 uptake is significantly reduced when dynamin-dependent endocytosis is inhibited. These findings strongly suggest that the FM1-43 uptake assay commonly used to assess repair capacity in skeletal muscle fibers measures a combination of dye influx through membrane lesions and a massive endocytic response to wounding and highlight a need for additional measures of membrane resealing in adult skeletal muscle. We show that calcium influx after wounding occurs *via* a largely dynamin-independent mechanism, and further highlight its utility in studying membrane repair by showing that dysferlin-deficiency results in increased influx of calcium after wounding. These findings highlight a previously unappreciated role for dynamin-dependent endocytosis in resting and wounded skeletal muscle and inform the membrane resealing process in adult skeletal muscle.

There is still a significant lack of knowledge with regards to which transport pathways are activated following wounding, and which membrane transport pathways contribute to membrane repair in adult skeletal muscle. While evidence for lysosomal or organelle exocytosis in muscle membrane repair exists [97, 114], the role of endocytosis in muscle membrane repair is largely unknown. The use of extracellular, lipophilic FM-dyes (FM1-43 and FM4-64) to indirectly measure resealing capacity has become the gold standard assay for membrane repair used as the primary method to identify putative membrane repair proteins based on the general principle that flow of

dye through lesions ("dye uptake") should be greatest in cells with impaired membrane resealing. However, FM-dyes are classically used to measure endocytosis following electrical stimulation or mechanical transection in neurons and the possibility that a similar mechanism exists in adult skeletal muscle has never been examined [115]. Our data in resting adult skeletal muscle fibers indicates that FM1-43 labeling is a gradual process that occurs over several minutes, a finding that is more consistent with dye uptake as an active process rather than simply passive labeling of the plasma membrane and t-tubules. Consistent with this interpretation, FM1-43 labeling is almost completely abolished in the absence of dynamin activity, suggesting that FM1-43 labeling occurs *via* delivery of extracellular dye into undefined intracellular compartments through dynamin-dependent endocytosis in adult skeletal muscle fibers.

A logical next step was to determine whether dynamin-mediated endocytosis is responsible for any or all of the dye uptake that occurs following acute membrane wounding in adult skeletal muscle. Our results suggest that the vast majority of wound-induced dye uptake is dependent upon dynamin activity, which supports the overall conclusion that dynamin-dependent endocytosis is responsible for much of the dye uptake that occurs following wounding. Dysferlin-deficient muscle as well as several other genetic models of muscle disease show a characteristic elevation of wound-induced FM1-43 uptake, which is the basis for the assertion that membrane resealing is impaired in this and other model systems [95, 116]. If FM1-43 is measuring endocytosis, then it is possible that the “membrane repair deficiency” phenotype commonly attributed to genetic models of muscle disease may actually be due to elevated wound-induced endocytosis.

Direct comparison of FM1-43 uptake to calcium influx after wounding suggests membrane repair is a much faster process than previously described by FM1-43 assays, and more consistent with previous estimates of resealing kinetics [37]. The time course of FM1-43 appears to have both a fast component, possibly due to entry through the lesion at the wound site and wound-induced endocytosis, and a slow component, primarily due to wound-induced, dynamin-dependent endocytosis. These results indicate that the two assays are measuring two separable entities, both of which could possibly play a role in membrane repair. Wounding dramatically increases cytoplasmic calcium, followed by a quick reversal in calcium concentrations measured by the fluorescent dyes loaded into cells. However, even several minutes after wounding, calcium remains elevated globally and compartmentalized in the cytoplasm immediately adjacent to the wound. Prolonged elevation of calcium in the cytoplasm near and distal to the wound could be a result of incomplete repair resulting in smaller, sustained calcium leak into the cytoplasm. Indeed, while we interpret the reversal of calcium levels as an indicator of resealing, it could also be interpreted as the time point where calcium efflux from the cytoplasm or *via* calcium reuptake into organelles lacking Fluo-4, exceeds calcium influx through the wound. Previous work has suggested that dysferlin may alter sarcoplasmic reticulum calcium handling [117] although Fluo-4 under the experimental conditions here should load the sarcoplasmic reticulum as well, and thus altered SR function may not explain the overall elevated calcium after sarcolemma wounding in dysferlin-deficient mice. The localized high levels of calcium could be the result of calcium compartmentalization by organelles proximal to the membrane wound. Recent work by Horn et. al. in differentiated myotubes showed that calcium influx after

laser wounding results in increased mitochondrial calcium that stimulates mitochondrial ROS production, which interestingly, has a positive effect on membrane repair [118]. This is consistent with the possibility that mitochondria or other organelles play a role in locally compartmentalizing calcium after membrane wounding, but the contribution of individual organelle compartments to calcium compartmentalization in adult fibers requires further experimentation. Finally, calcium influx through membrane wounds, plays an important but complicated role in regulating many aspects of muscle membrane repair. Calcium influx causes local muscle fiber contraction, and activates the lipid binding properties of dysferlin[60] and annexins[70] which may play an important role in bringing repair proteins and lipids to the membrane wound. Therefore, removing extracellular calcium in the extracellular buffer actually increases FM1-43 uptake[28], despite calcium being important for activating most forms of endocytosis in other cell types [119]. Prolonged excess intracellular calcium may also be detrimental to fibers by disrupting excitation coupling, overactivating the mitochondrial permeability transition pore and cell death, and/or downstream calcium-activated proteolysis, which may lead to muscle fiber degeneration [120].

Whether wound-induced endocytosis contributes to membrane resealing or is a detrimental consequence of sarcolemmal wounding is still unclear. One possibility is that membrane wounding activates both an endocytic (*via* dynamin) and an exocytic response which both contribute to membrane repair. In this case, mutant models could show if elevated dynamin-dependent endocytosis (FM1-43 uptake) is a compensatory response to facilitate membrane repair in the absence of other repair pathways (such as wound-induced exocytosis) (**Fig 2.8A**). Dynamin-2 mutant mouse models, expressing

mutations associated with centronuclear myopathy, have a complicated phenotype due to its additional role in t-tubule and triad biogenesis in muscle [121-123], but expression of dynamin-2 mutants does result in elevated resting calcium in muscle [124]. Alternatively, wound-induced dynamin-dependent endocytosis may be necessary to form an undefined population of membrane repair vesicles. In this scenario, elevated dynamin-dependent endocytosis (FM1-43 uptake) in mutant models may indicate defective fusion of nascent repair vesicles with the plasma membrane (**Fig 2.8B**). Indeed, accumulation of subsarcolemma vesicles is a common observation in electron micrographs of muscle fibers from dysferlin deficient muscular dystrophy patients and mice [95].

A balance of dynamin activity in muscle fibers is critical for muscle fiber health, as either genetic loss or over-expression of dynamin-2 results in muscle disease [125]. Furthermore, dynamin expression is elevated in a mouse model of myotubular myopathy and reducing dynamin levels restores muscle structure and function [126]. Therefore, it is possible that elevated dynamin-activity following wounding exacerbates muscle disease in membrane repair deficient skeletal muscles and reducing dynamin function may be beneficial for muscle function. However, further work needs to be done to characterize dynamin levels and potential post-translational modifications of dynamin that may give rise to enhanced dynamin-dependent endocytosis following wounding in the various genetic models of membrane repair deficiency. It is likely that dynamin acts in a separate pathway from that of dysferlin in membrane repair, as our data indicates that inhibition of dynamin activity does not appear to affect dysferlin transport following wounding (**Fig 2.4**). Thus, we propose the general model (shown in **Fig 2.8**) that a

localized influx of calcium through lesions activates dysferlin-mediated membrane repair, which feeds back to inhibit further calcium influx and minimizes activation of dynamin-dependent endocytosis. In the absence of dysferlin, dysferlin-mediated membrane repair is impaired, leading to an increased influx of calcium (**Fig 2.7**), over-activation of dynamin-dependent endocytosis and activation of downstream, potentially pathological, pathways.

Our data reveal a potential role for dynamin in wound-induced endocytosis in membrane repair and highlight modulation of dynamin levels or activity as a potential therapeutic approach for muscle disease. Furthermore, this study demonstrates that several independent measurements of membrane resealing capacity are needed and highlight that analysis of calcium dynamics in wounded cells may be a useful tool for studying sarcolemma resealing. Future work will focus on assessing inhibition of overactivated dynamin-activity as a therapeutic approach in dysferlin-deficiency.

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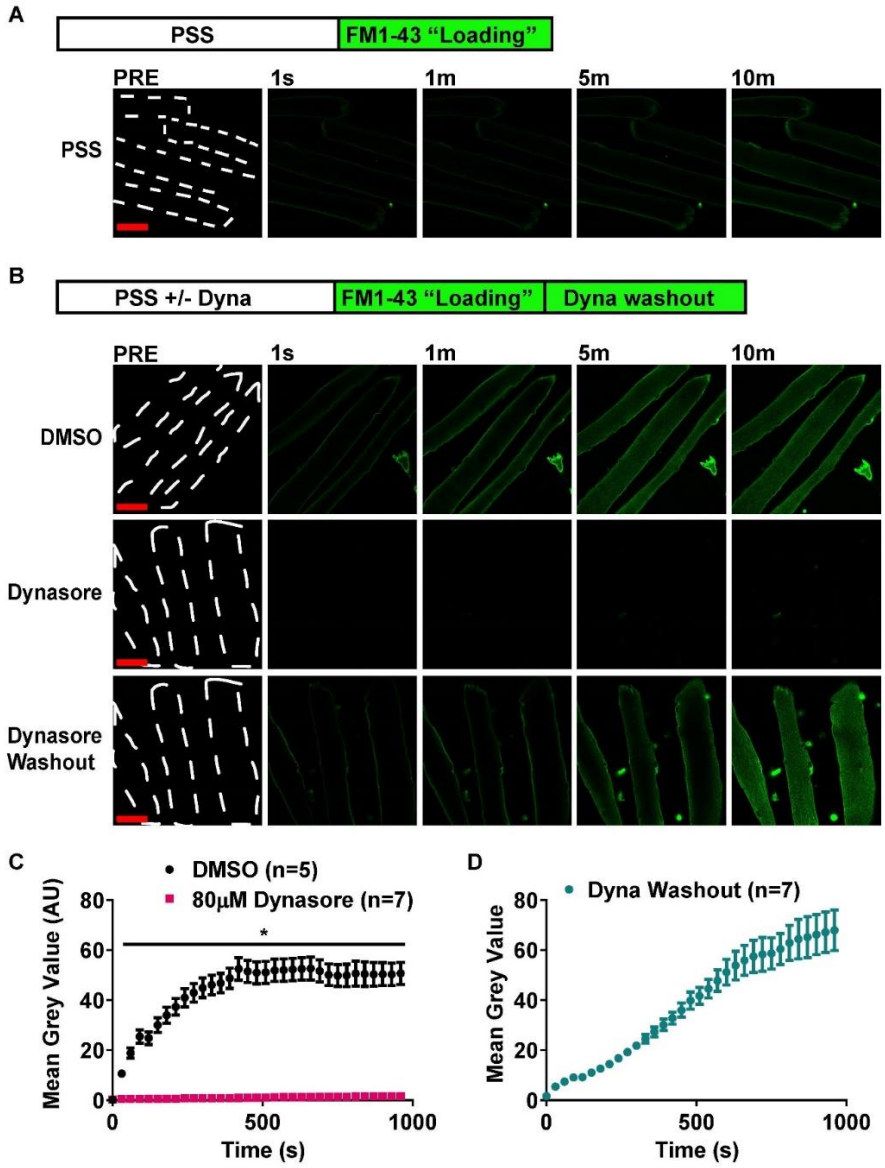

Figure 2.1. Resting adult skeletal muscle fibers take up FM1-43 *via* a dynamin-dependent mechanism. FM1-43 labeling occurs over several minutes in adult skeletal muscle fibers. FM1-43 uptake was assessed in untreated, resting adult C57BL/6 skeletal muscle fibers (**A**) and in resting adult skeletal muscle fibers following pre-treatment with DMSO or dynamin inhibitor Dynasore (**B**). Untreated and DMSO treated fibers take up extracellular FM1-43 with maximal labeling occurring within 10min (**A** and **B** top, respectively, quantified in **C**). Pre-treatment with 80μM Dynasore completely inhibits FM1-43 uptake in resting skeletal muscle fibers (**B**, middle, quantified in **C**). Dynasore-treated muscle fibers take up FM1-43 following removal of Dynasore from the extracellular solution (**C** bottom, quantified **D**). Statistical significance ($p<0.05$) between DMSO and Dynasore is denoted by (*****) for each timepoint under horizontal bar.

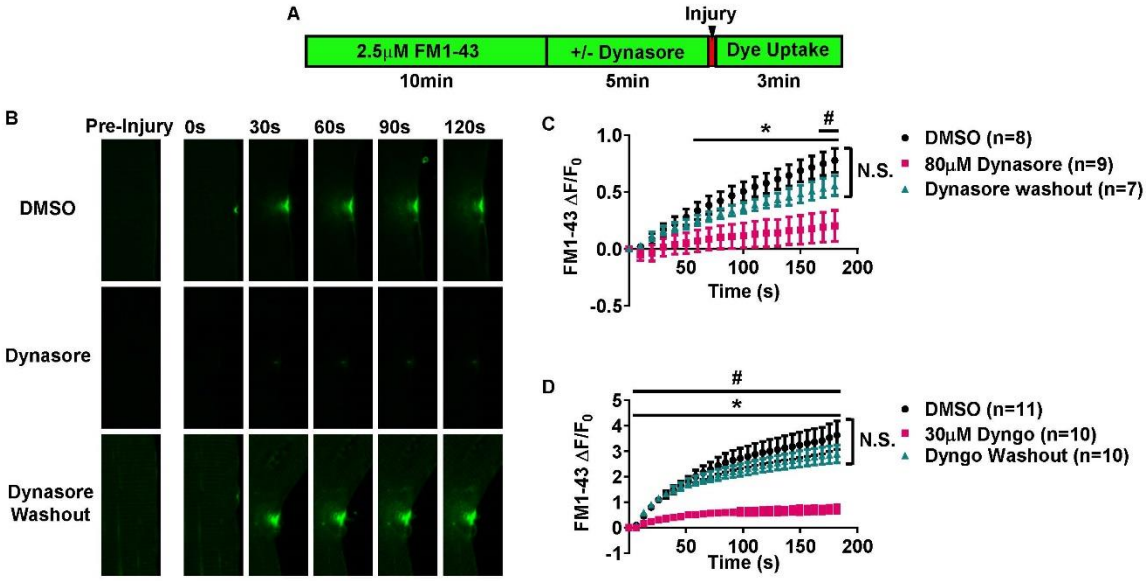

Figure 2.2. Wound-induced FM1-43 uptake is dynamin dependent in adult skeletal muscle fibers. C57BL/6 muscle fibers were loaded with FM1-43 normally, treated with DMSO +/- 80μM Dynasore and subjected to laser-induced wounding (**A**). Dynasore treatment (middle **B**, magenta squares in **C**) results in significantly reduced wound-induced FM1-43 uptake compared to DMSO treated control cells (top **B**, black circles in **C**). FM1-43 uptake in Dynasore-washout cells was not significantly different than DMSO treated controls, indicating a reversible effect of Dynasore treatment on wound-induced FM1-43 uptake in skeletal muscle cells (bottom **B**, teal triangles **C**). These results were independently confirmed using the same experimental setup (**A**) with an additional dynamin inhibitor, Dyngo4a, again showing that dynamin inhibition results in significantly reduced wound-induced FM1-43 uptake compared to DMSO controls, which is reversible upon Dyngo4A washout (**C**). Statistical significance ($p<0.05$) between DMSO and Dynasore/Dyngo4a is denoted by (*****) and between Dynasore/Dyngo4a and Washout by (#) for each timepoint under horizontal bar. **N.S.** designates no statistical difference ($p>0.05$) between DMSO and Washout conditions in both **C** and **D**.

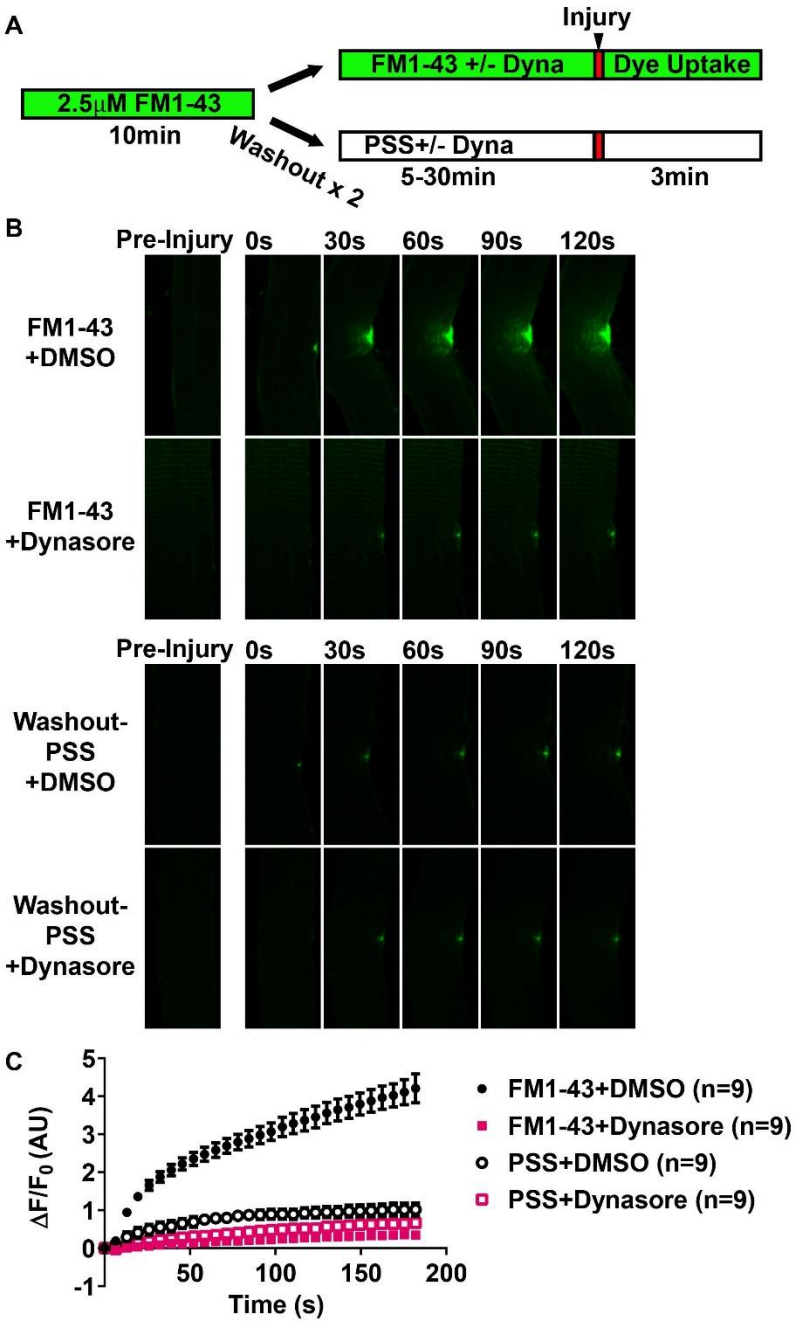

Figure 2.3. FM1-43 uptake after injury is dependent upon extracellular FM1-43. Isolated skeletal muscle fibers from adult C57BL/6 mice were loaded with 2.5μM FM1-43 for ~10min and wounded in the presence or absence of Dynasore (**A**). Washout conditions were removed of extracellular FM1-43 and cells then wounded in the presence or absence of Dynasore. Representative images of all conditions show that FM1-43 washout severely decreases FM1-43 fluorescence after wounding, however there is a rapid accumulation of dye at the wound that is uninhibited by Dynasore treatment (**B,** quantified, **C**).

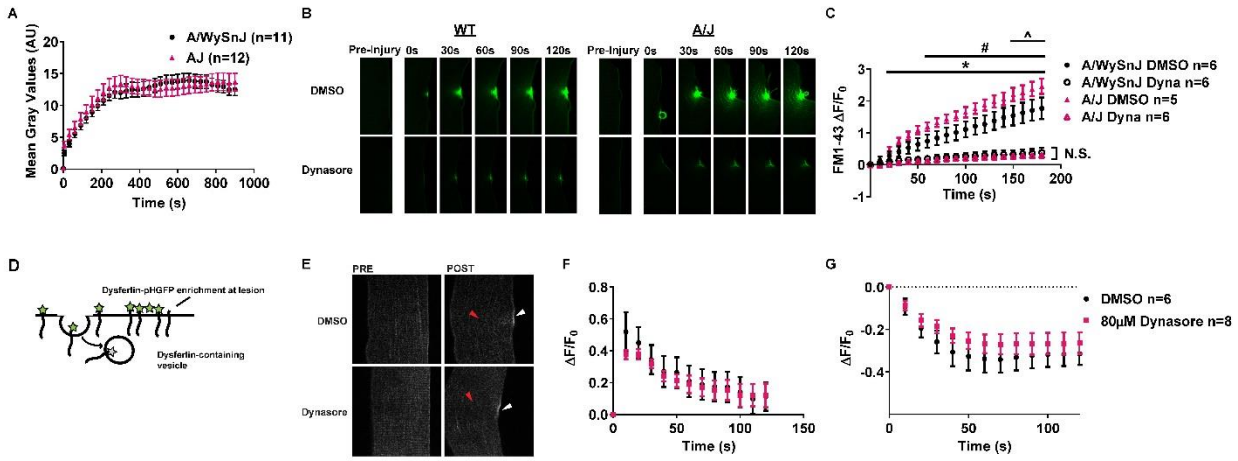

Figure 2.4. Dynamin is required for increased wound-induced uptake of FM1-43 in dysferlin-null muscle fibers. Basal FM1-43 uptake is unchanged in dysferlin-null A/J muscle fibers compared to A/WySnJ wild-type controls **(A)**. DMSO-treated, dysferlin null muscle fibers (representative images **B**, magenta triangles in **C**) take up more FM1-43 dye following wounding than wild-type controls (representative images **B**, black circles in **C**). Dynasore treatment significantly reduces wound-induced FM1-43 uptake in wild-type and dysferlin-null muscle fibers (representative images **B**, open black circles and open magenta triangles in **C**, respectively). Statistical significance ($p<0.05$) between A/J DMSO and A/J Dyna is denoted by (*****) and between A/WySnJ DMSO and A/WySnJ Dyna by (#) and between A/WySnJ DMSO and A/J DMSO by (**^**) for each timepoint under horizontal bar. **N.S.** designates no statistical difference ($p>0.05$) between A/WySnJ Dyna and A/J Dyna. The dysf-pHGFP TG reporter mouse provides a real-time assessment of dysferlin localization in adult skeletal muscle fibers (**D**). Recruitment of dysferlin to lesions (white arrowhead **E**, quantified **F**) and endocytosis of dysferlin following wounding (red arrowhead **E**, quantified **G**) are unchanged in Dynasore-treated cells, indicating that dynamin activity is not required for dysferlin transport following wounding.

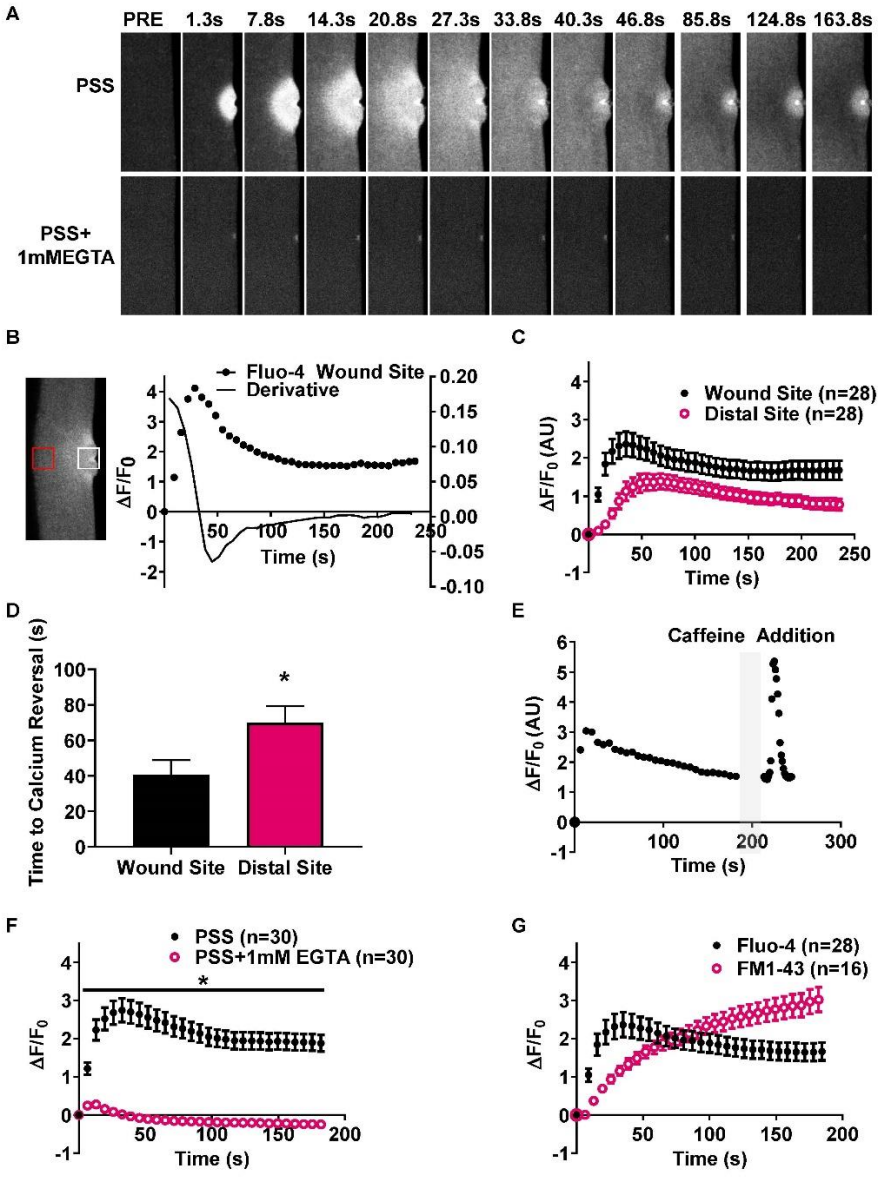

Figure 2.5. Calcium uptake into the cell as measured by Fluo-4 relative fluorescence shows membrane repair is a much faster process than as previously indicated by FM1-43 wounding studies. Isolated wild-type C57BL/6 muscle fibers were loaded with 3μM Fluo-4 for 1hr and then injured with a high-powered laser, representative images shown (top **A**). Calcium increases at the wound site were quantified (white box in **B)** which showed that injury causes a rapid increase in calcium at the wound site (one representative trace shown **B**, summary data from one representative experiment **C**). Calcium increases at the wound are followed by an increase in cytoplasmic calcium at sites distal to the wound (red box in **B**, and magenta open circles **C**). Calcium at the wound peaks and reverses at the wound at approximately 40s post injury (solid line **B**, summary data in **D**), but cytoplasmic calcium reversal is significantly delayed to approximately 60s **(D)** A bolus of 10mM caffeine to injured fibers, one representative trace shown, stimulates calcium release from the SR that exceeds peak calcium after injury **(E)**. Removing calcium from extracellular media and chelating any trace calcium with 1mM EGTA prevents the increase in cytosolic calcium after injury (representative images **A** bottom, quantified **F**). While calcium uptake reverses within the first minute following wounding, the concentration of FM1-43 rises in the cell for more than 2 minutes, and then continues to increase at a constant rate even after 2 minutes (**G**)**.**

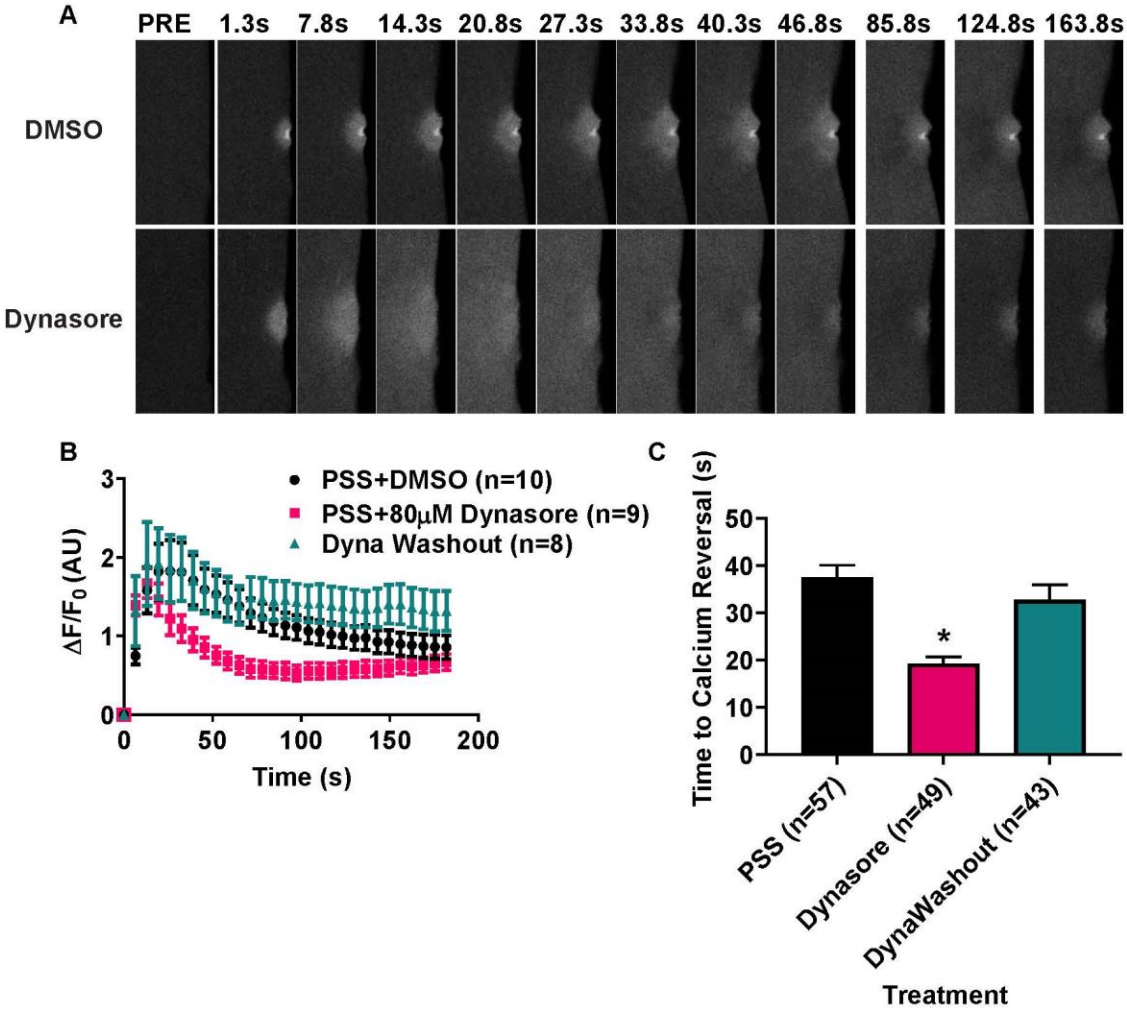

Figure 2.6. Dynamin inhibition does not affect calcium uptake after injury. Isolated wild-type C57BL/6 muscle fibers were loaded with 3μM Fluo-4 for 1hr and treated either with a vehicle (DMSO, top panel) or 80μM Dynasore (bottom panel) prior to injury showing calcium uptake after injury is uninhibited by Dynasore treatment (representative images **A**, quantified **B**). Quantification of the time at which the calcium signal peaks and reverses across 6 independent replicates shows Dynasore treated cells have a faster time to peak calcium compared to vehicle control (**C**). Statistical significance ($p<0.05$) between Dynasore and both PSS and Washout is denoted by (*****).

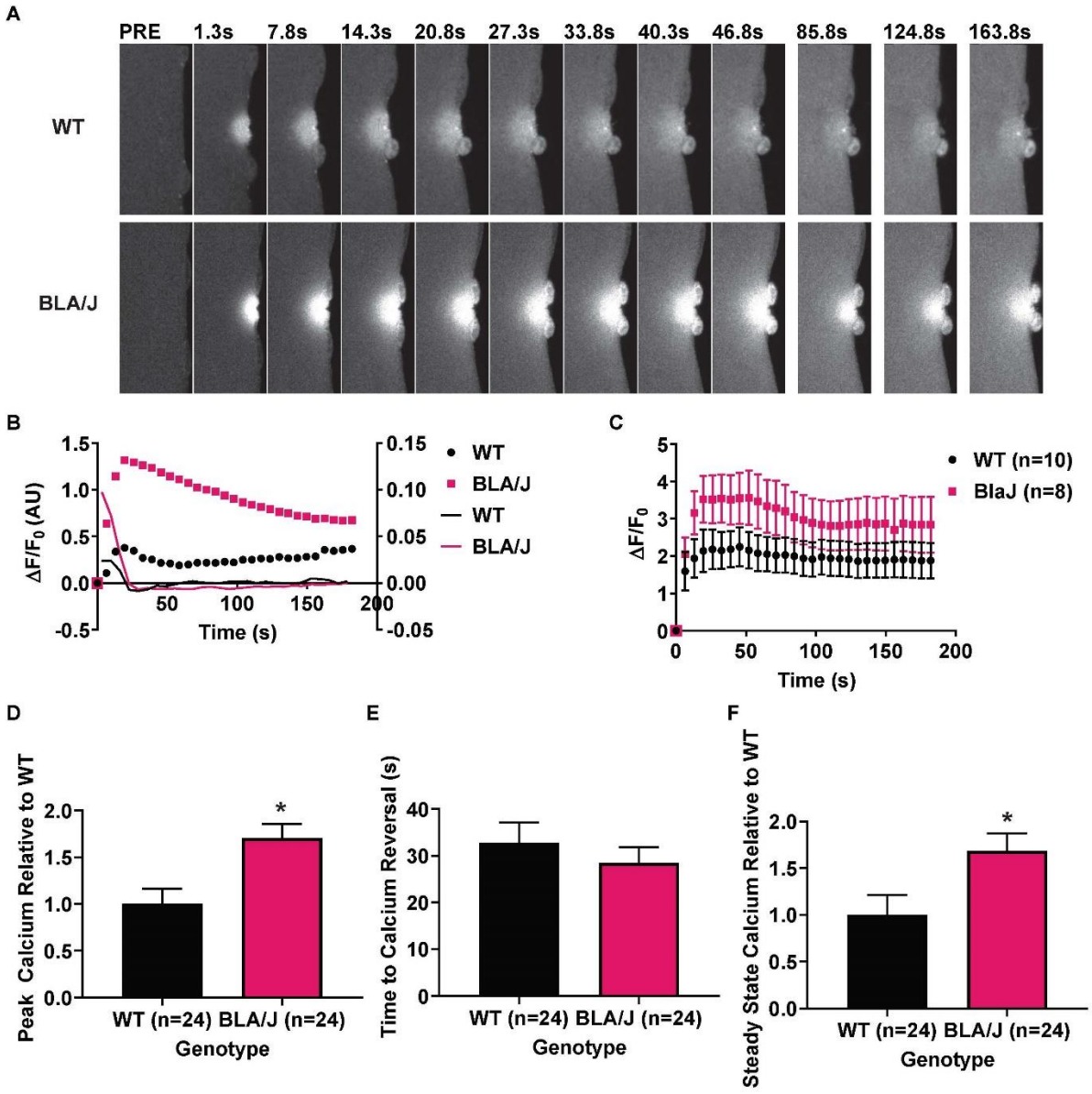

Figure 2.7. Dysferlin-deficient muscle fibers have persistently elevated calcium after injury. Isolated muscle fibers from dysferlin-deficient BLA/J mice and wild-type littermate controls (C57BL/6) were loaded with 3μM Fluo-4 for 1hr and injured with a high-powered laser (representative images **A**). Calcium concentrations after injury are increased in dysferlin-deficient fibers (individual traces **B**, summary of one experiment **C**), Peak calcium was normalized to WT peak calcium and pooled among three independent replicates shown that peak calcium uptake is increased in dysferlin-deficient cells (**D**), but the time to calcium reversal is similar in WT and dysferlin-deficient cells (solid line **D**, summary **E**). The steady state calcium at the end of imaging for three independent replicates was normalized to WT steady state calcium, which is also greater in dysferlin deficient cells (**F**).

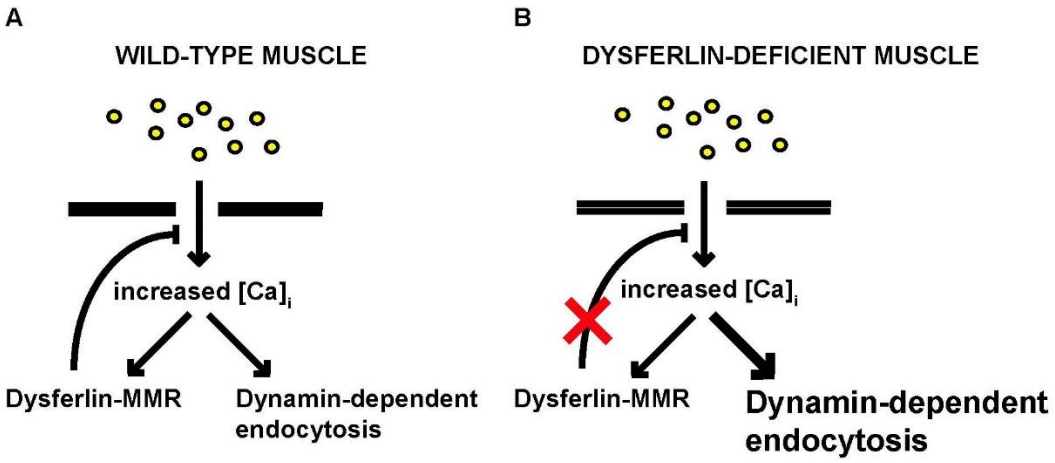

Figure 2.8. General model of dynamin-mediated endocytosis and dysferlin-mediated membrane repair in skeletal muscle. In normal muscle, sarcolemma wounding leads to localized calcium influx and activation of dysferlin-mediated membrane repair, preventing over-activation of dynamin-mediated endocytosis (**A**). In dysferlin-deficient muscle wherein dysferlin-mediated repair is impaired, prolonged calcium influx through unsealed lesions leads to increased activation of dynamin-dependent endocytosis which may compensate for impaired dysferlin-MMR or contribute to disease pathology (**B**)

CHAPTER 3

Subsarcolemmal Cytoskeleton in Dysferlin-Mediated Muscle Membrane Repair

ABSTRACT

Plasma membrane repair is critically important to maintain cellular membrane integrity and especially important in mechanically active tissues like skeletal and cardiac muscle. The mechanisms of plasma membrane repair remain unclear, but previous work from our laboratory has shown that following injury, one critical membrane repair protein, dysferlin, is rapidly recruited from the lateral sarcolemma to the wound site, and this can be inhibited by pharmacological agents that disrupt the actin cytoskeleton. However, the actin isoform responsible for dysferlin recruitment remains unknown. Cytoplasmic γ-actin localizes to the costameres of the muscle fiber and is one of the major actin isoforms that forms the subsarcolemmal actin cytoskeleton. Genetic disruption of γ-actin in mouse muscle causes a mild degenerative myopathy with some shared, mildly progressive phenotypes as dysferlin deficiency. Therefore, in the current study, we studied mice with genetic loss of γ-actin in differentiated muscle to investigate whether γ-actin may be a critical actin isoform in dysferlin recruitment and thus efficient membrane repair. Muscle fibers isolated from the *flexor digitorum brevis* of γ-actin

knockout mice show increased uptake of extracellular FM1-43 and increased uptake of calcium following injury, both indicative of a defect in membrane repair. While we hypothesized γ-actin may play a role in trafficking dysferlin to the site of an injury, our data suggest that dysferlin recruitment is unaffected by the genetic loss of γ-actin. Overall, our results suggest that γ-actin may have an important role in membrane repair in muscle which may contribute to the myopathy observed in the γ-actin-deficient mouse model.

INTRODUCTION

Plasma membrane repair is critical to maintaining the integrity of cellular membranes in the event of a plasma membrane breach. While membrane repair is thought to occur in nearly all cell types, the repair process becomes increasingly important in mechanically active cells, particularly those under high external load, such as skeletal or cardiac muscle fibers [127-130]. Frequent muscle stretch and contraction has been shown to result in acute damage to the plasma membrane that is quickly repaired, preventing significant muscle fiber death and loss [10]. In many forms of muscular dystrophy, muscles become more susceptible to mechanical injury through loss of proteins that maintain membrane stability [20, 131, 132]. However, in certain types of muscular dystrophy, disruption of the membrane repair process also compromises the preservation of sarcolemma integrity and leads to muscle degeneration and progressive muscle wasting [31, 32, 68]. While many putative membrane repair proteins have been identified, the mechanism of membrane repair remains unclear [28, 68, 73].

The current model of membrane repair in skeletal muscle posits that calcium entry through membrane wounds stimulates the rapid recruitment of critical membrane repair proteins, such as dysferlin and the annexins, to the site of membrane lesions *via* the actin cytoskeleton [64, 74]. It has also been shown that membrane repair proteins, such as MG53 and dysferlin, are sequestered into intracellular vesicles that are hypothesized to traffic back to membrane wounds wherein vesicle fusion replenishes plasma membrane lipids and restores barrier function to the membrane [33, 64, 133]. While the formation of intracellular vesicles after injury has been directly observed in skeletal muscle, it is still unclear how these vesicles are trafficked, how membrane resealing occurs, and how it may be facilitated by these membrane compartments and proteins.

Multiple membrane repair proteins have been shown to rely upon the actin cytoskeleton for efficient translocation to membrane wounds [64, 74]. Though, to date, all tools and technologies employed while investigating the role of actin in membrane repair have not had the capability to distinguish between different isoforms of actin. Studies aimed at identifying cellular distribution and function of separate isoforms of actin suggest that each isoform may have distinct, non-redundant functionality, even in cases of high sequence homology [134]. Therefore, isoforms of actin could contribute individually to the membrane repair process, warranting further investigation.

In skeletal muscle, three isoforms of actin are expressed, but display distinct localization: skeletal muscle α-actin, and cytoplasmic β- and γ-actin. The skeletal muscle α-actin is largely associated with the thin filaments of the contractile apparatus of the muscle. Cytoplasmic β- and γ-actins localize to the costameres of adult skeletal muscle

and are the major actin isoforms that form the subsarcolemmal actin cytoskeleton where they have been shown to bind to dystrophin and the dystrophin glycoprotein complex as well as other proteins [135]. When mutated, all three actin isoforms result in a progressive myopathy [136-138]. Mutations in α-actin are associated with Nemaline Myopathy. Genetic loss of β-actin in muscle of mice results in dysregulation of dystrophin and susceptibility to contraction-induced damaged similar to that found in dystrophies associated with mutations in the dystrophin-glycoprotein complex (DGC) [137, 138]. Interestingly, genetic disruption of γ-actin in muscle of mice does not result in alterations in DGC expression or localization directly, but does cause a mild degenerative myopathy through an, as of yet, unknown mechanism [136]. Muscle-specific knockout of γ-actin, while mild, shares some phenotypic similarity with muscular dystrophies associated with dysferlin-mutations, including a higher proportion of centrally nucleated fibers and more modest increases in serum creatine kinase compared to dystrophin deficiencies [136]. Therefore, the purpose of this study was to clarify the role of actin in membrane repair and to identify the contribution of one actin isoform, γ-actin, to membrane repair. We hypothesized that the subsarcolemmal cytoskeleton, and in particular, γ-actin, may regulate dysferlin function after membrane injury and its genetic loss would disrupt dysferlin trafficking, resulting in a membrane repair defect.

In the current study, we use *in vitro* laser injury to observe actin remodeling after injury in adult skeletal muscle fibers and investigate the role of actin, specifically the γ isoform, in membrane repair. We show that the subsarcolemma cytoskeleton is dynamic at membrane wounds and muscle-specific loss of γ-actin is consistent with a membrane

repair defect. However, while we note membrane repair may be affected by the loss of γ-actin, the trafficking of the critical membrane repair protein, dysferlin, is unaffected. These data highlight the isoform diversity among the actin gene family, establish γ-actin as a contributing member of the membrane repair machinery, and suggest a potential mechanism for the myopathy observed in mouse models of muscle-specific knockout of γ-actin.

MATERIALS AND METHODS

Animals: WT floxed controls and muscle specific γ-actin knockout animals (Actg1$^{-/-}$) muscle-specific knockout animals were obtained as a gift from Dr. James Ervasti. For dysferlin-pHGFP (dysf-pHGFP) experiments, Actg1 $^{-/-}$ animals were bred to the dysf-pHGFP reporter mouse developed by our laboratory to produce floxed control and Actg1$^{-/-}$ littermates expressing a dysferlin-pHluorin transgene [64]. All experiments were performed in mice between 8-12 weeks of age, prior to the onset of significant muscle disease. All procedures were carried out in accordance with IACUC protocols at the University of Michigan.

Muscle fiber isolation: Single muscle fibers were isolated from the adult mouse *flexor digitorum brevis* (FDB) using methods consistent with published protocols [139]. Briefly, FDB muscle was separated from surrounding connective tissue and excised from the adult mouse foot and digested in MEM+0.2% collagenase for approximately four hours at 37°C with gentle agitation. Following digestion, single muscle fibers were mechanically separated with forceps and further detached by gentle trituration with glass pipettes of decreasing pore size. Single fibers were then plated on glass bottomed

dishes coated with 10% Matrigel and allowed to adhere for 20 minutes prior to experimentation.

Membrane Injury Experiments: Single muscle fibers were injured according to previously published protocols from our laboratory [64]. All live cell injury experiments were completed using a Leica TCS SP8 MP equipped with a temperature-controlled chamber. Fibers were imaged through a 63x oil objective at 2x optical zoom and set to excite at 488nm and detect at 580-620nm (FM1-43) or 498-525nm (Fluo-4, dysf-pHGFP). For all injury experiments, three images of the uninjured single fiber were collected and used to calculate the baseline fluorescence and then a 2x2μm injury was produced on the lateral sarcolemma with a high powered multi-photon laser at a fixed wavelength of 890nm at ~80% power. Images were collected for 2-3min following injury.

FM1-43 Uptake Assay: Isolated muscle fibers were incubated in physiological saline (PSS: 15mM Hepes, 145mM NaCl, 5.6mM KCl, 2.2mM $CaCl_2$, 0.5mM $MgCl_2$, and 5.6mM dextrose, pH7.4)+2.5μM FM1-43 (Invitrogen: T3163) for 10min and then injured for 30minutes to 1hr with 2.5μM FM1-43 maintained throughout imaging. *Calcium Uptake Assay*: Isolated muscle fibers were incubated in MEM+10%FBS+3μM Fluo-4 (Invitrogen: F-14201) for 1hr. Loaded fibers were washed with MEM+10%FBS for 10min and then switched to PSS prior to imaging and injured for no longer than 1hr.

Dysferlin-pHGFP Assays: Isolated muscle fibers from dysf-pHGFP animals were injured in PSS to visualize dysf-pHGFP recruitment to membrane wounds. Dysferlin-containing endosomes were visualized similarly to our previously published work by injuring muscle fibers in PSS followed by immediately treating injured muscle fibers with PSS+50mM NH_4Cl and imaging vesicles at a high resolution [64].

Image Analysis**.** *FM1-43 Analysis:* FM1-43 uptake after injury was analyzed using the Analyze Particles macro in Fiji [140, 141]. Data are represented as the integrated density of uptake, which accounts for both the mean intensity and area of uptake to best represent the magnitude of fluorescence but also account for any diffusion of dye from the wound. A detailed protocol is as follows:

1. Before you begin:
    a. Duplicate original time course
    b. Set measurements to measure mean gray values, area, and integrated density (Analyze – Set Measurements)
2. Apply an identical threshold to all duplicated injury time courses.
3. Select the Analyze Particles macro in the Analyze menu of FIJI
    a. Designate that the particles size be between 3 and Infinity to limit the amount of falsely identified particles.
    b. Note no circularity to the particles
    c. Show masks and add particles to an ROI manager
    d. Run the macro
4. Remove any falsely identified ROIs from the ROI manager
5. Display ROIs on the original time course
6. Select all ROIs and measure indicated parameters
7. FM1-43 uptake is graphed as the integrated density (MeanxArea) vs. Time (s)

Fluo-4 Analysis: Fluorescence intensity of Fluo-4 was quantified at the wound within a fixed region of interest that encompassed the area of greatest intensity within a particular wounded fiber. Fluorescence intensity within the whole cell was measured in

a similar manner to FM1-43. A Default threshold was applied and the analyze particles macro employed. The particle size was designated as any particle greater than 30μm which resulted in the whole cell being identified as a single region of interest. Fluorescence intensity was than normalized to baseline fluorescence intensity ($(F_t-F_0)/F_0$) to account for any variability in dye loading between fibers. *Dysferlin-pHGFP Analysis:* Recruitment of dysferlin to membrane wounds was measured as the fluorescence intensity within a region specifically on the membrane, ~2x8μm, and normalized to baseline fluorescence. Dysferlin endocytosis was measured as the decrease in fluorescence intensity of a 10x10μm area within the t-tubules of the myofiber and normalized to baseline fluorescence.

SiR-Actin and Photobleaching Experiments: Isolated muscle fibers were simultaneously loaded with 3μM Fluo-4 and 25nM SiR-Actin for 1hr in MEM+10%FBS. Prior to injury, dyes were washed out with MEM+10%FBS, and media changed to PSS+25nM SiR-Actin ± calcium. In the no calcium condition, any trace calcium was chelated by 1mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA). For photobleaching experiments, prior to injury a 60μm span of labeled filaments was photobleached by three successive iterations of a 652nm laser at 100% power with 16 line averages per frame, while the z-plane was modulated manually to photobleach within a 6μm depth of the fiber. After confirmation of sufficient photobleach, fibers were injured as described and the fluorescence intensity within a 10x10μm box at the wound was measured and plotted as the increase in intensity from baseline ($\Delta F=F_T-F_0$).

Western Blot: Skeletal muscle KCL-washed membranes or whole lysates were purified from pooled hindlimb samples of adult mice. Protein content was determined using *DC*

protein assay and 100μg of protein was separated on a 3-15% polyacrylamide gel and transferred to polyvinylidene fluoride membranes. Membranes were blocked in 5% wt/vol nonfat dry milk for 1hr and incubated with the appropriate primary antibody overnight: Hamlet-NCL (Novacastra/Leica: Hamlet-CE; 1:1000), Annexin 1 (ThermoFisher:71-3400; 1:2000), Annexin 2 (Abcam: ab41803; 1:2000), Annexin 6 (Abcam:ab31026; 1:2000), followed by HRP-conjugated secondaries and chemiluminescent detection using an Alpha Innotech Western blot documentation system. Quantification of integrated density values of band intensities was performed using AlphaEaseFC software.

Cryosectioning and Immunohistochemistry: For dysf-pHGFP localization, Tibialis Anterior muscles were excised from anesthetized mice and frozen in liquid nitrogen-cooled isopentane. Eight-micron thin fresh cryosections were fixed with 3% paraformaldehyde. For H&E, eight-micron thin fresh cryosections were fixed with formalin and stained with hematoxylin and eosin.

Statistics: All data represented are mean+SEM. Significance was determine using Student's *t* test set with $\alpha<0.05$.

RESULTS

Calcium-dependent polymerization of new actin filaments occurs at the injury site. Previous work from our lab and others has indicated that actin may be a critical regulator of membrane repair and membrane injury. Previously, injury has been shown to enhance localization of actin near site of membrane disruption [64, 74]. To further elucidate the role of F-actin in membrane repair, we utilized a method to measure actin

polymerization at the wound using a filamentous actin-specific, fluorescent dye, SiR-Actin that is amenable to live cell labeling and imaging [142]. Actin filaments in adult isolated skeletal muscle fibers were labeled with SiR-Actin, which was found to be highly enriched at sarcomere structures. After injury, sarcomeres in close proximity to the wound shorten significantly, eventually becoming indiscernible from a distinct actin structure at the injury site (**Figure 3.1A**). Sarcomeres distant to the injury are largely unaffected, remaining at a resting sarcomere length of ~2μm. Actin at the wound continues to accumulate for approximately 30-40s following injury and remains stably localized to the membrane lesion for minutes following injury (**Figure 3.1B**). These data suggest that actin filaments rapidly respond to membrane injury, but also highlight that sarcomere structures may be disrupted following a membrane injury and may require additional remodeling even after the membrane has resealed.

Calcium influx through membrane wounding is considered to be critical to the activation of membrane repair machinery and efficient membrane repair. Furthermore, calcium has also been shown to play a regulatory role in actin dynamics and remodeling [143-145]. Therefore, we tested whether calcium influx through membrane wounds could modulate the dynamics of actin remodeling after injury. Actin filaments in adult isolated skeletal muscle fibers were labeled with SiR-Actin and injured in the presence of absence of extracellular calcium. Removing extracellular calcium prior to and during injury prevents both sarcomere shortening at the wound and completely prevents the formation of any actin structure at the wound (**Figure 3.1C**), suggesting that actin remodeling following injury is dependent upon extracellular calcium influx following injury.

While a distinct structure of polymerized actin forms at the wound, shortening of SiR-labeled, actin-containing sarcomeres within the wound area obscures the visibility of any newly polymerized actin filaments. Therefore, the previous assay is unable to detect whether the actin accumulated at the wound is composed of newly polymerized filaments or composed of previously labeled sarcomeres shortening into the area surrounding the wound. To address this, we utilized fluorescence recovery after photobleach (FRAP) prior to injury to visualize only new actin filaments after injury. As previously, actin filaments were labeled in isolated adult muscle fibers with SiR-Actin, and then switched to physiological saline (PSS)±25nM SiR-Actin. The labeled filaments within 60μm on either side of the wound were photobleached and then fibers were either injured or uninjured (**Figure 3.2A**). The initial labeling of filaments by SiR-Actin saturated most dye binding sites, but some minimal re-labeling of photobleached filaments occurs in the absence of injury by excess SiR-Actin (**Figure 3.2B** top, **3.2C** magenta triangle). However, the intensity of actin fluorescence at the wound is greatest in injured muscle fibers in the presence of excess SiR-Actin (**Figure 3.2B** middle, **3.2C** black circle). Importantly, the fluorescence intensity of actin at the wound in the absence of extracellular dye is minimal (**Figure 3.2B** bottom, **3.2C** teal square). These data taken together suggest the actin structure at the wound labeled by extracellular SiR-Actin is composed of newly polymerized actin filaments with little contribution from relabeling of filaments by excess dye and little contribution from previously labeled filaments.

Genetic loss of γ-actin in skeletal muscle leads to an increased uptake in cell-impermeable dye and prolonged influx of calcium following injury. Actin is a critical component of the membrane repair machinery that has shown to be essential for recruiting membrane repair proteins to membrane wounds for efficient wound closure [47, 64, 74]. We sought to investigate the contribution of one isoform of the subsarcolemmal cytoskeleton, γ-actin, to membrane repair using a muscle-specific knockout of γ-actin (Actg1$^{-/-}$). Isolated muscle fibers from Actg1$^{-/-}$ mice and floxed littermate control animals (WT) were injured in the presence of FM1-43, a cell-impermeable, lipophilic dye that has been used extensively to monitor membrane wound healing and identify putative membrane repair proteins. After injury, Actg1$^{-/-}$ muscle fibers initially uptake similar amounts of FM1-43 compared to WT, but shortly after injury, FM1-43 uptake into Actg1$^{-/-}$ fibers significantly increases (**Figure 3.3A**, quantified **3.3B, C**). We have shown previously that FM1-43 entry into injured muscle fiber is largely dependent upon endocytosis of extracellular dye (under review). Therefore, the increase in FM1-43 uptake in Actg1$^{-/-}$ could be a result of a membrane repair defect or a result of an increase in wound-induced endocytosis.

To separate wound-induced endocytosis from any membrane repair defect, we monitored calcium influx after injury. We have shown previously that calcium uptake can supplement FM1-43 uptake measurements to describe the dynamic process of membrane repair (under review). Individual muscle fibers isolated from Actg1$^{-/-}$ mice and floxed control animals were loaded with 3μM Fluo-4. The baseline fluorescence between WT and Actg1$^{-/-}$ is similar, suggesting both WT and Actg1$^{-/-}$ fibers have similar amounts of dye in the cytoplasm prior to injury (**Figure 3.4B**). After injury, calcium

floods both WT and Actg1$^{-/-}$ fibers, but muscle fibers that do not express γ-actin show a persistent increase in calcium directly at the wound (**Figure 3.4A**, quantified **3.4C**). This is further exemplified by calcium concentrations in the cytoplasm beyond the wound (**Figure 3.4D**). We note that while WT muscle fibers show a rapid increase followed by a quick reversal toward baseline calcium, cytoplasmic calcium concentrations in Actg1$^{-/-}$ fibers continue to increase. Interestingly, calcium in the cytoplasm of WT cells begins to approach baseline, though we note it remains slightly elevated; however, in Actg1$^{-/-}$ fibers, calcium is persistently, significantly increased even minutes following injury compared to WT(**Figure 3.4E**), consistent with a membrane repair defect.

Importantly, we show that, consistent with published results, Actg1$^{-/-}$ muscle fibers do not suffer more severe injuries due to the genetic loss of γ-actin [146]. Stratifying the injured muscle fibers by the severity of injury shows similar percentages of fibers in WT and Actg1$^{-/-}$ fibers do not recover from injury (**Figure 3.5A**, quantified **3.5B**). The fibers scored as severely injured or uninjured were excluded from analysis in this study. In addition, measuring the size of the injury immediately after laser ablation shows both WT and Actg1$^{-/-}$ muscle fibers show similar injury sizes (scale bar **Figure 3.5A**, quantified **3.5C**). These data indicate that the increased uptake of FM1-43 and the increased uptake of calcium following injury is not due to increased size or severity of injury in Actg1$^{-/-}$ muscle. Overall, these data suggest that Actg1$^{-/-}$ muscle fibers have an increased uptake of extracellular contents immediately following injury likely due to inefficient wound closure that contributes to persistently increased cytoplasmic calcium in adult Actg1$^{-/-}$ muscle fibers.

Actg1$^{-/-}$ muscle express membrane repair proteins are expressed at similar levels to WT. To probe the mechanism of the membrane repair defect caused by the absence of γ-actin, we tested whether membrane repair proteins were differentially expressed in skeletal muscle of Actg1$^{-/-}$ mice. Western blot analysis of membrane repair proteins-dysferlin, annexinA6, annexinA2, and annexinA1-shows that all full-length proteins are expressed at similar levels to WT when normalized to a loading control (**Figure 3.6A**, quantified **3.6B**). We do note that Western blot for annexin1 and annexin2 shows multiple bands at various molecular weights, suggesting alternatively processed versions of the full-length protein in skeletal muscle. The smaller molecular weight band of annexin1 (~15kDa) is significantly increased in Actg1$^{-/-}$ skeletal muscle and the smaller molecular weight band of annexin2 (~25kDa) is significantly decreased. Neither of these lower molecular weight annexins have been reported as important to membrane repair, though proteolysis of both annexin1 and annexin2 have been described as increasing the calcium sensitive, lipid-binding activity of the annexins [147, 148]. Therefore, potential further study of the processing of the annexins in the context of membrane repair could prove interesting.

Dysferlin recruitment to membrane wounds and dysferlin endocytosis occurs independent of γ-actin. Data from our laboratory has shown that following injury, dysferlin is both recruited from the lateral membrane and recruited into intracellular vesicles. Dysferlin recruitment to membrane wounds is dependent upon actin polymerization and actin has been shown to be critical to multiple forms of endocytosis [64, 149, 150]. Therefore, we hypothesized that the membrane repair defects noted in γ-

actin-deficient muscle may be due to defective dysferlin recruitment to membrane wounds or defective dysferlin endocytosis following injury. To test this, dysferlin-pHGFP (dysf-pHGFP) reporter mice developed by our laboratory were bred to muscle-specific Actg1$^{-/-}$ mice. We show that the dysf-pHGFP transgene is expressed at similar levels and properly localized to the plasma membrane with no effects on overall muscle health, as depicted by H&E staining, in both floxed littermate transgenic animals and Actg1$^{-/-}$ transgenic animals (**Figure 3.7A, 3.7B**). Following injury, we show that isolated adult skeletal muscle fibers from Actg1$^{-/-}$ mice and floxed controls both show similar levels of dysferlin recruitment to membrane wounds (**Figure 3.7C**) [64].

Our laboratory has shown that dysferlin is rapidly endocytosed following injury, which can be measured as a decrease in fluorescence of the dysf-pHGFP reporter [64]. We note that both WT and Actg1$^{-/-}$ muscle fibers show a similar decrease in dysf-pHGFP fluorescence rapidly after a membrane injury (**Figure 3.7D**). Furthermore, when injured muscle fibers from dysf-pHGFP animals are treated with NH4Cl, dysferlin-containing endosomes can be visualized within the cytoplasm of both WT and Actg1-/- (data not shown). In these studies, no defects in dysferlin trafficking to membrane wounds or subsequent dysferlin endocytosis have been identified, suggesting an independent mechanism for γ-actin in supporting efficient membrane repair.

DISCUSSION

Our results demonstrate the dynamic activity of the subsarcolemmal cytoskeleton after membrane injury and highlight a previously unidentified role for one of the major isoforms of the subsarcolemmal cytoskeleton, γ-actin, in membrane repair. We show

that actin polymerization at the wound is a proximal event to wound closure. In addition, our data suggest significant disruption of sarcomere structures occurs as a result of injury. Neither the disruption of sarcomere structures nor the accumulation of actin at membrane wounds occurs in the absence of calcium. Data suggests that extracellular calcium is required for membrane resealing and calcium has been shown to contribute to depolymerization of filaments [151]. Therefore it is possible that disassembly of sarcomere structures plays a role in membrane repair, possibly to decrease tension immediately following injury, or to allow access of intracellular vesicles to the open pore, similar to what has been described in *Drosophila* embryo and *Xenopus* oocyte models [50, 152, 153]. However, it is also possible that sarcomere disassembly is merely a consequence of the injury itself. Regardless, these data demonstrate that additional mechanisms for long term remodeling of the muscle fiber likely exist in order to prevent significant detriment to force production.

We also show that one isoform in particular, γ-actin, may be critical to efficient membrane repair. We show that loss of γ-actin results in significantly increased FM1-43 and calcium uptake after injury. The most notable difference in uptake occurs late in the injury time course, which could suggest a sustained membrane leak that results from inefficient repair. Though the mechanism by which γ-actin participates in membrane repair remains unclear, we show it is not explained by the loss of trafficking of dysferlin to membrane wounds. We show that in γ-actin knockout muscle fibers, dysferlin recruitment, which has been shown to be dependent upon actin polymerization, is unaffected by genetic loss of γ-actin. Therefore, future work should be devoted to identifying the role of γ-actin in membrane repair. The membrane repair defect caused

by genetic loss of γ-actin could be the result of defective trafficking of other membrane repair proteins not tested here, or γ-actin could possibly stabilize the membrane repair complex or the wound itself, or γ-actin could have a completely independent role in membrane repair.

Dysferlin has not been shown to interact directly with actin, but several of the annexins have. In fact, Annexin A5 has been shown to assemble into a lattice structure hypothesized to help stabilize the wound and prevent wound expansion [79-81]. Moreover, annexin A5 has been shown to have binding specificity for γ-actin [154]. Therefore, it is possible that γ-actin could regulate the trafficking of annexins to membrane wounds or the annexins could function with costameric γ-actin to prevent wound expansion and facilitate wound closure. Furthermore, γ-actin is expressed primarily in the costamere of the adult muscle fiber, necessary structures that stabilize the membrane during muscle contraction. We observe significant contraction following injury as a result of significant calcium influx. It's possible that γ-actin and costamere structures are responsible for stabilizing membrane at the wound, providing necessary tension to facilitate both endocytosis and vesicle fusion, as well as preventing significant retraction of the newly formed pore [155-158].

While the membrane repair defect is relatively mild comparatively, there are several possible explanations for these results. The most obvious of such, is potential compensation by other isoforms of actin when γ-actin is mutated. Though there is significant evidence that β- and γ-actin are not fully compensatory, these isoforms only differ in 4 amino acids [159]. The delay in membrane repair in our results suggests a

role for γ-actin independent of β-actin, but any compensation by other isomers of actin may reduce the phenotype severity. Therefore, it cannot be ruled out that compensation may occur, especially given the fact that γ-actin knockout in muscle results in the upregulation of additional actin isoforms, maintaining an equal amount of total actin between WT and Actg1$^{-/-}$ muscle [136]. Future studies should focus on highlighting the contributions of both β and γ-actin. It would be equally interesting to see if there is functional dependence between β- and γ-actin. While it has not been demonstrated *in vivo*, *in vitro* experiments show β- and γ-actin can copolymerize to form a heterofilament [160]. Therefore, beyond compensation by β-actin, there is potential that actin filament composition could affect membrane repair efficiency, which also could suggest that the ratio of β- to γ-actin in the cytoplasm is important. These data presented here suggest a multifaceted role for actin in membrane repair and highlight the need for more research on the specificity and function of specific isoforms of actin, particularly in membrane repair.

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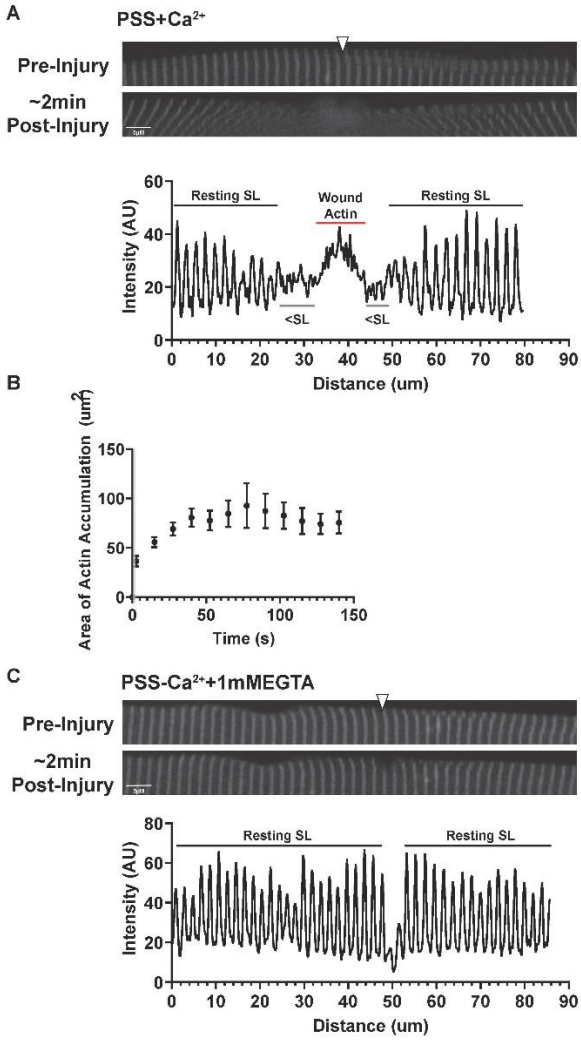

Figure 3.1. Actin filaments accumulate at the site of injury in a calcium-dependent manner. Isolated muscle fibers were loaded with 25nM SiR-Actin for 1hr and injured (white arrowhead **A**). Sarcomere length (SL) is represented by a line profile just below the sarcolemma (**B**). The area of the actin structure at the wound site was quantified over time (**C**). Isolated muscle fibers loaded with 25nM SiR-Actin injured in the absence of calcium and the presence of 1mM EGTA (white arrowhead **D**) and sarcomere length in the absence of calcium is represented by a line profile just below the sarcolemma (**E**). Data represent one independent replicate (n=11 fibers). Error bars shown as the SEM. Results were confirmed by 3 independent replicate experiments.

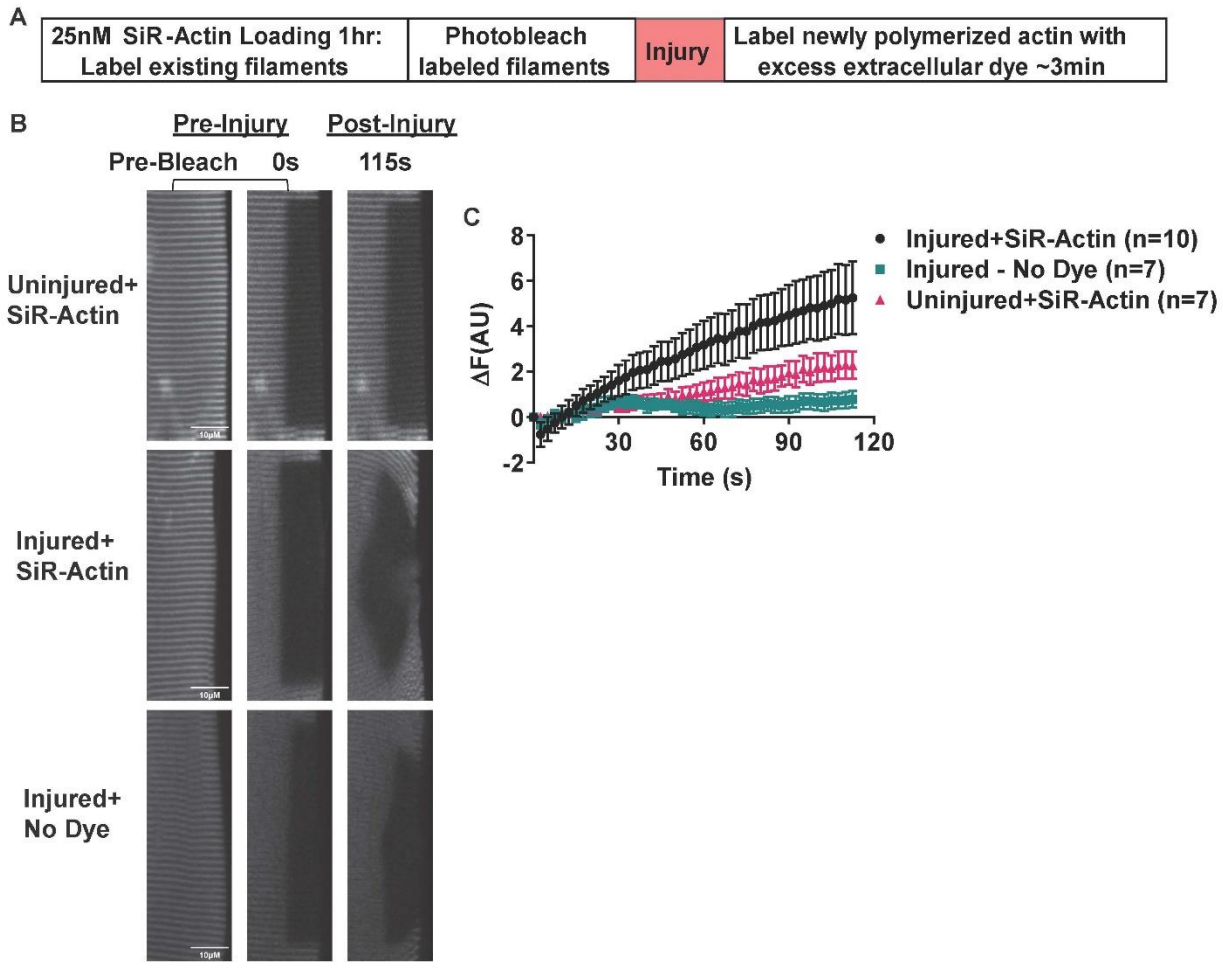

Figure 3.2. New actin filaments polymerize at the wound site. Isolated muscle fibers were loaded with 25nM SiR-Actin for 1hr. Labeled filaments were photobleached and injured in the presence or absence of extracellular dye. (outline **A**, representative images **B**). Actin fluorescence is quantified and plotted as the ΔF (**C**). Error bars represent SEM and data is pooled from three independent experiments.

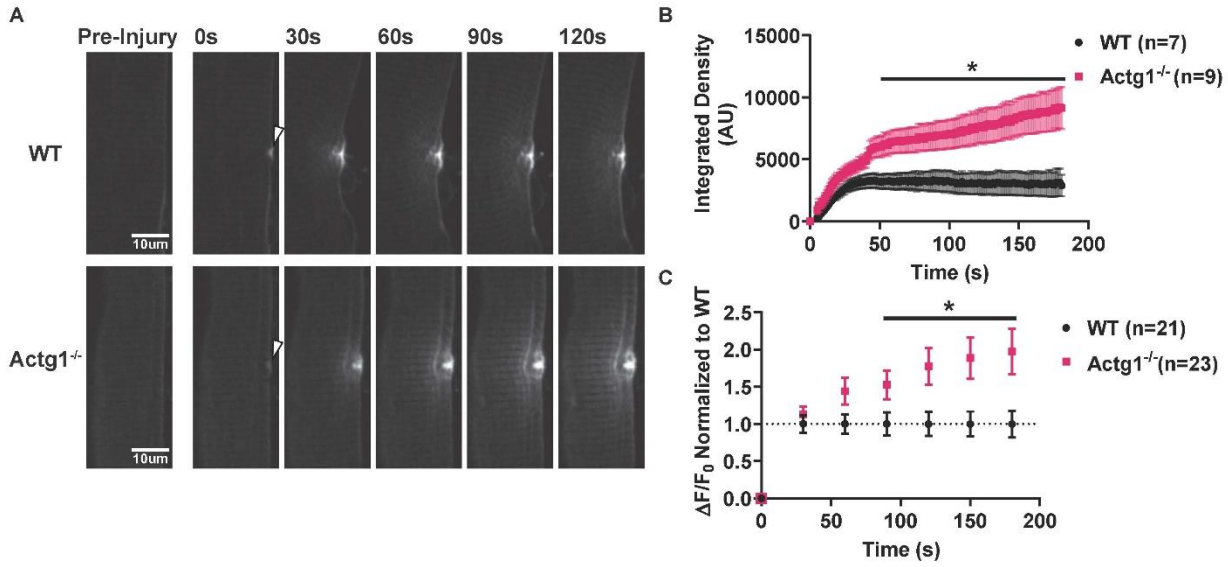

Figure 3.3. Actg1$^{-/-}$ muscle fibers show an increased uptake of FM1-43 following laser injury. Isolated muscle fibers from adult WT and Actg1$^{-/-}$ mice were loaded with 2.5μM FM1-43 for ten minutes and then injured in the presence of 2.5μM FM1-43 (representative images **A**). The integrated density of FM1-43 uptake after injury is plotted over time in one representative experiment (**B**). Data from independent replicates was normalized to the WT uptake of FM1-43 and then pooled. Dashed line represents WT=1 (**C**). Error bars represent SEM and statistical significance ($p<0.05$) between WT and Actg1$^{-/-}$ is denoted by (*) for all point under horizontal bar.

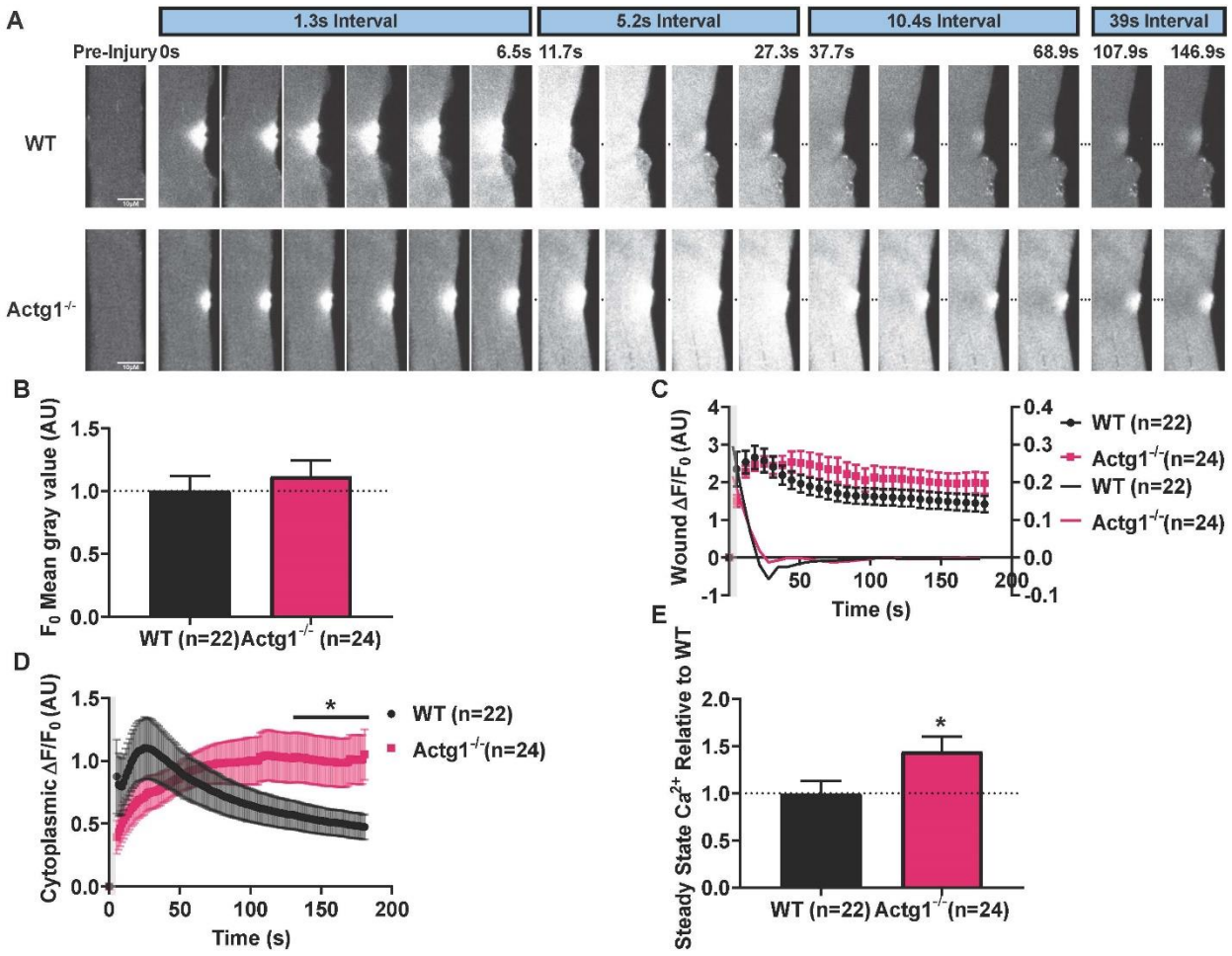

Figure 3.4. Actg1$^{-/-}$ muscle fibers show an increased uptake of calcium following laser injury. Isolated muscle fibers from adult WT and Actg1$^{-/-}$ mice were loaded with 3μM Fluo-4 for one hour, extracellular dye was removed, and then fibers injured (representative images **A**). The intensity of baseline dye fluorescence was normalized to the WT baseline fluorescence (dashed line, **B**) showing baseline Fluo-4 fluorescence is not significantly different ($p>0.05$) across the two genotypes (**B**). The normalized intensity of calcium dye directly at the wound shows calcium uptake into Actg1$^{-/-}$ muscle fibers following injury remains elevated at the wound compared to wildtype (magenta squares **C**) and the maximum fluorescence occurs at a later timepoint in Actg1$^{-/-}$ muscle fibers than WT as described by the first derivative of the time course (solid lines **C**). The normalized intensity of calcium dye within the whole cell shows calcium also continues to increase in the cytoplasm in injured Actg1$^{-/-}$ muscle fibers, whereas wildtype injured muscle fibers show a rapid increase in calcium, followed by a quick reversal toward baseline (**D**). The steady state calcium at the end of imaging was normalized to WT steady state calcium (dashed line, **E**) showing persistent elevation of calcium in injured Actg1$^{-/-}$ muscle fibers (**E**). All error bars represent SEM, significance is determined by Student's *t* test and (*) represents $p<0.05$. These data are pooled from four independent experiments.

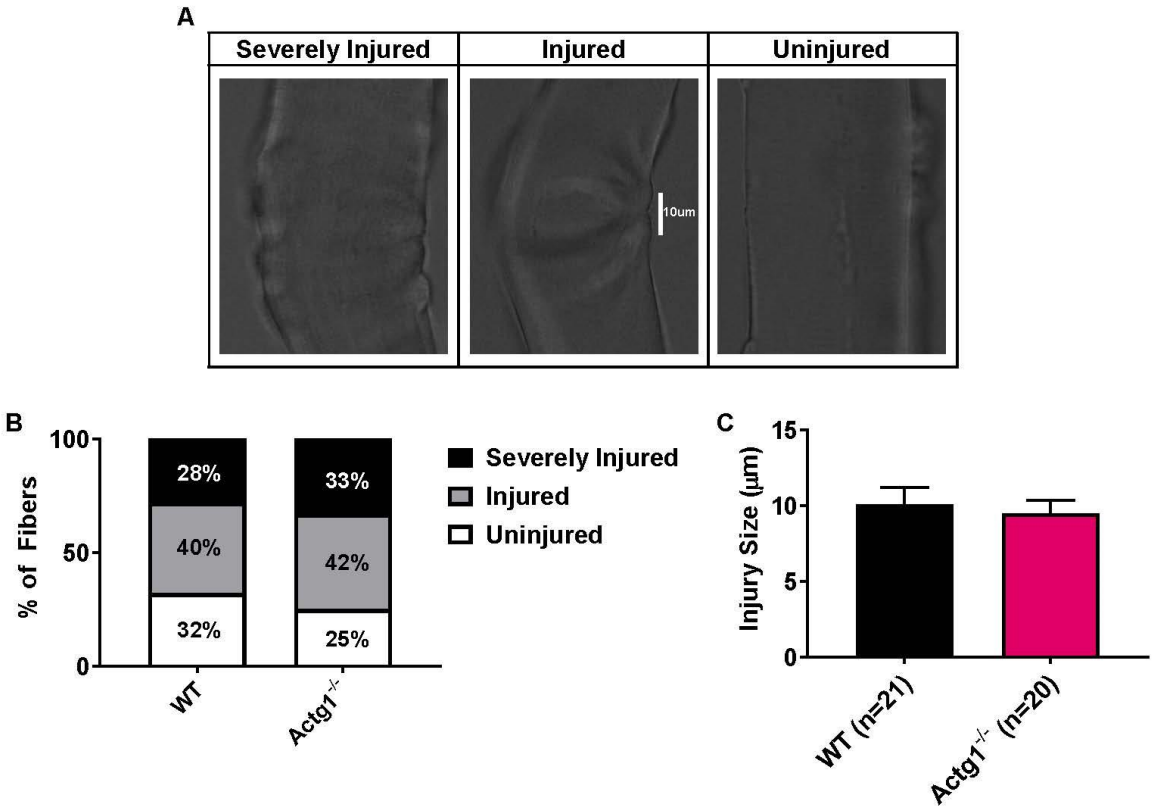

Figure 3.5. Injuries in WT and Actg1$^{-/-}$ muscle fibers are similar in size and severity. The population of injured fibers were stratified based on the degree of contraction induced by injury showing the percentage of injured fibers is similar in WT and Actg1$^{-/-}$ muscle and Actg1$^{-/-}$ muscle does not have a disproportionate number of severely injured fibers (**A**, quantified **B**). In addition, the deformation of the membrane was measured in microns (scale bar **A**, quantified **C**) in both wildtype and Actg1$^{-/-}$ injured muscle fibers showing that the size of the laser injury is not increased in injured Actg1$^{-/-}$ muscle fibers compared to wildtype ($p>0.05$). Error bars represent SEM and data is pooled from four independent experiments.

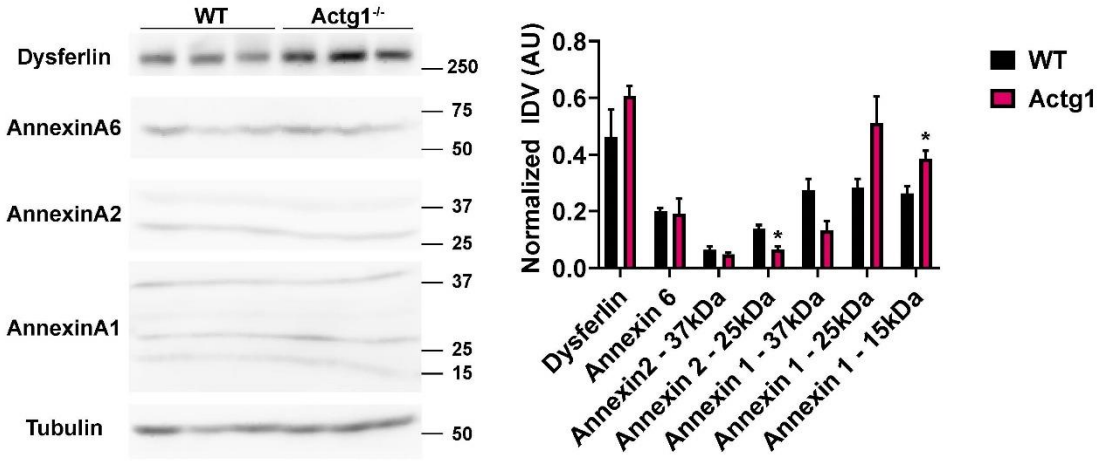

Figure 3.6. Membrane Repair Proteins are expressed, but unchanged, in Actg1$^{-/-}$ hindlimb. Western blot of pooled hindlimb whole lysate from both WT and Actg1$^{-/-}$ adult mice show the expression of known membrane repair proteins -dysferlin, annexin 6, annexin 2, and annexin 1- are all expressed at similar levels to floxed littermate controls (**A**, quantified **B**; n=3 per genotype).

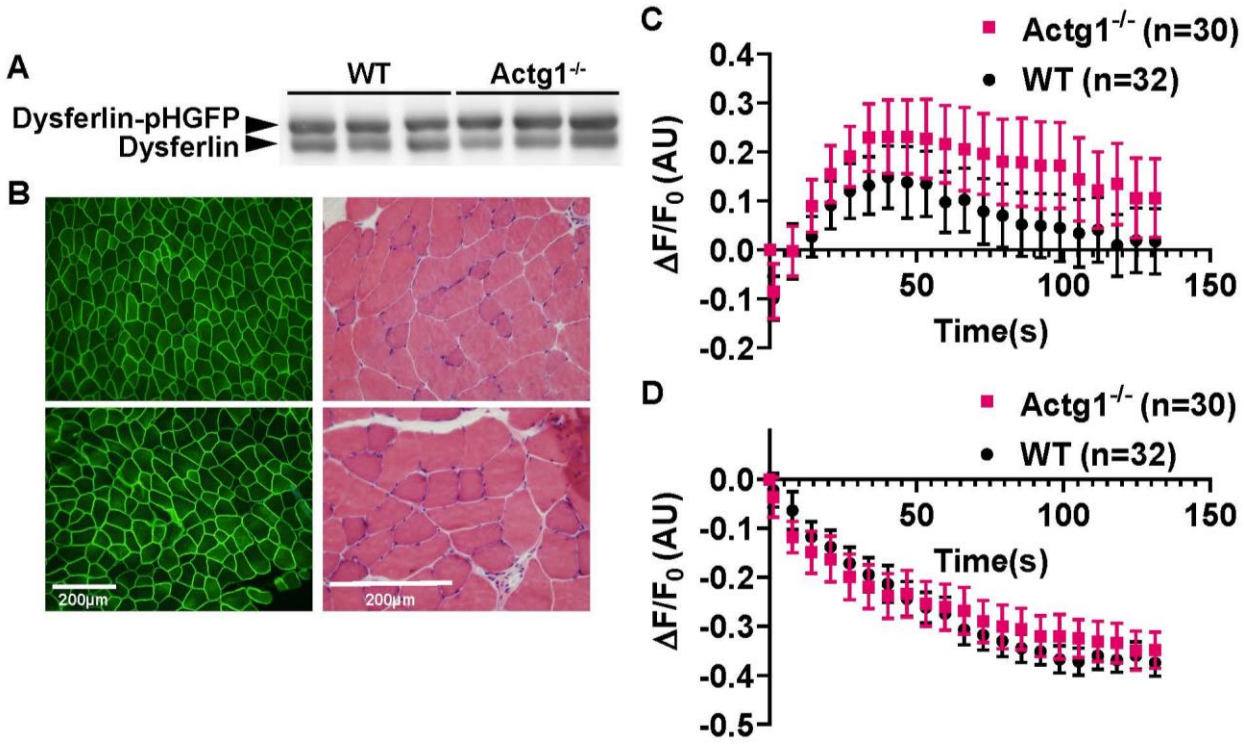

Figure 3.7. Dysferlin recruitment to membrane wounds and dysferlin endocytosis are unaffected by genetic loss of γ-actin. Dysf-pHGFP reporter gene was detected in the membrane fraction of skeletal muscle lysates of Actg1$^{-/-}$ hindlimb muscle by western blot using Hamlet-NCL antibody (**A**) and also detectable by fluorescence at the plasma membrane of skeletal muscle fibers of the quadricep muscle cut in cross-section of both floxed control and Actg1$^{-/-}$ animals (**B**). H&E staining shows the expression of the dysf-pHGFP reporter has no effect on overall muscle health in WT or Actg1$^{-/-}$ mice (**B**). Adult muscle fibers were isolated from dysf-pHGFP expressing floxed control animals and dysf-pHGFP expressing Actg1$^{-/-}$ animals, were injured, and the fluorescence intensity of dysf-pHGFP recruitment to the membrane wound is shown to be unimpaired in Actg1$^{-/-}$ fibers ($p>0.05$) (**C**). Dysf-pHGFP fluorescence is quenched as dysferlin is recruited into acidic intracellular vesicles, denoted by a decrease in dysf-pHGFP cellular fluorescence, that is also similar in floxed control and Actg1$^{-/-}$ injured muscle fibers, suggesting no defects in wound-induced dysferlin endocytosis ($p>0.05$) (**D**). Error bars represent the SEM of pooled data of four independent experiments.

CHAPTER 4

Conclusions and Future Directions

Striated muscle is a highly dynamic tissue that can on occasion experience significant damage from contractile activity, particularly activity under high external load [6]. This damage can be severe, such as a large-scale muscle tear. Alternatively, more subtle microscopic injuries can occur at the cellular level that lead to transient sarcolemma permeability. As a result, individual muscle fibers require both reinforcement and repair of the plasma membrane to maintain tissue integrity. Multiple types of muscular dystrophy are characterized by increased membrane instability as a result of either mutations that render the sarcolemma susceptible to contraction-induced damage or mutations that disrupt the process of membrane repair [161]. The focus of this thesis was to understand the basic mechanisms of plasma membrane repair, though the studies herein could have implications for all instances of membrane instability.

Because defects in membrane stability and repair lead to the devastating consequences of muscular dystrophy in humans, these studies investigated plasma membrane repair specifically in striated muscle. The primary model of plasma membrane repair proposed in muscle involves the assembly of a repair complex at the wound that includes, but is not limited to, dysferlin, several of the annexins, and F-actin

[74]. The membrane repair complex at the wound is hypothesized to facilitate the fusion of membrane repair protein-containing intracellular vesicles to create a lipid patch and restore barrier function of the plasma membrane. Many studies on plasma membrane repair have identified putative membrane repair proteins, and several models of repair have been proposed (discussed in Chapter 1); however, the exact biophysical and biochemical mechanism of plasma membrane repair has not been identified. Therefore, this dissertation focused on understanding key modulators of membrane repair protein trafficking by investigating the contribution of wound-induced endocytosis and specific cytoskeletal proteins to membrane repair in striated muscle.

Sarcolemma Wounding Activates Dynamin Dependent Endocytosis in Striated Muscle

Multiple membrane repair proteins, including dysferlin, have been shown to be endocytosed following injury in a calcium-dependent manner [64, 65, 133]. However, the extent to which endocytosis is activated following injury and which endocytic pathway may be responsible for wound-induced dysferlin endocytosis had not been identified. To that end, we investigated the activation of dynamin-dependent endocytosis after plasma membrane injury as a potential mediator of dysferlin function and membrane repair in adult skeletal muscle.

A critical regulator of endocytosis, dynamin is a large GTPase that self-assembles into oligomers surrounding the budding neck of nascent endocytic vesicles wherein conformational changes driven by GTP hydrolysis constrict the neck and promote vesicle release [162]. Dynamin is involved in several modes of endocytosis,

including clathrin-dependent and caveolar endocytosis. Interestingly, mutations in Dynamin 2, the predominant isoform expressed in skeletal muscle, results in a centronuclear myopathy that, in part, is hypothesized to be the result of plasma membrane instability, though the mechanism still remains unclear [124, 163]. While the myopathy caused by dynamin mutations shares phenotypic similarity with dysferlin deficiency, it also has several distinct features, and therefore alternate mechanisms could be responsible. For example, mutations in Bin1 have also been shown to result in centronuclear myopathy and both Bin1 and dynamin have been associated with t-tubule biogenesis [121, 164]. Therefore, it is possible dynamin 2 contributes to similar functions as Bin1 and may share this as a mechanism of disease, if there is indeed a "common mechanism" in this disease. Interesting to the topic of this thesis, dynamin function at focal adhesions in some types of cells appears to both require actin to function, but also in turn affect actin assembly and drive polymerization [165]. While we hypothesized dysferlin and dynamin may be a part of the membrane repair pathway, it is important to note that dynamin may have numerous cellular functions, all of which could contribute to disease pathology.

The studies presented in Chapter 2 investigate the activation of dynamin-dependent endocytosis after membrane injury using pharmacological inhibitors of dynamin-dependent endocytosis prior to and following injury. Endocytosis was monitored using the lipophilic, tracer molecular FM1-43 in resting adult skeletal muscle fibers in the presence or absence of a dynamin inhibitor, Dynasore. Pre-treatment of adult muscle fibers with Dynasore completely inhibited FM1-43 staining at rest. These data suggest FM1-43 labeling is an active process that is nearly completely dependent

upon dynamin-dependent endocytic uptake of dye from the extracellular media. These data highlight that dynamin-dependent endocytosis is highly active in resting adult skeletal muscle and are in agreement with previous studies using FM1-43 as an endocytic marker for synapse vesicle turnover [166].

While FM1-43 is traditionally a marker of endocytosis, it has also been used extensively in the membrane repair field and elevated uptake of FM1-43 has become a hallmark of defective membrane repair. Canonical assays of membrane repair typically track the uptake of FM1-43 after injury, which, up until this point, had been thought to primarily occur through the membrane lesion itself [28]. However, the data presented here suggest that a massive endocytic response is activated following a membrane injury that can be severely diminished by dynamin inhibition. Therefore, we conclude that the majority of FM1-43 uptake after injury enters cells by a dynamin-dependent mechanism. Importantly, elevated FM1-43 uptake after injury is the gold-standard method for identifying a membrane repair defect and has been used extensively to identify putative membrane repair proteins, dysferlin being one of them [28, 74, 133]. However, we show that dynamin-inhibition severely blunts the uptake of FM1-43 in both wild-type and dysferlin-deficient muscle fibers, which completely removes any observable difference in FM1-43 uptake between the two genotypes. Previously, elevated uptake of FM1-43 was attributed to increased dye influx through prolonged membrane wounds and is the basis for the assertion that membrane repair is defective in this model [28]. However, these data suggest that, instead, dynamin-dependent endocytosis may be driving elevated uptake of FM1-43 in injured, dysferlin-deficient muscle fibers.

In Chapter 2, calcium influx was measured as a dynamin-independent method of monitoring membrane repair as we showed that Dynasore treatment had minimal effect on calcium uptake after injury. Calcium influx after injury showed a rapid increase followed by a quick reversal within the first minute after injury, which is in stark contrast to the biphasic time course of FM1-43 that displays an initial fast phase most consistent with uptake through the lesion, followed by a slower, persistent phase that likely occurs primarily through endocytic uptake mechanisms. Using dynamin-independent measures of calcium flux after injury, we also showed that dysferlin-deficient muscle fibers have increased calcium uptake immediately following injury; however, calcium reversal occurred within the same time scale as wildtype muscle fibers. These data present the interesting possibility that defective membrane repair results in increased calcium uptake in dysferlin-deficient muscle fibers that drives increased dynamin-dependent endocytosis, and, therefore, drives increased uptake of FM1-43 after injury.

These data indicate that dynamin-dependent endocytosis is highly active at rest and increased following sarcolemma injury in adult myofibers and describe overactive dynamin-dependent endocytosis as a potentially pathological mechanism in dysferlin deficiency. Importantly, these experiments critically evaluate the parameters that contribute to the uptake of extracellular FM1-43 and present calcium flux as a complimentary method of monitoring membrane repair after injury. FM1-43 uptake after injury is the gold-standard for identifying membrane repair defects and characterizing membrane repair proteins, therefore, the careful consideration of contributing factors to both FM1-43 uptake and calcium uptake outlined in Chapter 2 will prove valuable to the membrane repair field.

Future Directions

In our concluding remarks in Chapter 2, we present a model that hypothesizes calcium influx drives dynamin-dependent endocytosis of FM1-43 and that increased calcium influx after injury in membrane repair defective cells could lead to overactive wound-induced endocytosis of extracellular FM1-43. While our data are consistent with this model in that dysferlin-deficient cells have increased calcium after injury that remains elevated for several minutes after injury and there is some evidence that dynamin-dependent endocytosis may be calcium dependent, it was not directly tested that calcium is the driver of wound-induced endocytosis [167, 168]. Therefore, I propose to investigate how calcium regulates FM1-43 uptake in adult skeletal muscle fibers. The simple experiment would be to remove extracellular calcium, however calcium itself may regulate dysferlin-dependent function and resealing, and indeed we and others have shown that reducing extracellular calcium increases FM1-43 uptake. Therefore, I would suggest that if calcium drives endocytic uptake of FM1-43, increased intracellular calcium should be sufficient to increase extracellular FM1-43 uptake even in the absence of injury. I hypothesize that increasing intracellular calcium using either photo-releasable caged calcium or caffeine-induced release of stored calcium should drive endocytic uptake of FM1-43 in the absence of injury, and if the uptake is dynamin-dependent, uptake should be sensitive to Dynasore treatment. If true, these data would demonstrate that calcium is sufficient to activate dynamin-dependent endocytosis, which could drive endocytic uptake of FM1-43 after injury. It would also be interesting to test endocytic uptake of FM1-43 as a function of increasing calcium concentration. By titrating the amount of caged calcium released, which would be quantitatively monitored

using the ratiometric calcium dye Fura-2, increased FM1-43 uptake could be correlated with calcium concentration to determine whether a positive association between the two exists.

We could then probe this further in membrane repair deficient cells, such as muscle fibers isolated from dysferlin-deficient, BLA/J mice. If increased calcium that results from defective membrane repair drives dynamin-dependent FM1-43 uptake, clearing cytoplasmic calcium more quickly could decrease FM1-43 uptake after injury. Enhancing re-entry of calcium into the SR after injury could attenuate the prolonged uptake of FM1-43 in injured fibers. Recent work by Jaiswal laboratory in anoctamin 5 deficient human myoblasts supports this possibility, although it's clear from the published work that the responses in myoblasts and adult muscle fibers to laser wounding are very different [169]. Therefore, I propose to injure both WT and dysferlin-deficient adult muscle fibers in the presence or absence of the SERCA2 activator CDN1163 [170]. I would expect that treatment would decrease the prolonged elevation of cytoplasmic calcium at the injury site in both dysferlin-deficient muscle fibers, decrease the activation of dynamin-dependent endocytosis, and thus decrease FM1-43 uptake after injury in dysferlin-deficient muscle fibers to wildtype levels.

One caveat to this hypothesized model that I previously mentioned is that removing extracellular calcium increases FM1-43 after injury. Membrane repair proteins are generally calcium-dependent and therefore, this elevated dye uptake was attributed to increased diffusion of dye through the lesion as a result of a prolonged membrane breach. However, our results suggest that the majority of FM1-43 uptake after injury is due to wound-induced endocytosis with a smaller amount of uptake occurring through

the lesion itself. While there is some evidence that suggests dynamin-dependent endocytosis can be activated by calcium, it is possible that dynamin-dependent endocytosis of FM1-43 after injury is stimulated by some other factor besides calcium or is stimulated to a lesser extent by calcium. To test this, muscle fibers could be injured in the presence of both Dynasore and EGTA. According to dogma, EGTA would prevent the activation of membrane repair and lead to a prolonged membrane breach, increasing FM1-43 uptake. If uptake is primarily through the lesion alone in this condition, Dynasore should have no effect on uptake in the presence of EGTA, resulting in increased FM1-43 uptake compared to Dynasore treatment alone. Due to the methods of buffer exchanges, it can be challenging to reduce extracellular calcium to zero even in the presence of EGTA and prolonged exposure to low extracellular calcium over a long period of time can begin to lead to calcium dis-homeostasis and loss of cell viability so these experiments always have to be interpreted with caution.

Our data show that injury dramatically increases cytoplasmic calcium, which is quickly handled by internal storage systems or membrane pumps resulting in a quick reversal in calcium concentrations measured by the fluorescent dyes loaded into cells. However, even several minutes after injury, calcium remains elevated and compartmentalized in the cytoplasm, which leads to several hypotheses that require further investigation. Prolonged elevation of calcium in the cytoplasm near and distal to the wound could represent a consequence of sustained membrane leak after injury, play a role in the long-term remodeling of the membrane after injury, or could be a result of injury that has dramatic effects on over-all cell health even after the membrane is repaired.

Preliminary data in our laboratory suggests that calcium influx through the wound results in increased influx of calcium into the mitochondria. While our data show that no gross changes to mitochondrial structure or membrane potential occur in wild-type muscle fibers, this has not been investigated in models of defective membrane repair in which cytoplasmic calcium is increased. Experiments in injured myotubes suggest that calcium stimulates mitochondrial ROS production after injury, which interestingly, has a positive effect on membrane repair [171]. However, there is a delicate balance between beneficial and detrimental mitochondrial ROS production [172] . Therefore, I hypothesize that calcium influx through membrane wounds stimulates mitochondrial ROS production, which may be beneficial to wildtype muscle fiber repair, however, prolonged calcium increases, such as the case in dysferlin-deficiency, may increase mitochondrial ROS to harmful levels and contribute to the dystrophic muscle phenotype in this model. Therefore, I propose to use an intracellular marker of mitochondrial ROS, mitoSOX, to monitor ROS production after injury in wildtype and dysferlin-deficient muscle fibers. Consistent with published results, I expect that both wildtype and dysferlin-deficient muscle fibers would have an increase in mitochondrial ROS production, but the prolonged increase in cytoplasmic calcium in dysferlin-deficiency may result in increased ROS production compared to wildtype [118]. Furthermore, I would show that mitochondrial calcium drives ROS production after injury by inhibiting the mitochondrial uniporter (MCU) with Ruthenium Red or using genetic models of MCU knockout. I would also suggest that if dysferlin-deficiency results in increased ROS production, it represents a potential pathological mechanism for dysferlin-deficient muscular dystrophy. Therefore, I propose to attenuate mitochondrial ROS using

antioxidant or mitoTEMPO treatment to scavenge ROS after membrane injury and potentially rescue the membrane repair defect in dysferlin-deficient fibers [173]. Furthermore, mitochondrial antioxidant treatment, such as MitoQ, could be applied *in vivo* and whole muscle outcome measures, such as serum creatine kinase, muscle fiber size, and centrally located nuclei, could be used as a readout for improved muscle pathology as a result of treatment [174]. These data together would provide evidence that calcium stimulates mitochondrial ROS production that contributes to disease progression in dysferlin-deficiency.

Our data shows that calcium influx is quickly reversed on the order of seconds after injury, suggesting that membrane injury is likely repaired very quickly, preventing further influx of ions and cell-damaging extracellular fluid; however, it is also likely that long term remodeling of the plasma membrane and cytoskeletal components occurs long after the injury is repaired. Indeed, data from our laboratory shows that actin is stably localized at the wound for several minutes following injury (Chapter 3) and preliminary data (not shown) suggest microtubules also accumulate at the wound and are stably localized for several minutes. Interestingly, calcium concentrations also remain elevated in a restricted area in close proximity to the injury site for several minutes following injury. Since we have shown that actin accumulates at the wound in a calcium-dependent manner, I propose that prolonged elevation of calcium directly at the wound may account for the stability of both actin and microtubules structures in the wound area. The effects of prolonged, elevated calcium on long-term remodeling of the cytoskeleton could be investigated by lowering cytoplasmic calcium to baseline levels after the initial increase in calcium has reversed while also monitoring cytoskeletal

dynamics with fluorescent cytoskeletal probes, namely SiR-Actin and SiR-Tubulin. I would propose to release a caged calcium chelator after the initial post-injury calcium peak, which would promote calcium to return to baseline rapidly, and quantify changes to the size and intensity of actin or tubulin fluorescence at the wound. These data would highlight the importance of calcium as a signaling molecule in the long-term remodeling of the plasma membrane even after the injury is repaired.

Interestingly, there is some evidence that dynamin may regulate actin polymerization in some way, either through direct interactions with actin or interactions with actin binding proteins, but whether dynamin regulates actin polymerization during membrane repair is unclear [165, 175-177]. Therefore, I propose to investigate the possibility of endocytosis-independent functions of dynamin in membrane repair. We show in Chapter 3 that subsarcolemma actin is localized at the site of a plasma membrane wound quickly after injury, which is stabilized for several minutes after injury. Dynamin has been shown to participate in the bundling of actin filaments and to support the formation of stress fibers; therefore, I hypothesize that dynamin may positively affect the stabilization of actin filaments at the wound site [165]. Therefore, I would perform immunofluorescent staining against dynamin after injury to look for colocalization of dynamin and actin filaments at the wound. I have developed an assay to fix laser-injured cells for staining applications that would prove useful to these experiments. In addition, I would perform live cell imaging of actin after injury using SiR-Actin, similar to Figure 3.1, in the presence of dynasore to investigate whether dynamin plays a role in stabilizing the actin structure at the plasma membrane wound. Dynamin has also been shown to enhance actin polymerization by displacing the capping protein gelsolin,

therefore, to investigate whether dynamin may affect actin polymerization during membrane repair, I propose to inhibit dynamin with Dynasore and Dyngo4a and track actin polymerization using FRAP of SiR-Actin as described in Figure 3.2 [165]. If dynamin directly regulates actin polymerization and this contributes to membrane repair, I expect that very little actin polymerization would occur in the presence of dynamin inhibition.

From these studies, it is unclear which mode of endocytosis is activated by injury, especially given that Dynasore treatment inhibits both dynamin-dependent and dynamin-independent mechanisms of endocytosis [178]. Therefore, I propose that the studies presented in Chapter 2 could be complemented with studies using dominant negative mutant dynamin constructs to rule out any off-target effects of Dynasore treatment on other mechanisms of membrane trafficking. Dominant negative mutant constructs of dynamin have been previously described to prevent binding and hydrolysis of GTP and effectively inhibit dynamin-dependent endocytosis [179]. Using electroporation, I could overexpress these mutants in FDB muscle and measure FM1-43 uptake prior to and following injury to probe the extent to which dynamin-dependent endocytosis is activated following wounding. These studies would clarify whether the massive endocytic response following wounding is dynamin-specific and would narrow the focus to dynamin-dependent forms of endocytosis that could then be followed up with investigations into which dynamin-dependent endocytosis pathway, clathrin-mediated or caveolar endocytosis, is activated by membrane wounding in future experiments [180]. In addition, these constructs could be used in the experiments proposed above to clarify any endocytosis-independent roles of dynamin in membrane

repair and, again, potentially explain any off-target effects of Dynasore that may contribute to its effect on membrane repair.

Subsarcolemmal Cytoskeleton in Dysferlin-Mediated Membrane Repair

Multiple membrane repair proteins have been shown to rely upon the actin cytoskeleton for efficient translocation to membrane wounds. Though, to date, all tools and technologies employed while investigating the role of actin in membrane repair have not had the capability to distinguish between different isoforms of actin. For example, previously in our laboratory we have used the inhibitor Cytochalasin D to inhibit actin polymerization, but we attempted to use doses as to not affect sarcomeric actin. However, it is difficult in pharmacological experiments to definitively prove this and indeed, in our hands, Cytochalasin D did seem to inhibit sarcomere shortening and cell contraction after wounding. Therefore, investigations centered on the contribution of the subsarcolemma cytoskeleton to membrane repair required more specificity that could only be achieved through genetic models.

Studies aimed at identifying cellular distribution and function of separate isoforms of actin suggest that each isoform may have distinct, non-redundant functionality that distinguishes isoforms from each other, even in cases of high sequence homology. Therefore, individual isoforms of actin could contribute individually to the membrane repair process and warrant investigation. In skeletal muscle, three isoforms of actin are expressed, but display distinct localization. Of the three major isoforms of actin expressed in skeletal muscle - α, β, and γ – α-actin is primarily restricted to the thin filaments of the sarcolemma, but γ- and β-actin make up the subsarcolemmal actin

network. Perhaps unsurprisingly, mutations in all three result in muscle pathology, though each myopathy is phenotypically distinct. Mutations in α-actin results in nemaline rod myopathy that is associated with sarcomeric disruption [138]. Mouse models of muscle-specific knockout of β-actin are associated with increased susceptibility to contraction-induced damage, but muscle-specific knockout of γ-actin is not [136, 137, 146]. Interestingly, the muscle pathology shares phenotypic similarity with dysferlin-deficiency, namely increased serum CK, increased fiber diameter, and increased centrally nucleated fibers; however, the mechanism by which γ-actin results in a myopathy remains unclear [136]. We have previously shown that dysferlin is actively recruited by the actin cytoskeleton from the lateral sarcolemma to membrane wounds. Therefore, we hypothesized that the subsarcolemmal cytoskeleton, and in particular, γ-actin, may regulate dysferlin function after membrane injury and its genetic loss would disrupt dysferlin trafficking, resulting in a membrane repair defect.

The role of γ-actin in membrane repair was investigated in a muscle-specific knockout of γ-actin and multiple measures of membrane disruption were employed to ascertain the effect of its loss. Principally, membrane repair was assessed using a canonical assay of membrane repair in the field that tracks the uptake of the extracellular, lipophilic dye, FM1-43, through the membrane lesion. Our data showed that muscle-specific knockout of γ-actin indeed resulted in increased uptake of FM1-43 after injury consistent with a membrane repair defect. However, because we had previously shown that FM1-43 uptake after injury is highly dependent upon dynamin-dependent endocytosis, these results were confirmed by monitoring calcium influx after injury in WT and Actg1$^{-/-}$ muscle fibers. Doing so showed that genetic loss of γ-actin

resulted in increased cytoplasmic calcium after injury compared to wildtype. Taken together, these data demonstrate that muscle specific knockout of γ-actin resulted in a membrane repair defect and further, suggest a role for γ-actin in membrane repair.

We hypothesized that γ-actin may contribute to membrane repair through the regulation of dysferlin trafficking after injury, which was tested by tracking the subcellular localization of a dysferlin-phluorin transgene in injured adult Actg1$^{-/-}$ skeletal muscle fibers. Our data showed that dysferlin trafficking to membrane wounds and dysferlin endocytosis are both unaffected by genetic loss of γ-actin. Therefore, these data suggest that the subsarcolemma-specific isoform of actin, γ-actin, seems to play a role in membrane repair, as it's loss of function in adult muscle fibers leads to a membrane repair defect, but the role is likely independent of dysferlin.

Future Directions

The mechanisms by which γ-actin participates in membrane repair remain unclear. This could be, in part, due to compensation by other isoforms of actin in the γ-actin knockout model. Both β- and γ-actin are highly similar in amino acid sequence and it has been shown previously that muscle-specific knockout of γ-actin results in increased expression of other actin isoforms [134]. Therefore, compensation by other isoforms cannot be ruled out in our experiments and contribution of β-actin could potentially diminish the effects of loss of γ-actin on membrane repair. To address this, I would propose to repeat the experiments outlined in Chapter 3 in animals lacking both β- and γ-actin compared to each singularly mutated mouse model (Actg1$^{-/-}$, Actb1$^{-/-}$) with the expectation that double knockout would have an even more severe phenotype than either mutation alone. One challenge to these experiments is, according to the Ervasti

laboratory which made these mice, the breeding of these animals, even using muscle specific promoters, is challenging and few double knockout animals appear to survive early development, the reasons for which remain unclear. Unfortunately, without any isoform-specific inhibitors or isoform-specific fluorescent probes, it may be impossible to determine the contribution of each isoform individually, but these experiments may provide some insight into membrane repair.

While compensation by β-actin could explain decreased phenotype severity in our experiments, an alternate explanation is that the role of γ-actin in membrane repair is more nuanced and perhaps indirect. Consistent with this hypothesis, our data shows that while Actg1$^{-/-}$ fibers do have a membrane repair defect, the mechanism is independent of at least one principle membrane repair protein: dysferlin. Furthermore, actin polymerization at membrane wounds is unaffected by genetic loss of γ-actin. Taken together, these data suggest that γ-actin may not play a direct role in wound closure. Therefore, I hypothesize that, instead, γ-actin may play a structural role in stabilizing the wound to prevent undue wound expansion and to facilitate quick wound closure, which is also consistent with the canonical role of γ-actin as a stabilizing feature within the costamere. I propose to investigate this using the membrane marker CellBrite. CellBrite is a covalent membrane stain that labels the membrane quickly and can be removed from the media to limit endocytic uptake of dye. Preliminary data from our laboratory using Fluorescence Recovery after Photobleach (FRAP) of CellBrite at various distances from the wound suggests that lateral sarcolemma from >30μm away from the wound is pulled toward the wound site after injury. I would anticipate that if γ-actin stabilized wound closure, repeating this experiment in Actg1$^{-/-}$ muscle fibers would

result in less membrane being pulled toward the wound site, membrane retraction from the wound, or visually apparent gaps in the sarcolemma as a result of wound expansion, any of which would suggest that γ-actin plays a role in stabilizing the pore to promote efficient repair.

An additional line of evidence that points to a structural role of γ-actin in membrane repair is a potential interaction between γ-actin and Annexin V. Annexin V, an identified member of the plasma membrane repair cap in striated muscle, has been shown to preferentially bind γ-actin [74, 154]. Annexin V has been proposed to assemble into a submembrane lattice structure after an injury, which is proposed to stabilize the wound [80, 181]. Therefore, an interaction between AnnexinV and γ-actin after injury could serve to promote stability of the membrane repair complex, stability of the wound, or both. I would propose to do colocalization and co-immunoprecipitation studies between γ-actin and AnnexinV in injured myofibers, with the expectation that if an interaction between γ-actin and AnnexinV exists, both would be enriched in pull-down assays of injured fibers and colocalize at the site of injury. In addition, a fluorescent construct of AnnexinV could be electroporated into adult skeletal muscle of both wildtype and γ-actin knockout mice prior to injury, as previously described by the McNally laboratory, with the expectation that if AnnexinV is regulated by γ-actin, recruitment of AnnexinV to membrane wounds would be disrupted in Actg1$^{-/-}$ muscle fibers [74]. These data would inform the contribution of γ-actin to membrane repair and potentially contribute to the understanding of disease pathology in γ-actin knockout mice.

While these studies may clarify the role of γ-actin in membrane repair, it remains unclear what drives actin polymerization at the wound. As discussed in Chapter 1, many actin regulators have been identified as critical to membrane repair in *Xenopus* oocytes and *Drosophila* embryos, particularly the Rho GTPases and their downstream targets [47, 51, 52]. Recently, Rho GTPases were also implicated in wound repair in L6 myotubes, but whether similar regulatory mechanisms are activated in adult skeletal muscle fibers after wounding is still unclear [118]. I hypothesize that RhoA, Rac1, and/or Cdc42 may be required for efficient membrane resealing through regulation of wound-induced actin polymerization that drives dysferlin recruitment to the wound site. To investigate whether these GTPases are involved in membrane repair, both pharmacological and genetic approaches can be applied. To inhibit RhoA, muscle fibers could be treated with C3 exoenzyme, a specific inhibitor that ADP-ribosylates the active site of small GTPases of the RhoA subfamily [182, 183]. To inhibit Rac1 muscle fibers could be treated with NSC23766 to prevent Rac1's association with Rac-specific GEFs [184]. Lastly, to inhibit Cdc42, muscle fibers could be treated with the allosteric inhibitor ML 141, which, importantly, does not affect Rac1 function [185]. Firstly, whether wounding activates these GTPases could be tested using pull-down activation assays using GST beads against active forms of RhoA, Rac1, and Cdc42 followed by quantitative Western blot could be implemented on fibers that are bulk wounded by syringe flushing and then compared to unwounded cells in the presence or absence of pharmacological agents against RhoA, Rac1, and/or Cdc42 [28, 186, 187]. The activation of Rho GTPases could also be investigated in real time after injury using FRET biosensors. Recently, a transgenic mouse has been developed expressing a

RhoA FRET biosensor [188]. Similarly, FRET biosensors for Rac1 and Cdc42 have been developed as plasmids that could be electroporated into the FDB prior to isolation [189, 190]. Individual muscle fibers expressing these biosensors, either transgenically or through electroporation, could be injured using laser injury, and the FRET intensity specifically at the membrane wound could be measured as an indicator of Rho activation following injury.

To test whether RhoA, Rac1, and/or Cdc42 may be involved in membrane repair, pharmacological agents can be applied prior to injury. I propose to treat isolated muscle fibers with each Rho inhibitor or a vehicle control prior to wounding and then measure both uptake of FM1-43 and uptake of calcium as measures of prolonged membrane disruption. To link any observed defects in membrane repair to actin polymerization at membrane wounds, actin would be labeled with SiR-Actin in isolated muscle fibers, such as in Figure 3.1, and subjected to a laser-wounding in the presence of RhoA, Rac1, or Cdc42 inhibitors or a vehicle control. In addition, FRAP of SiR-Actin, similar to Figure 3.2, would also be used to investigate the inhibitors' effects on actin polymerization. In addition, Rho GTPase FRET biosensors could be used in conjunction with pharmacological inhibition to show that activation of RhoA, Rac1, and/or Cdc42 precedes actin polymerization and that inhibition sufficiently prevents activation, thereby preventing actin polymerization at membrane wounds. The FRET biosensors could also help rule out non-specificity of inhibitors or potential cross talk between different Rho signaling pathways.

The rapid rearrangement in the actin cytoskeleton directed by the activity of Rho GTPases may be important for dysferlin recruitment to the wound site or trafficking of

dysferlin-containing vesicles. To test this, isolated muscle fibers from the dysf-pHGFP reporter mouse could be treated either with the RhoA, Rac1, or Cdc42 inhibitors or a vehicle control. Mean fluorescence intensity of dysferlin at the wound site, rate of dysferlin appearance at wound site, and the degree of dysferlin endocytosis from distal sites could be quantified. In addition, wounded fibers could be treated with NH_4Cl to illuminate dysferlin-containing vesicles and the number of vesicles as well as the number of vesicle movements could be quantified to measure any effects of the inhibitors on dysferlin endocytosis. I hypothesize that RhoA, Rac1, and/or Cdc42 may be required for efficient membrane resealing due to the dysregulation of wound-induced actin polymerization that results in the dysregulation of dysferlin trafficking to the wound or its recruitment into vesicles.

To account for any off-target effects of Rho inhibitors, the above experiments can be repeated in genetic models. Both a Cdc42 and a Rac1 floxed mouse are available and could be crossed to available mice that express the cre recombinase gene driven by the human skeletal actin promoter to generate a muscle-specific knockout of these GTPases similar to that of γ-actin in Chapter3. [191-193] To investigate RhoA, a dominant negative RhoA-mCherry plasmid could be electroporated into FDB fibers and experiments performed on positively transfected fibers. [194] Based on this hypothesis, I expect that upon inhibition or genetic ablation of RhoA, Rac1, or Cdc42, time to resealing, actin polymerization, and the trafficking of dysferlin to the wound site will be either delayed or completely disrupted.

Conclusions

Sarcolemma instability represents a major mechanism of disease pathology across multiple forms of muscular dystrophy. Understanding the basic mechanisms of plasma membrane repair not only addresses an important biological process but may also help inform new targets and treatments for muscular dystrophies associated with both increased susceptibility to muscle damage as well as muscular dystrophies associated with decreased capacity for membrane repair. To that end, the studies presented herein provide important insight into the mechanisms of plasma membrane repair in striated muscle. In particular, these studies explore the impact of dynamin-dependent endocytosis after injury and provide evidence that dynamin-dependent endocytosis is highly active at rest and after injury in adult skeletal muscle fibers, which may contribute to pathology in dysferlin-deficiency. The studies in this dissertation also present limitations to current methods of measuring membrane repair and give careful consideration to alternate modes of monitoring wound closure, which will prove extremely valuable to the membrane repair field. Furthermore, additional studies outlined in this thesis provide evidence that the subsarcolemmal cytoskeleton is activated by membrane injury and one isoform of actin, γ-actin, is particularly important for repairing membrane damage. Taken together, these data highlight multiple damage-response pathways that likely need to each function efficiently to repair the sarcolemma and collectively contribute to plasma membrane repair specifically in striated muscle (**Figure 4.1**).

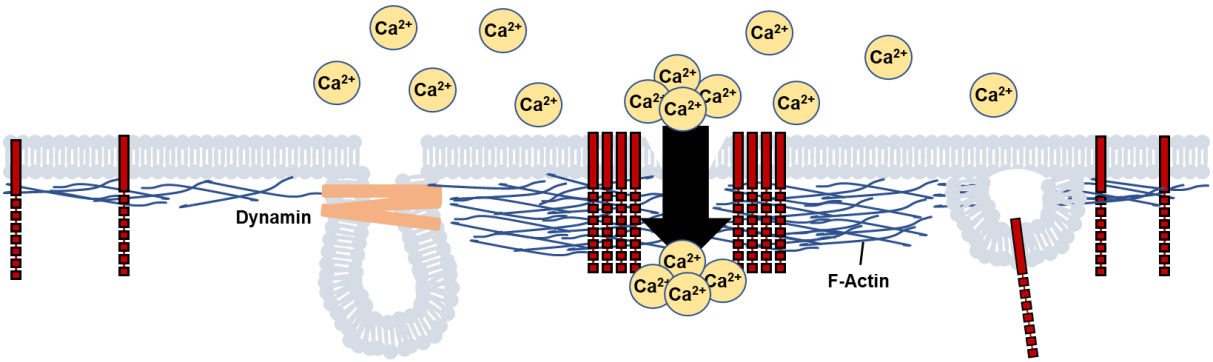

Figure 4.1 Proposed Model of Calcium-Activated Membrane Repair. Injury results in the rapid influx of calcium that we propose drives both dynamin-dependent endocytosis after injury and the polymerization of actin at membrane wounds. Actin polymerization drives the translocation of dysferlin to membrane wounds, but dysferlin endocytosis is independent of dynamin.

REFERENCES

1. Newham, D.J., D.A. Jones, and R.H.T. Edwards, *Plasma creatine kinase changes after eccentric and concentric contractions.* Muscle & Nerve, 1986. **9**(1): p. 59-63.

2. Newham, D.J., G. McPhail, K.R. Mills, and R.H.T. Edwards, *Ultrastructural changes after concentric and eccentric contractions of human muscle.* Journal of the Neurological Sciences, 1983. **61**(1): p. 109-122.

3. Tiidus, P.M. and C.D. Ianuzzo, *Effects of intensity and duration of muscular exercise on delayed soreness and serum enzyme activities.* Med Sci Sports Exerc, 1983. **15**(6): p. 461-5.

4. Friden, J., M. Sjostrom, and B. Ekblom, *Myofibrillar damage following intense eccentric exercise in man.* Int J Sports Med, 1983. **4**(3): p. 170-6.

5. Brooks, S.V., E. Zerba, and J.A. Faulkner, *Injury to muscle fibres after single stretches of passive and maximally stimulated muscles in mice.* J Physiol, 1995. **488 (Pt 2)**: p. 459-69.

6. McCully, K.K. and J.A. Faulkner, *Injury to skeletal muscle fibers of mice following lengthening contractions.* Journal of Applied Physiology, 1985. **59**(1): p. 119-126.

7. McCully, K.K. and J.A. Faulkner, *Characteristics of lengthening contractions associated with injury to skeletal muscle fibers.* Journal of Applied Physiology, 1986. **61**(1): p. 293-299.

8. Fridén, J. and R.L. Lieber, *Segmental muscle fiber lesions after repetitive eccentric contractions.* Cell and Tissue Research, 1998. **293**(1): p. 165-171.

9. Armstrong, R.B., R.W. Ogilvie, and J.A. Schwane, *Eccentric exercise-induced injury to rat skeletal muscle.* J Appl Physiol Respir Environ Exerc Physiol, 1983. **54**(1): p. 80-93.

10. McNeil, P.L. and R. Khakee, *Disruptions of muscle fiber plasma membranes. Role in exercise-induced damage.* Am J Pathol, 1992. **140**(5): p. 1097-109.

11. Ervasti, J.M., *Costameres: the Achilles' heel of Herculean muscle.* J Biol Chem, 2003. **278**(16): p. 13591-4.

12. Ramaswamy, K.S., M.L. Palmer, J.H. van der Meulen, A. Renoux, T.Y. Kostrominova, D.E. Michele, and J.A. Faulkner, *Lateral transmission of force is impaired in skeletal muscles of dystrophic mice and very old rats.* J Physiol, 2011. **589**(Pt 5): p. 1195-208.

13. Bloch, R.J. and H. Gonzalez-Serratos, *Lateral Force Transmission Across Costameres in Skeletal Muscle.* Exercise and Sport Sciences Reviews, 2003. **31**(2): p. 73-78.

14. Street, S.F., *Lateral transmission of tension in frog myofibers: a myofibrillar network and transverse cytoskeletal connections are possible transmitters.* J Cell Physiol, 1983. **114**(3): p. 346-64.

15. Rapoport, S.I., *Mechanical properties of the sarcolemma and myoplasm in frog muscle as a function of sarcomere length.* J Gen Physiol, 1972. **59**(5): p. 559-85.

16. CASELLA, C., *Tensile Force in Total Striated Muscle, Isolated Fibre and Sarcolemma.* Acta Physiologica Scandinavica, 1950. **21**(4): p. 380-401.

17. Hoffman, E.P., R.H. Brown, Jr., and L.M. Kunkel, *Dystrophin: the protein product of the Duchenne muscular dystrophy locus.* Cell, 1987. **51**(6): p. 919-28.

18. Jung, D., B. Yang, J. Meyer, J.S. Chamberlain, and K.P. Campbell, *Identification and characterization of the dystrophin anchoring site on beta-dystroglycan.* J Biol Chem, 1995. **270**(45): p. 27305-10.

19. Rybakova, I.N., J.R. Patel, and J.M. Ervasti, *The dystrophin complex forms a mechanically strong link between the sarcolemma and costameric actin.* The Journal of cell biology, 2000. **150**(5): p. 1209-1214.

20. Petrof, B.J., J.B. Shrager, H.H. Stedman, A.M. Kelly, and H.L. Sweeney, *Dystrophin protects the sarcolemma from stresses developed during muscle contraction.* Proceedings of the National Academy of Sciences, 1993. **90**(8): p. 3710-3714.

21. Consolino, C.M. and S.V. Brooks, *Susceptibility to sarcomere injury induced by single stretches of maximally activated muscles of mdx mice.* Journal of Applied Physiology, 2004. **96**(2): p. 633-638.

22. Dellorusso, C., R.W. Crawford, J.S. Chamberlain, and S.V. Brooks, *Tibialis anterior muscles in mdx mice are highly susceptible to contraction-induced injury.* J Muscle Res Cell Motil, 2001. **22**(5): p. 467-75.

23. Mokri, B. and A.G. Engel, *Duchenne dystrophy: electron microscopic findings pointing to a basic or early abnormality in the plasma membrane of the muscle fiber.* Neurology, 1975. **25**(12): p. 1111-20.

24. Durbeej, M. and K.P. Campbell, *Muscular dystrophies involving the dystrophin–glycoprotein complex: an overview of current mouse models.* Current Opinion in Genetics & Development, 2002. **12**(3): p. 349-361.

25. Chan, S., S.I. Head, and J.W. Morley, *Branched fibers in dystrophic mdx muscle are associated with a loss of force following lengthening contractions.* American Journal of Physiology-Cell Physiology, 2007. **293**(3): p. C985-C992.

26. Liu, J., M. Aoki, I. Illa, C. Wu, M. Fardeau, C. Angelini, C. Serrano, J.A. Urtizberea, F. Hentati, M.B. Hamida, S. Bohlega, E.J. Culper, A.A. Amato, K. Bossie, J. Oeltjen, K. Bejaoui, D. McKenna-Yasek, B.A. Hosler, E. Schurr, K. Arahata, P.J. de Jong, and R.H. Brown, *Dysferlin, a novel skeletal muscle gene, is mutated in Miyoshi myopathy and limb girdle muscular dystrophy.* Nature Genetics, 1998. **20**(1): p. 31-36.

27. Bashir, R., S. Britton, T. Strachan, S. Keers, E. Vafiadaki, M. Lako, I. Richard, S. Marchand, N. Bourg, Z. Argov, M. Sadeh, I. Mahjneh, G. Marconi, M.R. Passos-Bueno, E.d.S. Moreira, M. Zatz, J.S. Beckmann, and K. Bushby, *A gene related to Caenorhabditis elegans spermatogenesis factor fer-1 is mutated in limb-girdle muscular dystrophy type 2B.* Nature Genetics, 1998. **20**(1): p. 37-42.

28. Bansal, D., K. Miyake, S.S. Vogel, S. Groh, C.C. Chen, R. Williamson, P.L. McNeil, and K.P. Campbell, *Defective membrane repair in dysferlin-deficient muscular dystrophy.* Nature, 2003. **423**(6936): p. 168-72.

29. Straub, V. and K.P. Campbell, *Muscular dystrophies and the dystrophin-glycoprotein complex.* Curr Opin Neurol, 1997. **10**(2): p. 168-75.

30. Selcen, D., G. Stilling, and A.G. Engel, *The earliest pathologic alterations in dysferlinopathy.* Neurology, 2001. **56**(11): p. 1472-1481.

31. Piccolo, F., S.A. Moore, G.C. Ford, and K.P. Campbell, *Intracellular accumulation and reduced sarcolemmal expression of dysferlin in limb--girdle muscular dystrophies.* Ann Neurol, 2000. **48**(6): p. 902-12.

32. Bansal, D. and K.P. Campbell, *Dysferlin and the plasma membrane repair in muscular dystrophy.* Trends Cell Biol, 2004. **14**(4): p. 206-13.

33. Davenport, N.R. and W.M. Bement, *Cell repair: Revisiting the patch hypothesis.* Communicative & integrative biology, 2016. **9**(6): p. e1253643-e1253643.

34. McNeil, P.L., S.S. Vogel, K. Miyake, and M. Terasaki, *Patching plasma membrane disruptions with cytoplasmic membrane.* J Cell Sci, 2000. **113 (Pt 11)**: p. 1891-902.

35. Miyake, K. and P.L. McNeil, *Vesicle accumulation and exocytosis at sites of plasma membrane disruption.* Journal of Cell Biology, 1995. **131**(6): p. 1737-1745.

36. Terasaki, M., K. Miyake, and P.L. McNeil, *Large Plasma Membrane Disruptions Are Rapidly Resealed by Ca2+-dependent Vesicle–Vesicle Fusion Events.* Journal of Cell Biology, 1997. **139**(1): p. 63-74.

37. 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.

38. Bi, G.Q., J.M. Alderton, and R.A. Steinhardt, *Calcium-regulated exocytosis is required for cell membrane resealing.* J Cell Biol, 1995. **131**(6 Pt 2): p. 1747-58.

39. Eddleman, C.S., M.L. Ballinger, M.E. Smyers, H.M. Fishman, and G.D. Bittner, *Endocytotic formation of vesicles and other membranous structures induced by Ca2+ and axolemmal injury.* J Neurosci, 1998. **18**(11): p. 4029-41.

40. Eddleman, C.S., M.L. Ballinger, M.E. Smyers, C.M. Godell, H.M. Fishman, and G.D. Bittner, *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.

41. Corrotte, M. and T. Castro-Gomes, *Chapter One - Lysosomes and plasma membrane repair*, in *Current Topics in Membranes*, L.O. Andrade, Editor. 2019, Academic Press. p. 1-16.

42. Reddy, A., E.V. Caler, and N.W. Andrews, *Plasma membrane repair is mediated by Ca(2+)-regulated exocytosis of lysosomes.* Cell, 2001. **106**(2): p. 157-69.

43. Castro-Gomes, T., M. Corrotte, C. Tam, and N.W. Andrews, *Plasma Membrane Repair Is Regulated Extracellularly by Proteases Released from Lysosomes.* PLoS One, 2016. **11**(3): p. e0152583.

44. Tam, C., V. Idone, C. Devlin, M.C. Fernandes, A. Flannery, X. He, E. Schuchman, I. Tabas, and N.W. Andrews, *Exocytosis of acid sphingomyelinase by wounded cells promotes endocytosis and plasma membrane repair.* Journal of Cell Biology, 2010. **189**(6): p. 1027-1038.

45. Corrotte, M., P.E. Almeida, C. Tam, T. Castro-Gomes, M.C. Fernandes, B.A. Millis, M. Cortez, H. Miller, W. Song, T.K. Maugel, and N.W. Andrews, *Caveolae internalization repairs wounded cells and muscle fibers.* Elife, 2013. **2**: p. e00926.

46. Thiery, J., D. Keefe, S. Saffarian, D. Martinvalet, M. Walch, E. Boucrot, T. Kirchhausen, and J. Lieberman, *Perforin activates clathrin- and dynamin-dependent endocytosis, which is required for plasma membrane repair and delivery of granzyme B for granzyme-mediated apoptosis.* Blood, 2010. **115**(8): p. 1582-93.

47. Boucher, E., L. Goldin-Blais, Q. Basiren, and C.A. Mandato, *Actin dynamics and myosin contractility during plasma membrane repair and restoration: Does one ring really heal them all?* Curr Top Membr, 2019. **84**: p. 17-41.

48. 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, 1999. **9**(11): p. 579-587.

49. 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.* J Cell Biol, 2011. **193**(3): p. 455-64.

50. Mandato, C.A. and W.M. Bement, *Actomyosin transports microtubules and microtubules control actomyosin recruitment during Xenopus oocyte wound healing.* Curr Biol, 2003. **13**(13): p. 1096-105.

51. Benink, H.A. and W.M. Bement, *Concentric zones of active RhoA and Cdc42 around single cell wounds.* The Journal of cell biology, 2005. **168**(3): p. 429-439.

52. Abreu-Blanco, M.T., J.M. Verboon, and S.M. Parkhurst, *Coordination of Rho family GTPase activities to orchestrate cytoskeleton responses during cell wound repair.* Current biology : CB, 2014. **24**(2): p. 144-155.

53. Burkel, B.M., H.A. Benink, E.M. Vaughan, G. von Dassow, and W.M. Bement, *A Rho GTPase signal treadmill backs a contractile array.* Developmental cell, 2012. **23**(2): p. 384-396.

54. Cenacchi, G., M. Fanin, L.B. De Giorgi, and C. Angelini, *Ultrastructural changes in dysferlinopathy support defective membrane repair mechanism.* J Clin Pathol, 2005. **58**(2): p. 190-5.

55. Shao, X., B.A. Davletov, R.B. Sutton, T.C. Sudhof, and J. Rizo, *Bipartite Ca2+-binding motif in C2 domains of synaptotagmin and protein kinase C.* Science, 1996. **273**(5272): p. 248-51.

56. Shao, X., C. Li, I. Fernandez, X. Zhang, T.C. Sudhof, and J. Rizo, *Synaptotagmin-syntaxin interaction: the C2 domain as a Ca2+-dependent electrostatic switch.* Neuron, 1997. **18**(1): p. 133-42.

57. Therrien, C., S. Di Fulvio, S. Pickles, and M. Sinnreich, *Characterization of lipid binding specificities of dysferlin C2 domains reveals novel interactions with phosphoinositides.* Biochemistry, 2009. **48**(11): p. 2377-84.

58. Davis, D.B., K.R. Doherty, A.J. Delmonte, and E.M. McNally, *Calcium-sensitive phospholipid binding properties of normal and mutant ferlin C2 domains.* J Biol Chem, 2002. **277**(25): p. 22883-8.

59. Marty, N.J., C.L. Holman, N. Abdullah, and C.P. Johnson, *The C2 domains of otoferlin, dysferlin, and myoferlin alter the packing of lipid bilayers.* Biochemistry, 2013. **52**(33): p. 5585-92.

60. Abdullah, N., M. Padmanarayana, N.J. Marty, and C.P. Johnson, *Quantitation of the calcium and membrane binding properties of the C2 domains of dysferlin.* Biophys J, 2014. **106**(2): p. 382-9.

61. Fuson, K., A. Rice, R. Mahling, A. Snow, K. Nayak, P. Shanbhogue, A.G. Meyer, G.M. Redpath, A. Hinderliter, S.T. Cooper, and R.B. Sutton, *Alternate splicing of dysferlin C2A confers Ca(2)(+)-dependent and Ca(2)(+)-independent binding for membrane repair.* Structure, 2014. **22**(1): p. 104-15.

62. Azakir, B.A., S. Di Fulvio, S. Salomon, M. Brockhoff, C. Therrien, and M. Sinnreich, *Modular dispensability of dysferlin C2 domains reveals rational design for mini-dysferlin molecules.* J Biol Chem, 2012. **287**(33): p. 27629-36.

63. Xu, L., S. Pallikkuth, Z. Hou, G.A. Mignery, S.L. Robia, and R. Han, *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.

64. McDade, J.R., A. Archambeau, and D.E. Michele, *Rapid actin-cytoskeleton-dependent recruitment of plasma membrane-derived dysferlin at wounds is critical for muscle membrane repair.* Faseb j, 2014. **28**(8): p. 3660-70.

65. McDade, J.R. and D.E. Michele, *Membrane damage-induced vesicle-vesicle fusion of dysferlin-containing vesicles in muscle cells requires microtubules and kinesin.* Hum Mol Genet, 2014. **23**(7): p. 1677-86.

66. Swaggart, K.A., A.R. Demonbreun, A.H. Vo, K.E. Swanson, E.Y. Kim, J.P. Fahrenbach, J. Holley-Cuthrell, A. Eskin, Z. Chen, K. Squire, A. Heydemann, A.A. Palmer, S.F. Nelson, and E.M. McNally, *Annexin A6 modifies muscular dystrophy by mediating sarcolemmal repair.* Proceedings of the National Academy of Sciences of the United States of America, 2014. **111**(16): p. 6004-6009.

67. Lennon, N.J., A. Kho, B.J. Bacskai, S.L. Perlmutter, B.T. Hyman, and R.H. Brown, *Dysferlin Interacts with Annexins A1 and A2 and Mediates Sarcolemmal Wound-healing.* Journal of Biological Chemistry, 2003. **278**(50): p. 50466-50473.

68. Cai, C., H. Masumiya, N. Weisleder, N. Matsuda, M. Nishi, M. Hwang, J.K. Ko, P. Lin, A. Thornton, X. Zhao, Z. Pan, S. Komazaki, M. Brotto, H. Takeshima, and J. Ma, *MG53 nucleates assembly of cell membrane repair machinery.* Nat Cell Biol, 2009. **11**(1): p. 56-64.

69. Matsuda, C., Y.K. Hayashi, M. Ogawa, M. Aoki, K. Murayama, I. Nishino, I. Nonaka, K. Arahata, and R.H.B. Jr, *The sarcolemmal proteins dysferlin and caveolin-3 interact in skeletal muscle.* Human Molecular Genetics, 2001. **10**(17): p. 1761-1766.

70. Gerke, V., C.E. Creutz, and S.E. Moss, *Annexins: linking Ca2+ signalling to membrane dynamics.* Nat Rev Mol Cell Biol, 2005. **6**(6): p. 449-61.

71. Cagliani, R., F. Magri, A. Toscano, L. Merlini, F. Fortunato, C. Lamperti, C. Rodolico, A. Prelle, M. Sironi, M. Aguennouz, P. Ciscato, A. Uncini, M. Moggio, N. Bresolin, and G.P. Comi, *Mutation finding in patients with dysferlin deficiency and role of the dysferlin interacting proteins annexin A1 and A2 in muscular dystrophies.* Hum Mutat, 2005. **26**(3): p. 283.

72. Lennon, N.J., A. Kho, B.J. Bacskai, S.L. Perlmutter, B.T. Hyman, and R.H. Brown, Jr., *Dysferlin interacts with annexins A1 and A2 and mediates sarcolemmal wound-healing.* J Biol Chem, 2003. **278**(50): p. 50466-73.

73. Roostalu, U. and U. Strahle, *In vivo imaging of molecular interactions at damaged sarcolemma.* Dev Cell, 2012. **22**(3): p. 515-29.

74. Demonbreun, A.R., M. Quattrocelli, D.Y. Barefield, M.V. Allen, K.E. Swanson, and E.M. McNally, *An actin-dependent annexin complex mediates plasma membrane repair in muscle.* The Journal of cell biology, 2016. **213**(6): p. 705-718.

75. Leikina, E., A. Defour, K. Melikov, J.H. Van der Meulen, K. Nagaraju, S. Bhuvanendran, C. Gebert, K. Pfeifer, L.V. Chernomordik, and J.K. Jaiswal, *Annexin A1 Deficiency does not Affect Myofiber Repair but Delays Regeneration of Injured Muscles.* Scientific Reports, 2015. **5**(1): p. 18246.

76. McNeil, A.K., U. Rescher, V. Gerke, and P.L. McNeil, *Requirement for annexin A1 in plasma membrane repair.* J Biol Chem, 2006. **281**(46): p. 35202-7.

77. Defour, A., S. Medikayala, J.H. Van der Meulen, M.W. Hogarth, N. Holdreith, A. Malatras, W. Duddy, J. Boehler, K. Nagaraju, and J.K. Jaiswal, *Annexin A2 links poor myofiber repair with inflammation and adipogenic replacement of the injured muscle.* Human molecular genetics, 2017. **26**(11): p. 1979-1991.

78. Swaggart, K.A., A.R. Demonbreun, A.H. Vo, K.E. Swanson, E.Y. Kim, J.P. Fahrenbach, J. Holley-Cuthrell, A. Eskin, Z. Chen, K. Squire, A. Heydemann, A.A. Palmer, S.F. Nelson, and E.M. McNally, *Annexin A6 modifies muscular dystrophy by mediating sarcolemmal repair.* Proc Natl Acad Sci U S A, 2014. **111**(16): p. 6004-9.

79. Carmeille, R., F. Bouvet, S. Tan, C. Croissant, C. Gounou, K. Mamchaoui, V. Mouly, A.R. Brisson, and A. Bouter, *Membrane repair of human skeletal muscle cells requires Annexin-A5.* Biochim Biophys Acta, 2016. **1863**(9): p. 2267-79.

80. Bouter, A., C. Gounou, R. Berat, S. Tan, B. Gallois, T. Granier, B.L. d'Estaintot, E. Poschl, B. Brachvogel, and A.R. Brisson, *Annexin-A5 assembled into two-dimensional arrays promotes cell membrane repair.* Nat Commun, 2011. **2**: p. 270.

81. Voges, D., R. Berendes, A. Burger, P. Demange, W. Baumeister, and R. Huber, *Three-dimensional structure of membrane-bound annexin V. A correlative electron microscopy-X-ray crystallography study.* J Mol Biol, 1994. **238**(2): p. 199-213.

82. Alvarez-Guaita, A., S. Vilà de Muga, D.M. Owen, D. Williamson, A. Magenau, A. García-Melero, M. Reverter, M. Hoque, R. Cairns, R. Cornely, F. Tebar, T. Grewal, K. Gaus, J. Ayala-Sanmartín, C. Enrich, and C. Rentero, *Evidence for annexin A6-dependent plasma membrane remodelling of lipid domains.* British journal of pharmacology, 2015. **172**(7): p. 1677-1690.

83. Hayes, M.J., D. Shao, M. Bailly, and S.E. Moss, *Regulation of actin dynamics by annexin 2.* The EMBO Journal, 2006. **25**(9): p. 1816-1826.

84. Boye, T.L., K. Maeda, W. Pezeshkian, S.L. Sønder, S.C. Haeger, V. Gerke, A.C. Simonsen, and J. Nylandsted, *Annexin A4 and A6 induce membrane curvature and constriction during cell membrane repair.* Nature Communications, 2017. **8**(1): p. 1623.

85. Defour, A., J.H. Van der Meulen, R. Bhat, A. Bigot, R. Bashir, K. Nagaraju, and J.K. Jaiswal, *Dysferlin regulates cell membrane repair by facilitating injury-triggered acid sphingomyelinase secretion.* Cell Death & Disease, 2014. **5**(6): p. e1306-e1306.

86. Codding, S.J., N. Marty, N. Abdullah, and C.P. Johnson, *Dysferlin Binds SNAREs (Soluble N-Ethylmaleimide-sensitive Factor (NSF) Attachment Protein Receptors) and Stimulates Membrane Fusion in a Calcium-sensitive Manner.* The Journal of biological chemistry, 2016. **291**(28): p. 14575-14584.

87. Cocucci, E., G. Racchetti, P. Podini, M. Rupnik, and J. Meldolesi, *Enlargeosome, an exocytic vesicle resistant to nonionic detergents, undergoes endocytosis via a nonacidic route.* Molecular biology of the cell, 2004. **15**(12): p. 5356-5368.

88. Benaud, C., B.J. Gentil, N. Assard, M. Court, J. Garin, C. Delphin, and J. Baudier, *AHNAK interaction with the annexin 2/S100A10 complex regulates cell membrane cytoarchitecture.* The Journal of cell biology, 2004. **164**(1): p. 133-144.

89. Huang, Y., S.H. Laval, A.v. Remoortere, J. Baudier, C. Benaud, L.V.B. Anderson, V. Straub, A. Deelder, R.R. Frants, J.T.d. Dunnen, K. Bushby, and S.M.v.d. Maarel, *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.

90. Cai, C., N. Weisleder, J.-K. Ko, S. Komazaki, Y. Sunada, M. Nishi, H. Takeshima, and J. Ma, *Membrane repair defects in muscular dystrophy are linked to altered interaction between MG53, caveolin-3, and dysferlin.* The Journal of biological chemistry, 2009. **284**(23): p. 15894-15902.

91. Matsuda, C., K. Miyake, K. Kameyama, E. Keduka, H. Takeshima, T. Imamura, N. Araki, I. Nishino, and Y. Hayashi, *The C2A domain in dysferlin is important for association with MG53 (TRIM72).* PLoS Curr, 2012. **4**: p. e5035add8caff4.

92. Weisleder, N., N. Takizawa, P. Lin, X. Wang, C. Cao, Y. Zhang, T. Tan, C. Ferrante, H. Zhu, P.J. Chen, R. Yan, M. Sterling, X. Zhao, M. Hwang, M. Takeshima, C. Cai, H. Cheng, H. Takeshima, R.P. Xiao, and J. Ma, *Recombinant MG53 protein modulates therapeutic cell membrane repair in treatment of muscular dystrophy.* Sci Transl Med, 2012. **4**(139): p. 139ra85.

93. Allen, D.G., N.P. Whitehead, and S.C. Froehner, *Absence of Dystrophin Disrupts Skeletal Muscle Signaling: Roles of Ca2+, Reactive Oxygen Species, and Nitric Oxide in the Development of Muscular Dystrophy.* Physiol Rev, 2016. **96**(1): p. 253-305.

94. 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.

95. Bansal, D., K. Miyake, S.S. Vogel, S. Groh, C.-C. Chen, R. Williamson, P.L. McNeil, and K.P. Campbell, *Defective membrane repair in dysferlin-deficient muscular dystrophy.* Nature, 2003. **423**(6936): p. 168-172.

96. Cai, C., H. Masumiya, N. Weisleder, N. Matsuda, M. Nishi, M. Hwang, J.-K. Ko, P. Lin, A. Thornton, X. Zhao, Z. Pan, S. Komazaki, M. Brotto, H. Takeshima, and J. Ma, *MG53 nucleates assembly of cell membrane repair machinery.* Nat Cell Biol, 2009. **11**(1): p. 56-64.

97. Chakrabarti, S., K.S. Kobayashi, R.A. Flavell, C.B. Marks, K. Miyake, D.R. Liston, K.T. Fowler, F.S. Gorelick, and N.W. Andrews, *Impaired membrane resealing and autoimmune myositis in synaptotagmin VII–deficient mice.* The Journal of Cell Biology, 2003. **162**(4): p. 543-549.

98. Zhu, H., P. Lin, G. De, K.-h. Choi, H. Takeshima, N. Weisleder, and J. Ma, *Polymerase Transcriptase Release Factor (PTRF) Anchors MG53 Protein to Cell Injury Site for Initiation of Membrane Repair.* Journal of Biological Chemistry, 2011. **286**(15): p. 12820-12824.

99. Demonbreun, A.R. and E.M. McNally, *Plasma Membrane Repair in Health and Disease.* Curr Top Membr, 2016. **77**: p. 67-96.

100. Ryan, T.A., H. Reuter, and S.J. Smith, *Optical detection of a quantal presynaptic membrane turnover.* Nature, 1997. **388**(6641): p. 478-82.

101. 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.

102. Bi, G.-Q., R.L. Morris, G. Liao, J.M. Alderton, J.M. Scholey, and R.A. Steinhardt, *Kinesin- and Myosin-driven Steps of Vesicle Recruitment for Ca2+-regulated Exocytosis.* The Journal of Cell Biology, 1997. **138**(5): p. 999-1008.

103. Bitoun, M., S. Maugenre, P.-Y. Jeannet, E. Lacene, X. Ferrer, P. Laforet, J.-J. Martin, J. Laporte, H. Lochmuller, A.H. Beggs, M. Fardeau, B. Eymard, N.B. Romero, and P. Guicheney, *Mutations in dynamin 2 cause dominant centronuclear myopathy.* Nat Genet, 2005. **37**(11): p. 1207-1209.

104. Durieux, A.-C., A. Vignaud, B. Prudhon, M.T. Viou, M. Beuvin, S. Vassilopoulos, B. Fraysse, A. Ferry, J. Lainé, N.B. Romero, P. Guicheney, and M. Bitoun, *A centronuclear myopathy-dynamin 2 mutation impairs skeletal muscle structure and function in mice.* Human Molecular Genetics, 2010. **19**(24): p. 4820-4836.

105. Thiery, J., D. Keefe, S. Saffarian, D. Martinvalet, M. Walch, E. Boucrot, T. Kirchhausen, and J. Lieberman, *Perforin activates clathrin- and dynamin-dependent endocytosis, which is required for plasma membrane repair and delivery of granzyme B for granzyme-mediated apoptosis*. Vol. 115. 2010. 1582-1593.

106. Lostal, W., M. Bartoli, N. Bourg, C. Roudaut, A. Bentaïb, K. Miyake, N. Guerchet, F. Fougerousse, P. McNeil, and I. Richard, *Efficient recovery of dysferlin deficiency by dual adeno-associated vector-mediated gene transfer.* Human Molecular Genetics, 2010. **19**(10): p. 1897-1907.

107. McDade, J.R., A. Archambeau, and D.E. Michele, *Rapid actin-cytoskeleton–dependent recruitment of plasma membrane–derived dysferlin at wounds is critical for muscle membrane repair.* The FASEB Journal, 2014. **28**(8): p. 3660-3670.

108. Park, K.H., N. Weisleder, J.S. Zhou, K. Gumpper, X.Y. Zhou, P. Duann, J.J. Ma, and P.H. Lin, *Assessment of Calcium Sparks in Intact Skeletal Muscle Fibers.* Jove-Journal of Visualized Experiments, 2014(84).

109. 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.

110. Cai, C., N. Weisleder, J.-K. Ko, S. Komazaki, Y. Sunada, M. Nishi, H. Takeshima, and J. Ma, *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.

111. McCluskey, A., J.A. Daniel, G. Hadzic, N. Chau, E.L. Clayton, A. Mariana, A. Whiting, N.N. Gorgani, J. Lloyd, A. Quan, L. Moshkanbaryans, S. Krishnan, S. Perera, M. Chircop, L. von Kleist, A.B. McGeachie, M.T. Howes, R.G. Parton, M. Campbell, J.A. Sakoff, X. Wang, J.Y. Sun, M.J. Robertson, F.M. Deane, T.H. Nguyen, F.A. Meunier, M.A. Cousin, and P.J. Robinson, *Building a better dynasore: the dyngo compounds potently inhibit dynamin and endocytosis.* Traffic, 2013. **14**(12): p. 1272-89.

112. Covian-Nares, J.F., S.V. Koushik, H.L. Puhl, III, and S.S. Vogel, *Membrane wounding triggers ATP release and dysferlin-mediated intercellular calcium signaling.* J Cell Sci, 2010. **123**(11): p. 1884-1893.

113. Mellgren, R.L., K. Miyake, I. Kramerova, M.J. Spencer, N. Bourg, M. Bartoli, I. Richard, P.A. Greer, and P.L. McNeil, *Calcium-dependent plasma membrane repair requires m- or [mu]-calpain, but not calpain-3, the proteasome, or caspases.* Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 2009. **1793**(12): p. 1886-1893.

114. Cheng, X., X. Zhang, Q. Gao, M. Ali Samie, M. Azar, W.L. Tsang, L. Dong, N. Sahoo, X. Li, Y. Zhuo, A.G. Garrity, X. Wang, M. Ferrer, J. Dowling, L. Xu, R. Han, and H. Xu, *The intracellular Ca2+ channel MCOLN1 is required for sarcolemma repair to prevent muscular dystrophy.* Nat Med, 2014. **20**(10): p. 1187-1192.

115. Betz, W.J., G.S. Bewick, and R.M.A.P. Ridge, *Intracellular Movements of Fluorescently Labeled Synaptic Vesicles in Frog Motor-Nerve Terminals during Nerve-Stimulation.* Neuron, 1992. **9**(5): p. 805-813.

116. Han, R., D. Bansal, K. Miyake, V.P. Muniz, R.M. Weiss, P.L. McNeil, and K.P. Campbell, *Dysferlin-mediated membrane repair protects the heart from stress-induced left ventricular injury.* The Journal of Clinical Investigation, 2007. **117**(7): p. 1805-1813.

117. Kerr, J.P., A.P. Ziman, A.L. Mueller, J.M. Muriel, E. Kleinhans-Welte, J.D. Gumerson, S.S. Vogel, C.W. Ward, J.A. Roche, and R.J. Bloch, *Dysferlin stabilizes stress-induced Ca2+ signaling in the transverse tubule membrane.* Proceedings of the National Academy of Sciences, 2013. **110**(51): p. 20831-20836.

118. Horn, A., J.H. Van der Meulen, A. Defour, M. Hogarth, S.C. Sreetama, A. Reed, L. Scheffer, N.S. Chandel, and J.K. Jaiswal, *Mitochondrial redox signaling enables repair of injured skeletal muscle cells.* Science Signaling, 2017. **10**(495): p. eaaj1978.

119. Wu, X.-S., B.D. McNeil, J. Xu, J. Fan, L. Xue, E. Melicoff, R. Adachi, L. Bai, and L.-G. Wu, *Ca2+ and calmodulin initiate all forms of endocytosis during depolarization at a nerve terminal.* Nature Neuroscience, 2009. **12**(8): p. 1003-1010.

120. Burr, A.R. and J.D. Molkentin, *Genetic evidence in the mouse solidifies the calcium hypothesis of myofiber death in muscular dystrophy.* Cell Death & Differentiation, 2015. **22**(9): p. 1402-1412.

121. Chin, Y.H., A. Lee, H.W. Kan, J. Laiman, M.C. Chuang, S.T. Hsieh, and Y.W. Liu, *Dynamin-2 mutations associated with centronuclear myopathy are hypermorphic and lead to T-tubule fragmentation.* Hum Mol Genet, 2015. **24**(19): p. 5542-54.

122. Cowling, B.S., A. Toussaint, L. Amoasii, P. Koebel, A. Ferry, L. Davignon, I. Nishino, J.L. Mandel, and J. Laporte, *Increased expression of wild-type or a centronuclear myopathy mutant of dynamin 2 in skeletal muscle of adult mice leads to structural defects and muscle weakness.* Am J Pathol, 2011. **178**(5): p. 2224-35.

123. Gibbs, E.M., A.E. Davidson, W.R. Telfer, E.L. Feldman, and J.J. Dowling, *The myopathy-causing mutation DNM2-S619L leads to defective tubulation in vitro and in developing zebrafish.* Dis Model Mech, 2014. **7**(1): p. 157-61.

124. Fraysse, B., P. Guicheney, and M. Bitoun, *Calcium homeostasis alterations in a mouse model of the Dynamin 2-related centronuclear myopathy.* Biology open, 2016. **5**(11): p. 1691-1696.

125. Cowling, B.S., A. Toussaint, L. Amoasii, P. Koebel, A. Ferry, L. Davignon, I. Nishino, J.-L. Mandel, and J. Laporte, *Increased Expression of Wild-Type or a Centronuclear Myopathy Mutant of Dynamin 2 in Skeletal Muscle of Adult Mice Leads to Structural Defects and Muscle Weakness.* The American Journal of Pathology. **178**(5): p. 2224-2235.

126. Cowling, B.S., T. Chevremont, I. Prokic, C. Kretz, A. Ferry, C. Coirault, O. Koutsopoulos, V. Laugel, N.B. Romero, and J. Laporte, *Reducing dynamin 2*

expression rescues X-linked centronuclear myopathy. The Journal of Clinical Investigation, 2014. **124**(3): p. 1350-1363.

127. Gajic, O., J. Lee, C.H. Doerr, J.C. Berrios, J.L. Myers, and R.D. Hubmayr, *Ventilator-induced cell wounding and repair in the intact lung.* Am J Respir Crit Care Med, 2003. **167**(8): p. 1057-63.

128. Rodriguez, A., P. Webster, J. Ortego, and N.W. Andrews, *Lysosomes behave as Ca2+-regulated exocytic vesicles in fibroblasts and epithelial cells.* J Cell Biol, 1997. **137**(1): p. 93-104.

129. Leung, C., C. Yu, M.I. Lin, C. Tognon, and P. Bernatchez, *Expression of myoferlin in human and murine carcinoma tumors: role in membrane repair, cell proliferation, and tumorigenesis.* Am J Pathol, 2013. **182**(5): p. 1900-9.

130. Sonnemann, K.J. and W.M. Bement, *Wound repair: toward understanding and integration of single-cell and multicellular wound responses.* Annual review of cell and developmental biology, 2011. **27**: p. 237-263.

131. Gumerson, J.D. and D.E. Michele, *The dystrophin-glycoprotein complex in the prevention of muscle damage.* Journal of biomedicine & biotechnology, 2011. **2011**: p. 210797-210797.

132. Han, R., M. Kanagawa, T. Yoshida-Moriguchi, E.P. Rader, R.A. Ng, D.E. Michele, D.E. Muirhead, S. Kunz, S.A. Moore, S.T. Iannaccone, K. Miyake, P.L. McNeil, U. Mayer, M.B.A. Oldstone, J.A. Faulkner, and K.P. Campbell, *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.

133. Weisleder, N., H. Takeshima, and J. Ma, *Mitsugumin 53 (MG53) facilitates vesicle trafficking in striated muscle to contribute to cell membrane repair.* Communicative & integrative biology, 2009. **2**(3): p. 225-226.

134. Perrin, B.J. and J.M. Ervasti, *The actin gene family: function follows isoform.* Cytoskeleton (Hoboken), 2010. **67**(10): p. 630-4.

135. Herman, I.M., *Actin isoforms.* Curr Opin Cell Biol, 1993. **5**(1): p. 48-55.

136. Sonnemann, K.J., D.P. Fitzsimons, J.R. Patel, Y. Liu, M.F. Schneider, R.L. Moss, and J.M. Ervasti, *Cytoplasmic gamma-actin is not required for skeletal muscle development but its absence leads to a progressive myopathy.* Dev Cell, 2006. **11**(3): p. 387-97.

137. Prins, K.W., J.A. Call, D.A. Lowe, and J.M. Ervasti, *Quadriceps myopathy caused by skeletal muscle-specific ablation of beta(cyto)-actin.* J Cell Sci, 2011. **124**(Pt 6): p. 951-7.

138. Nowak, K.J., D. Wattanasirichaigoon, H.H. Goebel, M. Wilce, K. Pelin, K. Donner, R.L. Jacob, C. Hubner, K. Oexle, J.R. Anderson, C.M. Verity, K.N. North, S.T. Iannaccone, C.R. Muller, P. Nurnberg, F. Muntoni, C. Sewry, I. Hughes, R. Sutphen, A.G. Lacson, K.J. Swoboda, J. Vigneron, C. Wallgren-Pettersson, A.H. Beggs, and N.G. Laing, *Mutations in the skeletal muscle alpha-actin gene in patients with actin myopathy and nemaline myopathy.* Nat Genet, 1999. **23**(2): p. 208-12.

139. Shefer, G. and Z. Yablonka-Reuveni, *Isolation and culture of skeletal muscle myofibers as a means to analyze satellite cells.* Methods Mol Biol, 2005. **290**: p. 281-304.

140. Schindelin, J., I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, S. Preibisch, C. Rueden, S. Saalfeld, B. Schmid, J.-Y. Tinevez, D.J. White, V. Hartenstein, K. Eliceiri, P. Tomancak, and A. Cardona, *Fiji: an open-source platform for biological-image analysis.* Nature Methods, 2012. **9**(7): p. 676-682.

141. Schindelin, J., C.T. Rueden, M.C. Hiner, and K.W. Eliceiri, *The ImageJ ecosystem: An open platform for biomedical image analysis.* Mol Reprod Dev, 2015. **82**(7-8): p. 518-29.

142. Lukinavičius, G., L. Reymond, E. D'Este, A. Masharina, F. Göttfert, H. Ta, A. Güther, M. Fournier, S. Rizzo, H. Waldmann, C. Blaukopf, C. Sommer, D.W. Gerlich, H.-D. Arndt, S.W. Hell, and K. Johnsson, *Fluorogenic probes for live-cell imaging of the cytoskeleton.* Nature Methods, 2014. **11**(7): p. 731-733.

143. Saneyoshi, T. and Y. Hayashi, *The Ca2+ and Rho GTPase signaling pathways underlying activity-dependent actin remodeling at dendritic spines.* Cytoskeleton (Hoboken), 2012. **69**(8): p. 545-54.

144. Neely, M. and M. Gesemann, *Disruption of microfilaments in growth cones following depolarization and calcium influx.* The Journal of Neuroscience, 1994. **14**(12): p. 7511-7520.

145. Cheng, X., X. Zhang, L. Yu, and H. Xu, *Calcium signaling in membrane repair.* Seminars in cell & developmental biology, 2015. **45**: p. 24-31.

146. Prins, K.W., D.A. Lowe, and J.M. Ervasti, *Skeletal muscle-specific ablation of gamma(cyto)-actin does not exacerbate the mdx phenotype.* PLoS One, 2008. **3**(6): p. e2419.

147. Ando, Y., S. Imamura, Y.M. Hong, M.K. Owada, T. Kakunaga, and R. Kannagi, *Enhancement of calcium sensitivity of lipocortin I in phospholipid binding induced by limited proteolysis and phosphorylation at the amino terminus as analyzed by phospholipid affinity column chromatography.* J Biol Chem, 1989. **264**(12): p. 6948-55.

148. Chuah, S.Y. and C.J. Pallen, *Calcium-dependent and phosphorylation-stimulated proteolysis of lipocortin I by an endogenous A431 cell membrane protease.* J Biol Chem, 1989. **264**(35): p. 21160-6.

149. Mooren, O.L., B.J. Galletta, and J.A. Cooper, *Roles for actin assembly in endocytosis.* Annu Rev Biochem, 2012. **81**: p. 661-86.

150. Wu, X.S., S.H. Lee, J. Sheng, Z. Zhang, W.D. Zhao, D. Wang, Y. Jin, P. Charnay, J.M. Ervasti, and L.G. Wu, *Actin Is Crucial for All Kinetically Distinguishable Forms of Endocytosis at Synapses.* Neuron, 2016. **92**(5): p. 1020-1035.

151. Miyake, K., P.L. McNeil, K. Suzuki, R. Tsunoda, and N. Sugai, *An actin barrier to resealing.* Journal of Cell Science, 2001. **114**(19): p. 3487-3494.

152. Abreu-Blanco, M.T., J.M. Verboon, and S.M. Parkhurst, *Coordination of Rho family GTPase activities to orchestrate cytoskeleton responses during cell wound repair.* Curr Biol, 2014. **24**(2): p. 144-55.

153. Miyake, K., P.L. McNeil, K. Suzuki, R. Tsunoda, and N. Sugai, *An actin barrier to resealing.* J Cell Sci, 2001. **114**(Pt 19): p. 3487-94.

154. Tzima, E., P.J. Trotter, M.A. Orchard, and J.H. Walker, *Annexin V relocates to the platelet cytoskeleton upon activation and binds to a specific isoform of actin.* Eur J Biochem, 2000. **267**(15): p. 4720-30.

155. Wen, P.J., S. Grenklo, G. Arpino, X. Tan, H.S. Liao, J. Heureaux, S.Y. Peng, H.C. Chiang, E. Hamid, W.D. Zhao, W. Shin, T. Nareoja, E. Evergren, Y. Jin, R. Karlsson, S.N. Ebert, A. Jin, A.P. Liu, O. Shupliakov, and L.G. Wu, *Actin dynamics provides membrane tension to merge fusing vesicles into the plasma membrane.* Nat Commun, 2016. **7**: p. 12604.

156. Zhelev, D.V. and D. Needham, *Tension-stabilized pores in giant vesicles: determination of pore size and pore line tension.* Biochim Biophys Acta, 1993. **1147**(1): p. 89-104.

157. Joseph, J.G. and A.P. Liu, *Mechanical Regulation of Endocytosis: New Insights and Recent Advances.* Advanced Biosystems, 2020. **n/a**(n/a): p. 1900278.

158. Porat-Shliom, N., O. Milberg, A. Masedunskas, and R. Weigert, *Multiple roles for the actin cytoskeleton during regulated exocytosis.* Cellular and molecular life sciences : CMLS, 2013. **70**(12): p. 2099-2121.

159. Bunnell, T.M. and J.M. Ervasti, *Structural and functional properties of the actin gene family.* Crit Rev Eukaryot Gene Expr, 2011. **21**(3): p. 255-66.

160. Bergeron, S.E., M. Zhu, S.M. Thiem, K.H. Friderici, and P.A. Rubenstein, *Ion-dependent polymerization differences between mammalian beta- and gamma-nonmuscle actin isoforms.* J Biol Chem, 2010. **285**(21): p. 16087-95.

161. Cooper, S.T. and S.I. Head, *Membrane Injury and Repair in the Muscular Dystrophies.* The Neuroscientist, 2014. **21**(6): p. 653-668.

162. Ferguson, S.M. and P. De Camilli, *Dynamin, a membrane-remodelling GTPase.* Nature Reviews Molecular Cell Biology, 2012. **13**(2): p. 75-88.

163. Durieux, A.-C., B. Prudhon, P. Guicheney, and M. Bitoun, *Dynamin 2 and human diseases.* Journal of Molecular Medicine, 2010. **88**(4): p. 339-350.

164. Toussaint, A., B.S. Cowling, K. Hnia, M. Mohr, A. Oldfors, Y. Schwab, U. Yis, T. Maisonobe, T. Stojkovic, C. Wallgren-Pettersson, V. Laugel, A. Echaniz-Laguna, J.L. Mandel, I. Nishino, and J. Laporte, *Defects in amphiphysin 2 (BIN1) and triads in several forms of centronuclear myopathies.* Acta Neuropathol, 2011. **121**(2): p. 253-66.

165. Gu, C., S. Yaddanapudi, A. Weins, T. Osborn, J. Reiser, M. Pollak, J. Hartwig, and S. Sever, *Direct dynamin-actin interactions regulate the actin cytoskeleton.* The EMBO journal, 2010. **29**(21): p. 3593-3606.

166. Amaral, E., S. Guatimosim, and C. Guatimosim, *Using the Fluorescent Styryl Dye FM1-43 to Visualize Synaptic Vesicles Exocytosis and Endocytosis in Motor Nerve Terminals*, in *Light Microscopy: Methods and Protocols*, H. Chiarini-Garcia and R.C.N. Melo, Editors. 2011, Humana Press: Totowa, NJ. p. 137-148.

167. Lai, M.M., J.J. Hong, A.M. Ruggiero, P.E. Burnett, V.I. Slepnev, P. De Camilli, and S.H. Snyder, *The calcineurin-dynamin 1 complex as a calcium sensor for synaptic vesicle endocytosis.* J Biol Chem, 1999. **274**(37): p. 25963-6.

168. María Cabeza, J., J. Acosta, and E. Alés, *Dynamics and Regulation of Endocytotic Fission Pores: Role of Calcium and Dynamin.* Traffic, 2010. **11**(12): p. 1579-1590.

169. Chandra, G., A. Defour, K. Mamchoui, K. Pandey, S. Mishra, V. Mouly, S. Sreetama, M. Mahad Ahmad, I. Mahjneh, H. Morizono, N. Pattabiraman, A.K. Menon, and J.K. Jaiswal, *Dysregulated calcium homeostasis prevents plasma*

membrane repair in Anoctamin 5/TMEM16E-deficient patient muscle cells. Cell Death Discovery, 2019. **5**(1): p. 118.

170. Dahl, R., *A new target for Parkinson's disease: Small molecule SERCA activator CDN1163 ameliorates dyskinesia in 6-OHDA-lesioned rats.* Bioorganic & Medicinal Chemistry, 2017. **25**(1): p. 53-57.

171. Horn, A., J.H. Van der Meulen, A. Defour, M. Hogarth, S.C. Sreetama, A. Reed, L. Scheffer, N.S. Chandel, and J.K. Jaiswal, *Mitochondrial redox signaling enables repair of injured skeletal muscle cells.* Sci Signal, 2017. **10**(495).

172. Orrenius, S., B. Zhivotovsky, and P. Nicotera, *Regulation of cell death: the calcium-apoptosis link.* Nat Rev Mol Cell Biol, 2003. **4**(7): p. 552-65.

173. Trnka, J., F.H. Blaikie, R.A. Smith, and M.P. Murphy, *A mitochondria-targeted nitroxide is reduced to its hydroxylamine by ubiquinol in mitochondria.* Free Radic Biol Med, 2008. **44**(7): p. 1406-19.

174. Oyewole, A.O. and M.A. Birch-Machin, *Mitochondria-targeted antioxidants.* The FASEB Journal, 2015. **29**(12): p. 4766-4771.

175. Lee, E. and P. De Camilli, *Dynamin at actin tails.* Proceedings of the National Academy of Sciences, 2002. **99**(1): p. 161-166.

176. Taylor, M.J., M. Lampe, and C.J. Merrifield, *A feedback loop between dynamin and actin recruitment during clathrin-mediated endocytosis.* PLoS Biol, 2012. **10**(4): p. e1001302.

177. Krueger, E.W., J.D. Orth, H. Cao, and M.A. McNiven, *A dynamin-cortactin-Arp2/3 complex mediates actin reorganization in growth factor-stimulated cells.* Mol Biol Cell, 2003. **14**(3): p. 1085-96.

178. Park, R.J., H. Shen, L. Liu, X. Liu, S.M. Ferguson, and P. De Camilli, *Dynamin triple knockout cells reveal off target effects of commonly used dynamin inhibitors.* J Cell Sci, 2013. **126**(Pt 22): p. 5305-12.

179. Damke, H., D.D. Binns, H. Ueda, S.L. Schmid, and T. Baba, *Dynamin GTPase domain mutants block endocytic vesicle formation at morphologically distinct stages.* Mol Biol Cell, 2001. **12**(9): p. 2578-89.

180. Kumari, S., S. Mg, and S. Mayor, *Endocytosis unplugged: multiple ways to enter the cell.* Cell Research, 2010. **20**(3): p. 256-275.

181. Lin, Y.-C., C. Chipot, and S. Scheuring, *Annexin-V stabilizes membrane defects by inducing lipid phase transition.* Nature Communications, 2020. **11**(1): p. 230.

182. Genth, H., R. Gerhard, A. Maeda, M. Amano, K. Kaibuchi, K. Aktories, and I. Just, *Entrapment of Rho ADP-ribosylated by Clostridium botulinum C3 exoenzyme in the Rho-guanine nucleotide dissociation inhibitor-1 complex.* J Biol Chem, 2003. **278**(31): p. 28523-7.

183. Vogelsgesang, M., A. Pautsch, and K. Aktories, *C3 exoenzymes, novel insights into structure and action of Rho-ADP-ribosylating toxins.* Naunyn Schmiedebergs Arch Pharmacol, 2007. **374**(5-6): p. 347-60.

184. Gao, Y., J.B. Dickerson, F. Guo, J. Zheng, and Y. Zheng, *Rational design and characterization of a Rac GTPase-specific small molecule inhibitor.* Proc Natl Acad Sci U S A, 2004. **101**(20): p. 7618-23.

185. Hong, L., S.R. Kenney, G.K. Phillips, D. Simpson, C.E. Schroeder, J. Nöth, E. Romero, S. Swanson, A. Waller, J.J. Strouse, M. Carter, A. Chigaev, O. Ursu, T. Oprea, B. Hjelle, J.E. Golden, J. Aubé, L.G. Hudson, T. Buranda, L.A. Sklar, and A. Wandinger-Ness, *Characterization of a Cdc42 protein inhibitor and its use as a molecular probe.* J Biol Chem, 2013. **288**(12): p. 8531-43.

186. Tang, D.D. and S.J. Gunst, *The small GTPase Cdc42 regulates actin polymerization and tension development during contractile stimulation of smooth muscle.* J Biol Chem, 2004. **279**(50): p. 51722-8.

187. Yang, S.A., C.L. Carpenter, and C.S. Abrams, *Rho and Rho-kinase mediate thrombin-induced phosphatidylinositol 4-phosphate 5-kinase trafficking in platelets.* J Biol Chem, 2004. **279**(40): p. 42331-6.

188. Nobis, M., D. Herrmann, S.C. Warren, S. Kadir, W. Leung, M. Killen, A. Magenau, D. Stevenson, M.C. Lucas, N. Reischmann, C. Vennin, J.R.W. Conway, A. Boulghourjian, A. Zaratzian, A.M. Law, D. Gallego-Ortega, C.J. Ormandy, S.N. Walters, S.T. Grey, J. Bailey, T. Chtanova, J.M.W. Quinn, P.A. Baldock, P.I. Croucher, J.P. Schwarz, A. Mrowinska, L. Zhang, H. Herzog, A. Masedunskas, E.C. Hardeman, P.W. Gunning, G. Del Monte-Nieto, R.P. Harvey, M.S. Samuel, M. Pajic, E.J. McGhee, A.E. Johnsson, O.J. Sansom, H.C.E. Welch, J.P. Morton, D. Strathdee, K.I. Anderson, and P. Timpson, *A RhoA-FRET Biosensor Mouse for Intravital Imaging in Normal Tissue Homeostasis and Disease Contexts.* Cell Rep, 2017. **21**(1): p. 274-288.

189. Miskolci, V., B. Wu, Y. Moshfegh, D. Cox, and L. Hodgson, *Optical Tools To Study the Isoform-Specific Roles of Small GTPases in Immune Cells.* J Immunol, 2016. **196**(8): p. 3479-93.

190. Hanna, S., V. Miskolci, D. Cox, and L. Hodgson, *A new genetically encoded single-chain biosensor for Cdc42 based on FRET, useful for live-cell imaging.* PLoS One, 2014. **9**(5): p. e96469.

191. Miniou, P., D. Tiziano, T. Frugier, N. Roblot, M. Le Meur, and J. Melki, *Gene targeting restricted to mouse striated muscle lineage.* Nucleic Acids Res, 1999. **27**(19): p. e27.

192. Chen, L., G. Liao, L. Yang, K. Campbell, M. Nakafuku, C.Y. Kuan, and Y. Zheng, *Cdc42 deficiency causes Sonic hedgehog-independent holoprosencephaly.* Proc Natl Acad Sci U S A, 2006. **103**(44): p. 16520-5.

193. Glogauer, M., C.C. Marchal, F. Zhu, A. Worku, B.E. Clausen, I. Foerster, P. Marks, G.P. Downey, M. Dinauer, and D.J. Kwiatkowski, *Rac1 deletion in mouse neutrophils has selective effects on neutrophil functions.* J Immunol, 2003. **170**(11): p. 5652-7.

194. Demonbreun, A.R. and E.M. McNally, *DNA Electroporation, Isolation and Imaging of Myofibers.* J Vis Exp, 2015(106): p. e53551.