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Sarcolemma Wounding Activates Dynamin-Dependent Endocytosis in Striated Muscle

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Abbreviations

DMSO: Dimethyl Sulfoxide; Dysf-pHGFP: Dysferlin-pHLuorin; FBS: Fetal Bovine Serum; i.p.: Intraperitoneal; MP: Multiphoton; NRK Cells: Normal Rat Kidney Epithelial Cells; PSS: Physiological Saline Solution; WT: Wild-Type

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 that are 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 [1-3]. Mutations in several putative membrane transport proteins including dysferlin [4], annexin [5], MG53 [6], synaptotagmin-VII [7] and PTRF/Cavin-1 [8] have been shown in mouse models to result in muscle disease, potentially through reduced capacity to reseal the plasma membrane

following wounding [9]. 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 [4, 6, 8]. One important caveat of these studies is that FM-dyes can also be taken up by endocytosis [10], 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 [11-13]. On the basis of 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 [14], 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 [15, 16]. 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 [16], 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 [17]. 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.

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 [15, 17]. 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 [10]. 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 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 1B**). FM1-43 labeling in resting adult skeletal muscle fibers was not affected by DMSO-treatment, (**Fig 1B** top, quantified **1C**) but is almost completely abolished in the presence of dynasore (**Fig 1B** middle, quantified in **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 1B** bottom, quantified **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 [18]. The accumulation of dye fluorescence is commonly used as a gold standard assay to study membrane resealing and quantify

defective membrane repair [4, 6, 19]. 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 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 2B**, quantified **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 2C**) [20]. 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 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 3B**, quantified **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 extracllular 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 [4]. 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 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 4B**, quantified **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 4D**) [21]. Consistent with our previous report, surface localized dysferlin-pHGFP molecules adjacent to the lesion are rapidly recruited to the membrane wound (white arrow **Fig 4E**, quantified **4F**), whereas remaining dysferlin-pHGFP molecules are rapidly quenched in response to wounding (red arrow **Fig 4E**, quantified **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 4E**), or endocytosis following wounding (**Fig 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 [11, 22, 23]. 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 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 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. 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 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 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 6A,** quantified **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 7A-D**). However, while the peak amount of calcium is increased in BLA/J mice, calcium reverses similarly in both genotypes (solid line **Fig 7B, 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 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 [7, 24], 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 [25]. 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 [4, 26]. 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 [13]. 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 also 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 [27] 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 [28]. 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 compartmentalizaiton 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[29] and annexins[30] 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[31], despite calcium being important for activating most forms of endocytosis in other cell types [32]. 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 degeration [33].

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 8A**). Dynamin-2 mutant mouse models, expressing mutations associated with centranuclear myopathy, have a complicated phenotype due to it's additional role in t-tubule and triad biogenesis in muscle [34-36], but expression of dynamin-2 mutants does result in elevated resting calcium in muscle [37]. 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 8B**). Indeed, accumulation of subsarcolemma vesicles is a common observation in electron micrographs of muscle fibers from dysferlin deficient muscular dystrophy patients and mice [4].

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 [38]. Furthermore, dynamin expression is elevated in a mouse model of myotubular myopathy and reducing dynamin levels restores muscle structure and function [39]. 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 4**). Thus, we propose the general model (shown in **Fig 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 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.

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. [40]. Dysferlin-pHluorin (Dysf-pHGFP) transgenic mice were generated as previously described [21]. 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 [4, 21]. 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 [41]. 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.

Author contributions: J.R.M. and M.T.N. performed the research and analyzed the data. J.R.M., M.T.N., and D.E.M. designed the research and wrote the paper.

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537

538 **Figure Legends:**

539 **Figure 1. Resting adult skeletal muscle fibers take up FM1-43 *via* a dynamin-dependent mechanism.**
540 **FM1-43 labeling occurs over several minutes in adult skeletal muscle fibers.** FM1-43 uptake was
541 assessed in untreated, resting adult C57BL/6 skeletal muscle fibers (**A**) and in resting adult skeletal
542 muscle fibers following pre-treatment with DMSO or dynamin inhibitor dynasore (**B**). Untreated and
543 DMSO treated fibers take up extracellular FM1-43 with maximal labeling occurring within 10min (**A** and
544 **B** top, respectively, quantified in **C**). Pre-treatment with 80μM dynasore completely inhibits FM1-43
545 uptake in resting skeletal muscle fibers (**B**, middle, quantified in **C**). Dynasore-treated muscle fibers take
546 up FM1-43 following removal of dynasore from the extracellular solution (**C** bottom, quantified **D**). The
547 red scale bar indicates 50μm. Statistical comparisons were performed using an unpaired, two-tailed

Student's t-test. Statistical significance ($p<0.05$) between DMSO and Dynasore is denoted by (*****) for each timepoint under the horizontal bar. Error bars represent the standard deviation of the mean. Data is summarized from three independent loading plates.

Figure 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 comparisons were performed using a Two-Way ANOVA followed by posthoc Student's t-tests. 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**. Error bars represent the standard deviation of the mean. One representative experiment of three independent replicates (**B-C**) or two independent replicates (**D**) is shown.

Figure 3. FM1-43 uptake after wounding 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**). Statistical comparisons were performed using an unpaired, two-tailed Student's t-test (**A**) or Two-Way ANOVA followed by posthoc Student's t-tests (**C**). The threshold for statistical significance was not met in **A** ($p>0.05$), but statistical significance ($p<0.05$) between FM1-43+DMSO and all other traces is denoted by (*****) for each timepoint under the horizontal bar. Error bars represent the standard deviation of the mean. One representative experiment of two independent replicates is shown.

Figure 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 cirlces and open magenta triangles in **C**, respectively). Statistical comparisons were performed using Student's t-tests. Statstical 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. Statistical comparisons were made using an unpaired, two-tailed Student's t-tests and did not meet the threshold of significance ($p>0.05$). Error bars represent the standard deviation of the mean. One representative experiment of three independent replicates (**A**), one independent replicate (**B-C**), and two independent replicates (**D-G**) is shown.

Figure 5. Calcium influx 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 wounded with a high powered laser, representative images shown (top **A**). Calcium increases at the wound site were quantified, described by the white box in **B**, which showed that wounding 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 wound (solid line **B**, summary data in **D**), but cytoplasmic calcium reversal is significantly delayed to approximately 60s **(D)** A bolus of 10mM caffeine to wounded fibers, one representative trace shown, stimulates calcium release from the SR that exceeds peak calcium after wounding **(E)**. Removing calcium from extracellular media and chelating any trace

calcium with 1mM EGTA prevents the increase in cytosolic calcium following wounding (representative images **A** bottom, quantified **F**). While calcium flux 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**)**.** Statistical compaisons were performed using an unpaired, two-tailed Student's t-test. Statistical significance ($p<0.05$) is denoted by (*****). Error bars represent the standard error of the mean. One representative experiment of three independent replicates is shown.

Figure 6. Dynamin inhibition does not affect calcium influx after membrane wounding. 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 wounding shows calcium influx after wounding 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 comparisons were performed using a Two-Way ANOVA followed by posthoc Student's t-tests. Statistical significance ($p<0.05$) between Dynasore and both PSS and Washout is denoted by (*****). Error bars represent the standard error of the mean.

Figure 7. Dysferlin-deficiency muscle fibers have persistently elevated calcium after membrane wounding. Isolated muscle fibers from dysferlin-deficient BLA/J mice and wild-type littermate controls (C57BL/6) were loaded with 3□M Fluo-4 for 1hr and wounded with a high-powered laser (representative images **A**). Calcium concentrations after wounding 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 influx 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**). Statistical compaisons were performed using an unpaired, two-tailed Student's t-test. Statistical significance ($p<0.05$) between WT and BLA/J is denoted by (*****). Error bars represent the standard error of the mean.

Figure 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**).