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# Muscle Cells Fix Breaches by Orchestrating a Membrane Repair Ballet

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**Abstract.** Skeletal muscle undergoes many micro-membrane lesions at physiological state. Based on their sizes and magnitude these lesions are repaired via different complexes on a specific spatio-temporal manner. One of the major repair complex is a dysferlin-dependent mechanism. Accordingly, mutations in the *DYSF* gene encoding dysferlin results in the development of several muscle pathologies called dysferlinopathies, where abnormalities of the membrane repair process have been characterized in patients and animal models. Recent efforts have been deployed to decipher the function of dysferlin, they shed light on its direct implication in sarcolemma resealing after injuries. These discoveries served as a strong ground to design therapeutic approaches for dysferlin-deficient patients. This review detailed the different partners and function of dysferlin and positions the sarcolemma repair in normal and pathological conditions.

Keywords: Muscle, membrane, dystrophy, dysferlin, myopathy

## INTRODUCTION

Dysferlin is a transmembrane protein belonging to the ferlin family. It is coded by the gene *DYSF* located on chromosome 2p13.2. The dysferlin protein is comprised of multiple domains responsible for its various functionalities. Among these are multiple C2 domains, which are known to be calcium-sensitive and involved mainly in phospholipid interactions [1–3]. Mutations in *DYSF* [4, 5] cause a class of muscular disorders termed “dysferlinopathies”, including two principal muscular dystrophies: Limb Girdle Muscular Dystrophy type 2B (LGMD2B) and Miyoshi Myopathy (MM). Both disorders are inherited in an autosomal recessive way; LGMD2B affects mainly proximal muscle while MM manifests predominantly in distal muscle. Despite

variation in age of onset, patients with either disorder are mostly healthy and athletic at young ages without any clinical sign of pathology. Disease onset usually becomes evident only in the second decade, for example with difficulties in ambulation or climbing stairs. The disease progress slowly but frequently results in wheelchair dependency.

Dysferlinopathies are clinically and genetically heterogeneous, with a broad spectrum of severity from asymptomatic to marked functional disability [6–8]. Patients with LGMD2B and MM are notable phenotypically for exhibiting massive increases in blood creatine kinase, up to 100-fold compared to normal values, associated with severe muscle inflammation [9] that can be misdiagnosed as polymyositis [10–12]. Because of such misdiagnoses and the recent introduction of the international patient registry within only the last few years (<https://www.jain-foundation.org/patient-registration>), the worldwide prevalence and incidence of dysferlinopathies are hard to estimate with much accuracy. However, some

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reviews suggest that dysferlinopathies may be the second most frequent type of LGMD (25% of the LGMD cases) after LGMD2A, with a prevalence of up to 1/50,000 [13].

In this review we describe the function of dysferlin and its network; while dysferlin functions are not exclusively limited to membrane repair, we decided to focus the review on this role in light of the recent discoveries in the field.

## **DYSFERLIN AND ANNEXINS DIRECT THE MEMBRANE REPAIR PROCESS**

Due to its inherent function, skeletal muscle is a mechanically stressed organ and is thus exposed to frequent and sudden physiological tears at the membrane [14]. Membrane rupture is followed by a drastic increase in intracellular calcium levels, which in turn triggers plasma membrane repair (PMR) [15]. PMR is a multistep process that entails varied strategies aiming to repair the injury in a spatiotemporal manner. The cell creates an “emergency” patch to contain the damage (A on Fig), prior to other mechanisms (involving cytoskeletal and membrane remodeling) taking over the repair process. Calcium increase at the injury site lasts for several seconds (B on Fig), but it takes much longer to complete PMR (~120 sec for myoblasts and up to several minutes for muscle fibers) [16, 17]. More specifically, a calcium gradient is formed after the injury that triggers the PMR events (C on Fig). This sets off a chain reaction involving recruitment and/or recycling of Ca<sup>2+</sup>-sensor proteins [synaptotagmins, ferlins, calpains, annexins, apoptosis-linked gene (ALG)-2], and additional proteins (ESCRT-III complex, MG53), membrane lipids, as well as cytoskeletal and organellar reorganization [18–25], which is absolutely essential for effective membrane repair [26]. Ca<sup>2+</sup>-triggered exocytosis likewise diminishes membrane tension, another key determinant of cell membrane resealing and effective repair [27, 28]. Despite some variation in the succession of events taking place during PMR, Ca<sup>2+</sup> and ATP are indispensable [29] (D on Fig).

Cellular injuries also disturb the sarcomere, disorganize membrane lipid organization and composition, and influence the cytoskeletal dynamic [14]. Phosphatidylserine (PS) becomes aggregated around wound sites over time while actin binds PS [30, 31] (E on Fig). Transitory actin cytoskeleton rearrangement is in turn essential for repair [14, 32]. Indeed, cytoskeletal actin facilitates the localized transport of

internal vesicles to the wounded membrane, assisted by kinesin, while myosin motors promote exocytosis [33]. Also, consequent to the injury, proteins known to bind actin, like affixin, EDH2, and ANXA1, are increased at the lesion site together with F-actin in muscle cells [34–37] (A on Fig). Small GTPases (Rho, Rac, Cdc42) are also involved in distinct subcellular patterns by recruiting the cytoskeleton surrounding the lesion, as has been described in *Drosophila* or *Xenopus* oocytes [38–41] (F on Fig). PM depolarization is induced by the massive entry of calcium and the cleavage of two of actin’s partners, talin and vimentin, by calpains [42, 43] (G on Fig). Moreover, the disassembly of the microtubule mesh around the lesion, triggered by the calcium rise, may also influence the lysosomal transport and lipid traffic to the PM (H on Fig) [44, 45].

### *Intracellular vesicles*

In the wake of cellular wounding, cells must undergo an emergency response (plugging) involving the cross-linkage of membranous compartments or vesicles, such as lysosomes, enlargeosomes, or other components to the plasma membrane such as membrane-deforming proteins (I on Fig) [46–48]. An elevation of Ca<sup>2+</sup> in a 20 nm perimeter is required to trigger vesicle fusion. Following membrane rupture, there is an accumulation of small vesicles underneath the plasmalemma that function as the main reservoirs of Ca<sup>2+</sup> required for membrane fusion. Among them, lysosomes represent one of the first actively recruited reservoirs, as confirmed by the presence of luminal epitopes of LAMP-1 (a lysosome-specific marker) on the cell surface after injury [29, 47] (J on Fig). This is also suggested by the observation that lysosomes form a pre-existing punctate distribution beneath the membrane [49, 50]. Indeed, lysosomes have the ability to fuse with the plasma membrane through a two-step approach, they are first recruited underneath the membrane independently from calcium and then they fuse with the plasma membrane in response to an increase in calcium via synaptotagmin VII which is present at the surface of the lysosome [47] (H on Figure). Therefore, lysosomes and lysosome-related organelles have been shown to participate in the membrane repair process [51, 52]. Calcium-dependent lysosomal exocytosis has been shown in injured muscle fibers, decreasing membrane tension and facilitating PMR [27] (M’ on Figure).

It is also widely acknowledged that the specific manner in which PMR takes place is dependent

on the size of the lesion [29, 52]. Large injuries involve an increase of caveolar endocytosis [53] (K on Fig). Small lesions (<100 nm) meanwhile will trigger the ESCRT III complex, which promotes vesicle budding or membrane shedding via a complex involving apoptosis-linked gene 2 (ALG 2), ALG-2-interacting protein X (ALIX), ESCRT III and Vps4 [22, 25, 54] (L on Fig). Exocytosis releases acid sphingomyelinases (ASM), which will be turned into ceramide in the outer leaflet of the plasma membrane, leading to endosome formation [55, 56] (L on Fig). These domains change the binding affinities of annexins and caveolins for the plasmalemma, affect cytoskeleton dynamics, and bring about changes in plasmalemma architecture that play an important role in the progression of membrane repair [57, 58].

Intracellular vesicles undergo a rapid recycling in the region of the tears by a process of endocytosis/exocytosis turnover (M/M' on Fig). Indeed, exocytosis of lysosomes, relying on synaptotagmin VII and calcium [47], is rapidly followed by massive endocytosis of small endosome vesicles that merge to form a larger endosome before joining the endocytic pathway [59, 60] (M on Fig). However, in dysferlinopathies, absence of dysferlin prevents the tethering of lysosomes to the membrane, thereby delaying their exocytosis [61]. This is especially problematic since lysosome exocytosis is a fundamental process occurring in all kinds of PMR independently of the type of injury involved (mechanical, toxin-induced perforation or other causes) [62].

The endocytic vesicles look highly similar to caveolae when observed via electronic microscopy as they are similar in appearance and size [63]. This is plausible since caveolar internalization could also function as a reservoir. Certainly, caveolae are highly expressed in tissue under mechanical stress or in a disease context [53, 63–65]. They could also merge to form a larger vesicle prior to their endocytosis or flattening, suggesting that an enlarged reservoir is required for membrane integrity [15, 53, 59, 66]. Markers of lysosomes or endosomes are also expressed on the surface of caveolae when fused after internalization [53]. Stretch-induced membrane rupture can also disrupt T-tubules by pulling them out of position [67] (Q on Fig). MG53, ANXA1, and dysferlin have been shown to localize at the T-tubule during stretch-induced injury, suggesting a role for the T-tubule as another membrane reservoir [68]. Moreover, EDH2 translocates to the lesion site via a T-tubule-dependent trafficking mechanism [36].

Finally, one other PMR mechanism of note is membrane blebbing, a temporary detachment of sub-membranous cytoskeleton from the plasma membrane that occurs when the PMR process needs more time to be fully completed or when the initial attempts at rescue have failed [69]. These blebs, which involve ANXA1 as a mediator, can be definitively excluded from the membrane if necessary [69].

### *Dysferlin*

Dysferlin is a major mediator of PMR. Among other interactions, it has been reported to bind the cell membrane lipids phosphatidylserine (PS) and phosphatidylinositol bisphosphate (PIP2), and to colocalize with the SNARE protein syntaxin 4 and snap23 [70, 71]. Dysferlin-positive vesicles can fuse together via microtubule transport and accumulate underneath the membrane [72]. Additionally, it has been shown that dysferlin must be cleaved at its C-terminus by calpains to release an active fragment [composed of the last 2 C2 domains (out of 7) and the transmembrane domain] called minidysferlin-C72. Only the truncated dysferlin stays at the lesion site where it encircles the lesion [73, 74] (O on Fig). From there, as has been demonstrated, m- or  $\mu$ -calpain can facilitate repair of damaged plasma membrane [75]. Dysferlin is also expressed at the PM via its interaction with caveolin 3, the main component of the caveolae [76, 77]. Dysferlin and MG53 are present at the surface of membrane vesicles, recruited by an actin-cytoskeleton-dependent mechanism during membrane damage [32, 78] (N on Fig).

### *MG53*

Another protein found in caveolae and involved in membrane repair is MG53. This component binds directly to the PS at the plasma membrane or via Cavin-1/PTRF, probably in caveolae [79, 80]. MG53 and Cavin-1 may help in stabilizing the membrane by recruiting dysferlin and caveolin 3 [80, 81]. This mechanism also incorporates annexins (ANXA) 1 and 6 [82]. However, the direct involvement of this pathway in a physiological context is not clearly defined; it may involve prevention of cell death and therefore the maintenance of an environment favorable to membrane resealing with annexins as key factors [69, 82] (P in Fig). On the other hand, mutations in caveolin 3 and PTRF cause serious abnormalities in muscle where vesicles are often found underneath the PM [83, 84]. Finally, it has

been suggested that MG53 could also function outside of the cell since the protein can participate to the PMR effort after intravenous injection of recombinant MG53 in a mouse model [85, 86].

### *Annexins*

Annexins are a family of ubiquitously expressed soluble proteins binding negatively charged membrane lipids (mostly PS) in a Ca<sup>2+</sup>-dependent manner [87]. Among them, ANXA5 stands out since it can promote the formation of annexin-2D arrays below the membrane surface [88, 89] (A on Fig). Together with other components, ANXA (6, 1, 2) has been shown to promote several membrane-related processes like endocytosis or exocytosis, vesicle trafficking, and general membrane dynamics (including fusion, organization, and aggregation) [90]. ANXA2 seems to promote enlargeosomes' movement toward the PM where they can fuse via SNAP23, VAMP4, and syntaxin6 or via a connecting ANXA2/S100A10-AHNAK complex [91–93] (I on Fig). Another protein interacting with ANXA2, S100A11, could play a role in the mesh formation by facilitating the actin polymerization at the wounding site [94, 95]. Enlargeosomes have also been involved in PMR with an undefined role; their main marker, AHNAK, is re-localized to the PM after injury [96]. ANXAs are therefore one of the major players in the membrane repair process [91]. ANXA 6, 1, 5, and 2 in particular are recruited at the outset to prevent extension of the wound by promoting emergency fusion [88, 91, 97] (A on Fig).

Additional proteins (MCOLN1/TRMPL1 and ANO5) have also been linked to PMR, but their roles are as yet not fully understood (A on Fig). It is believed that MCOLN1 may be involved in lysosomal exocytosis by promoting lysosomes' trafficking to the membrane via a mechanism dependent on calcium [98]. The fact that ALG-2 and MCOLN1 interact in a calcium-dependent manner raises the question of possible involvement of MCOLN1 in membrane shedding [99]. In the same manner, ANO5 has been linked to PMR since it has been shown that recruitment of ANO5 vesicles to the membrane is triggered following the increase of calcium influx [100].

Altogether these results suggest that dysferlin and ANXAs, among other actors (caveolin 3, MG53, calpains, and others), are essential for the PMR process and more generally for muscle homeostasis, since damage to these components has been directly associated with pathologic processes or the exacerbation of

numerous diseases [4, 5, 101–104]. They are highly active during the events following membrane rupture via vesicle recruitment and endocytosis/exocytosis events (K and R on Fig). Finally, it has been proposed recently that damaged membrane components could also be sequestered and removed from the membrane instead of being resealed, and we believe that in this context, dysferlin and ANXAs would likely be among the participants involved in this process [55]. It should also be noted that the vesicles involved in PMR are eliminated by macrophages (S on Fig) that have been recruited to the wound site via the increase of phosphatidylserine at the membrane triggered by dysferlin [105].

### **CONCLUDING REMARKS**

PMR is an essential and conserved protective process found in multiple species and a variety of tissues. Several mechanisms have been proposed over the years but in light of recent progress in the field, it seems that the recycling of membrane vesicles in and out of the cells is crucial. Another consensus finding is that PMR is highly dependent on calcium and ATP and that the succession of events taking place has to be relatively fast (from an emergency response within seconds to a complete membrane repair within minutes). Studies suggest that the size and type of the lesion may trigger different mechanisms of repair, but one other possibility is that multiple mechanisms involved in the correction of the membrane damage can occur simultaneously within the same cell [106]. This hypothesis has served as the basis of our review, in which we seek to precisely depict the different players in the highly regulated PMR process in which we believe dysferlin plays a central role.

Furthermore, although a substantial body of knowledge about the PMR mechanisms has been attained in recent years, we are only beginning to fully comprehend the complexity of the events taking place, especially regarding the coordination of all the molecular participants. The knowledge accumulated in the past few years has been valuable to better understand the pathophysiology of numerous diseases involving defects in membrane repair. Nevertheless, numerous gaps still persist regarding our comprehension of the subtlety within each aspect of the pathology and in particular in dysferlinopathies where, although patients share a commonly mutated gene, the pathophysiology of the disease still varies significantly between patients independently of a

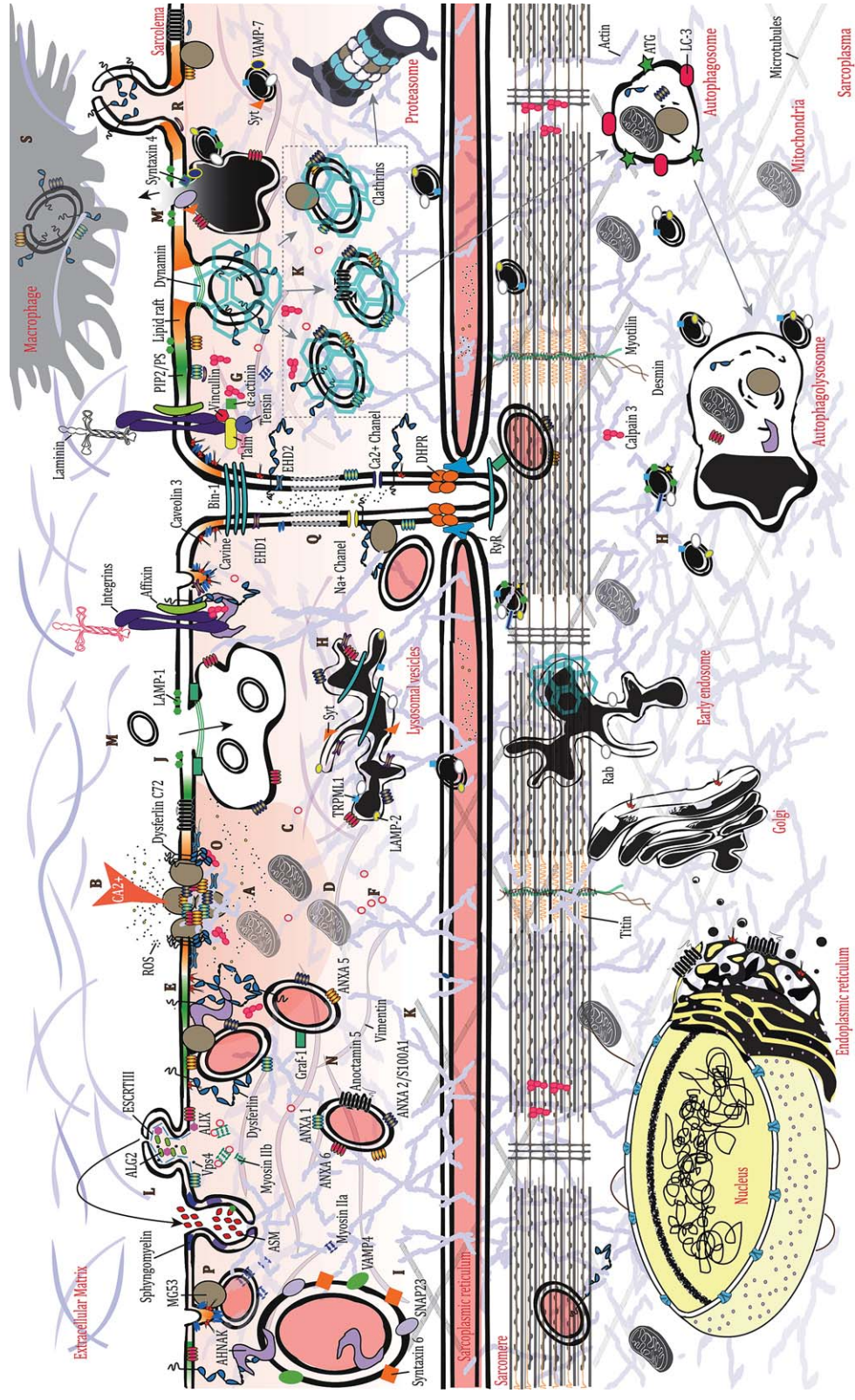


Fig. 1. Membrane repair in muscle cell, Schematic representation of the different events from an emergency patch to a functional recovery in muscle cell after mechanic lesion. See text for description.

given mutation for reasons not fully elucidated. Moreover, each domain of dysferlin has been shown to carry potential disease-causing mutations, resulting in a variety of natural histories in patients and demonstrating the importance of each domain in dysferlin's functions. Based on the finding that the c-terminal portion of the protein is essential for membrane repair [73, 74, 107], mutations in this region could have dramatic consequences for muscle homeostasis. Therefore, new experiments (based on live imaging and models using mutated proteins) are still needed to fill in the gaps and help conceive more appropriate therapeutics in disorders resulting from malfunctioning PMR mechanisms, such as muscular dystrophy and cardiomyopathy.

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