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Dysferlin and muscle membrane repair
Renzhi Han and Kevin P Campbell

The ability to repair membrane damage is conserved across eukaryotic cells and is necessary for the cells to survive a variety of physiological and pathological membrane disruptions. Membrane repair is mediated by rapid Ca^{2+}-triggered exocytosis of various intracellular vesicles, such as lysosomes and enlargeosomes, which lead to the formation of a membrane patch that reseals the membrane lesion. Recent findings suggest a crucial role for dysferlin in this repair process in muscle, possibly as a Ca^{2+} sensor that triggers vesicle fusion. The importance of membrane repair is highlighted by the genetic disease, dysferlinopathy, in which the primary defect is the loss of Ca^{2+}-regulated membrane repair due to dysferlin deficiency. Future research on dysferlin and its interacting partners will enhance the understanding of this important process and provide novel avenues to potential therapies.

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Introduction
The plasma membrane is a biological barrier between the extracellular and intracellular environments and is essential to the maintenance of cell integrity. Damage to this barrier leads to cell death; however, plasma membrane disruptions occur physiologically, and even frequently, in a variety of cells such as the milk-secreting epithelial cells of the mammary gland [1] and the myocytes of both skeletal [2] and cardiac muscle [3]. For more than half a century, it has been known that animal cells can survive after experimental perforation of their cell membranes (holes of >1000 μm²). It is now widely accepted that an active membrane repair mechanism is conserved in many types of mammalian cells and that it serves to reseal membrane lesions. Rapid Ca^{2+}-triggered endomembrane

exocytosis is a crucial step in the membrane resealing process [4,5] that enables cells to survive routine membrane disruptions.

Muscular dystrophies are a heterogeneous group of progressive muscle wasting disorders of genetic origin. A large number of muscular dystrophy genes encode components of the dystrophin–glycoprotein complex (DGC) that are directly or indirectly involved in linking the cytoskeleton to the surrounding basement membrane. Disruption of this link renders the muscle membrane abnormally susceptible to contraction-induced injury, and the accumulation of muscle membrane damage ultimately leads to muscle necrosis and weakness. This highlights the importance of maintaining plasma membrane integrity for normal physiological function and long-term survival of muscle cells. Several additional forms of muscular dystrophy arise from defects unrelated to the DGC. For example, although dysferlin is not a DGC component [6••], mutations in dysferlin gene are responsible for three clinically distinct muscular dystrophies: limb-girdle muscular dystrophy type 2B (LGMD2B), which is first characterized by proximal muscle weakness at onset [7]; Miyoshi myopathy (MM), a distal muscle disorder that preferentially affects the gastrocnemius muscle [7,8]; and a distal anterior compartment myopathy that is distinct from MM and progresses rapidly through the anterior tibial muscles [9].

Recent work has shown that loss of dysferlin compromises Ca^{2+}-dependent membrane repair in skeletal muscle [6••,10•]. Wild-type muscle fibers, in the presence of Ca^{2+}, can efficiently exclude the membrane impermeable dye FM 1-43 after laser wounding, but the dye continuously fills the muscle fiber through the wounding site when Ca^{2+} is omitted in the bath solution (Figure 1), suggesting that the wild-type muscle fiber possesses an efficient Ca^{2+}-dependent membrane resealing mechanism [6••]. Dysferlin-null muscle fibers fail to exclude the dye entry even in the presence of Ca^{2+} (Figure 1), strongly suggesting the Ca^{2+}-dependent membrane repair requires dysferlin [6••]. Loss of dysferlin-mediated membrane repair results in progressive myonecrosis [6••,10•]. In this review, we discuss recent advances in our understanding of dysferlin-mediated membrane repair in disease processes.

Ferlin-1-like proteins
Soon after the initial identification of dysferlin as the product of a gene mutated in MM, a new family of mammalian proteins, named "ferlin-1 like proteins", was predicted on the basis of structural similarity and sequence

Figure 1
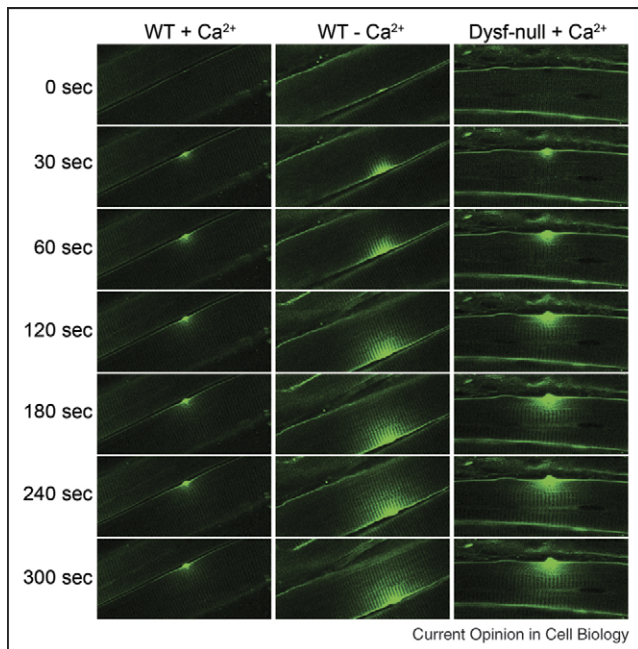
Defective Ca^{2+}-dependent membrane repair in dysferlin-deficient skeletal muscle cells. The sarcolemma of skeletal muscle fibers isolated from wild-type (WT) and dysferlin-deficient (Dysf-null) mice was wounded by full-power irradiation with a mode-locked infrared laser, in the presence of the membrane-impermeable dye FM 1-43. Fluorescence images were then taken every 10 s. In wild-type muscle fiber in the presence of Ca^{2+}, dye influx ceased by 30–60 s after laser-induced disruption of the sarcolemma (left panel), suggesting that the site of damage had been resealed. In wild-type muscle in the absence of Ca^{2+}, dye influx through the site of disruption continued over the entire time course of the experiment (middle panel), indicating a failure in the resealing mechanism. Likewise, in the dysferlin-null muscle fiber, dye filling occurred over the entire course of experiment (right panel), indicating that resealing failed, even in the presence of Ca^{2+}.

homology (Figure 2). These include dysferlin or Fer1L1 [7,8]; otoferlin or Fer1L2 [11,12]; myoferlin or Fer1L3 [13,14]; Fer1L4; Fer1L5 and Fer1L6. Each protein contains multiple C2 domains, anchors to the membrane via a single carboxy-terminal transmembrane domain (Figure 2a), and shows sequence homology to the *Caenorhabditis elegans* ferlin-1 (FER-1) gene. FER-1 expression is restricted to primary spermatocytes, where it is essential for maturation of motile spermatozoa. In homozygous FER-1 mutants, spermatid membranous organelles (MO) fail to fuse with the plasma membrane during spermatogenesis, resulting in nonmotile spermatozoa and infertility [15]. A single amino acid substitution in any of three FER-1 C2 domains is sufficient to disrupt MO fusion by altering the Ca^{2+} sensitivity of this protein [16]. A phylogenetic tree constructed from the alignment of individual mammalian ferlin C2 domain sequences shows that, in all ferlins, a given C2 domain is more similar to other C2 domains at similar positions in the other proteins than to C2 domains in

different positions of the same protein [16] (Figure 2b). These data suggest that multiple C2 domains may be required for protein activity.

Dysferlin is a 230 kDa protein that contains seven C2 domains. It is found in a variety of tissues including skeletal and cardiac muscle, kidney, placenta, lung, and brain, and it is most highly expressed in skeletal and cardiac muscle [7]. Interestingly, a single missense mutation in any of five dysferlin C2 domains (C2A, B, D, E and G) has been reported to cause muscular dystrophy [17] (Figure 2a), again suggesting nonredundancy among individual C2 domains. But mutations in these C2 domains may also lead to dysferlin misfolding and thus degradation [17,18]. The dysferlin C2A domain binds phospholipids in a Ca^{2+}-dependent manner [19], consistent with its role in skeletal muscle membrane repair [6$^{\bullet\bullet}$,10$^{\bullet}$]. This novel function is also supported by ultrastructural observations of dysferlin-deficient skeletal muscle: subsarcolemmal regions are characterized by prominent aggregations of small vesicles of unknown origin [6$^{\bullet\bullet}$,20,21]; the sarcolemma itself shows many gaps and microvilli-like projections rather than being continuous and smooth [6$^{\bullet\bullet}$,21]; and the basal lamina is multilayered in some regions [21]. In addition, dysferlin deficiency delays myoblast fusion/maturation *in vitro* [22], suggesting that dysferlin may also participate in muscle differentiation/regeneration.

Myoferlin shares the highest sequence homology to dysferlin. It is present at the sarcolemma of skeletal muscle but, unlike dysferlin, it is also enriched in the nucleus [14]. Although both myoferlin and dysferlin are expressed in skeletal muscle, they seem to participate in distinct cellular events. In contrast to dysferlin, myoferlin functions in myoblast fusion during muscle differentiation/maturation and myoferlin-null mice show muscle atrophy [23]. Also, a lack of compensatory overexpression of myoferlin in muscles with dysferlinopathy [24] supports the view that myoferlin and dysferlin have few overlapping functions. So far, myoferlin has not been linked to any human disease.

Otoferlin is predominately expressed in the cochlea, vestibule, and brain, although a low level of expression is seen in other tissues including lung, kidney, skeletal muscle, and heart [12]. Mutations in otoferlin are responsible for nonsyndromic deafness known as DFNB9 in humans [11,12]. Otoferlin was recently shown to be essential for a late step of synaptic vesicle exocytosis and may act as the major Ca^{2+} sensor triggering synaptic vesicle–plasma membrane fusion at the inner hair cell ribbon synapse [25$^{\bullet\bullet}$].

The Fer1L4, Fer1L5, and Fer1L6 proteins are predicted from the human and mouse genomic sequences but have not yet been characterized. In light of the association of dysferlin and otoferlin with human diseases, it is reasonable

Figure 2
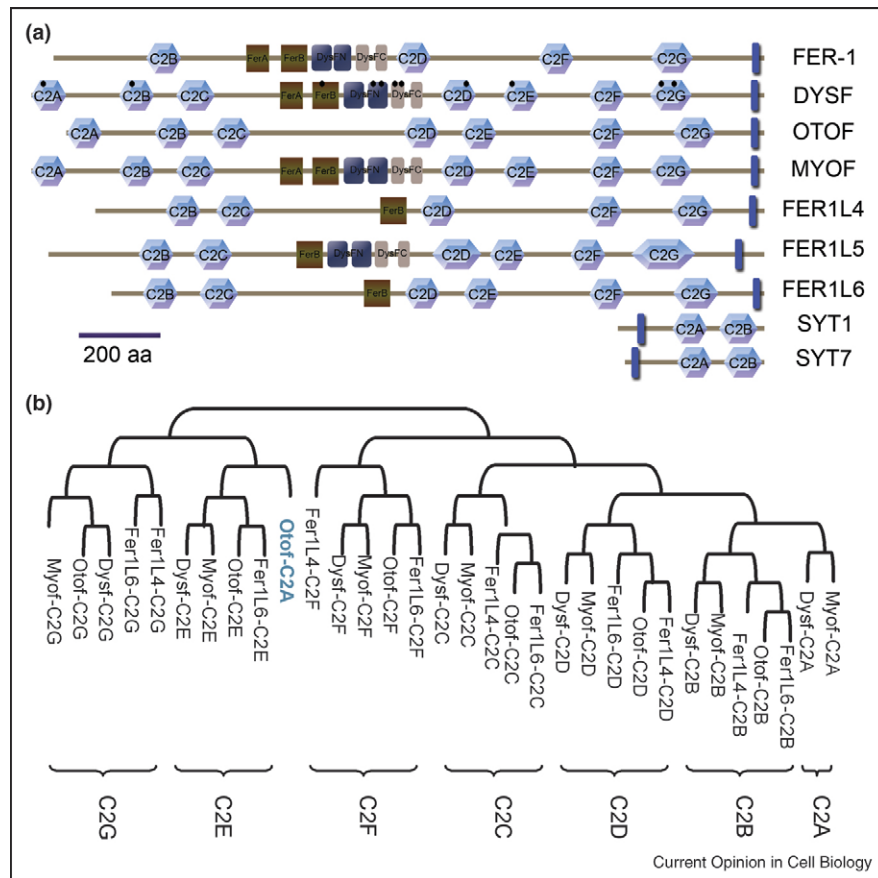
Structural characteristics of the ferlin-1-like proteins. FER-1 and mammalian ferlin-1-like proteins are aligned, with their domain composition indicated **(a)**. Most ferlins have five to seven C2 domains and a single carboxyl transmembrane domain. FER-1, dysferlin (Dysf), myoferlin (Myof), and Fer1L5 have FerA and FerB domains, and nested DysfN and DysfC domains, which are not present in otoferlin (Otof) and other ferlins. The black dots on dysferlin domains indicate the missense mutations that lead to muscular dystrophy. A phylogenetic tree **(b)** was constructed on the basis of the alignment of individual ferlin C2 domain sequences. The tree shows that the C2 domains at any similar positions of distinct proteins are more similar to each other than to those within the same protein.
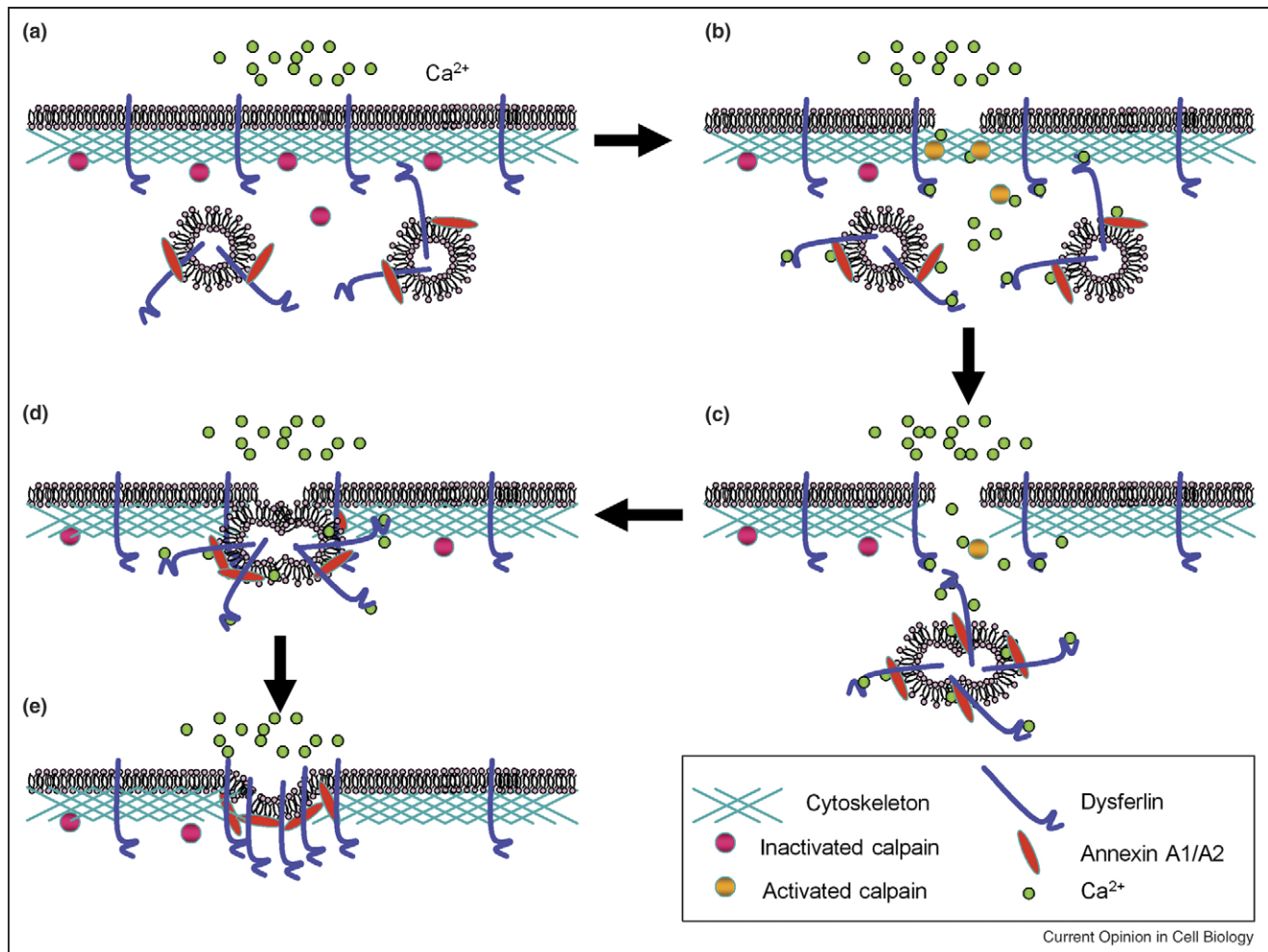
Mechanism of dysferlin-mediated membrane repair

Recent progress toward elucidating the mechanisms involved in Ca^{2+}-dependent membrane repair has led to the proposal of two mechanisms: the *lipid flow promotion* hypothesis and the *patch* hypothesis.

According to the lipid flow promotion hypothesis, hydrophobic lipids at the free edge of the membrane disruption site are energetically unfavorable in the aqueous environment, creating 'line tension'. The line tension may promote automatic lipid flow over this site to fuse to the complementary free membrane end, closing the disruption. The fact that the plasma membrane adheres to an underlying cortical cytoskeleton, which generates 'membrane tension' [26] opposing the 'line tension'

(reviewed in [27]), argues against this. However, if the membrane disruption was capable of somehow reducing the 'membrane tension', membrane resealing through 'line tension'-driven lipid flow would be a viable mechanism. Notably membrane disruption has been observed to cause a rapid Ca^{2+}-dependent reduction in 'membrane tension' [25••]. Consistent with the lipid flow hypothesis, treatment of damaged cells with surface active agents thought to reduce the 'membrane tension' also enhances resealing and cell survival [28,29]. With regard to the observed reduction in membrane tension in response to membrane disruption, this may result from depolymerization of the cortical cytoskeleton by Ca^{2+}-activated calpain proteolytic activity (Figure 3), as supported by the finding that the typical μ-calpain and m-calpain (also called calpain-1 and calpain-2, respectively) are required for efficient membrane repair [30,31•,32]. However, this membrane repair mechanism might only be applicable to relatively small disruptions (<1 μm diameter) [33].

Figure 3

Schematic model of dysferlin-mediated membrane repair. In normal muscle fiber, dysferlin is localized to the sarcolemma and cytoplasmic vesicles and interacts with annexins A1 and A2 **(a)**. Membrane disruption causes calcium flooding into the muscle fiber and creates a zone of high calcium around the disruption site **(b)**. The high calcium activates proteases such as calpain, which leads to the cleavage of cortical cytoskeleton proteins and thereby reduces membrane tension **(c)**. The high calcium also triggers the aggregation of dysferlin-carrying repair vesicles probably involving annexins and the migration of these vesicles toward the disruption site, where they fuse with one another and with the plasma membrane in the presence of localized high levels of calcium **(b–d)**. Dysferlin then triggers vesicle fusion with the plasma membrane, probably through SNAREs. Fusion of the repair vesicles with the plasma membrane results in a membrane patch being inserted across the site of membrane disruption and thereby reseals the disrupted plasma membrane **(e)**.

In the patch hypothesis for membrane repair, Ca^{2+} flooding through a membrane disruption is thought to evoke local vesicle–vesicle and vesicle–plasma membrane fusion events. As a result, a population of large vesicles accumulates underneath the disruption site, eventually creating a 'patch' of new membrane across the membrane gap via vesicle–vesicle and vesicle–membrane fusion (Figure 3). In support of this possibility, abnormally large vesicles rapidly accumulate, in a Ca^{2+}-dependent manner, at sites of membrane damage in several systems: endothelial cells [5], sea urchin eggs [34] and nerve axons [35]. These findings indicate that Ca^{2+}-triggered vesicle migration and vesicle–vesicle fusion events take place

immediately following disruption of the plasma membrane.

With regard to the kinds of vesicles that might be involved in membrane repair by the patch process, accumulated evidence suggests that yolk granules [34], lysosomes [36], enlargeosome [37], or vesicles generated by endocytosis of axolemma [35] are utilized depending on their abundance in different cells. Lysosomes are widely present and competent for Ca^{2+}-dependent regulated exocytosis and thus considered an ideal candidate for membrane repair. Indeed, inhibition of lysosome exocytosis has been reported to inhibit membrane resealing in NRK, CHO, L6E9,

3T3, and primary skin fibroblast cells [36], suggesting that Ca^{2+}-regulated exocytosis of lysosomes is probably involved in membrane repair. However, whether lysosomes are dispensable for membrane repair is a matter of debate. One group reported that a small molecule vacuolin-1 blocked the Ca^{2+}-dependent exocytosis of lysosomes induced by ionomycin or plasma membrane wounding, without affecting the process of resealing [38], whereas the inhibitory effect of vacuolin-1 on Ca^{2+}-regulated exocytosis of lysosomes was not confirmed by the other group [39]. The reason for this discrepancy is not yet clear. The specific organelles providing membrane for resealing in skeletal muscle remain to be determined. Skeletal muscle undergoes frequent damage, thus requiring frequent membrane resealing. If lysosomes were the predominant donor membranes for resealing, lysosomal enzymes would be expected to accumulate in the extracellular space, and this would probably have deleterious effects on the muscle. It is conceivable that skeletal muscle may use whatever Ca^{2+}-regulated exocytic vesicles available for resealing.

Membrane fusion requires several membrane proteins that could potentially be required for membrane resealing. These include SNARE proteins, a family of transmembrane proteins essential for most intracellular membrane fusion processes, and synaptotagmins (Syt), transmembrane proteins with two highly conserved C2 domains that serve as calcium sensors in the regulation of vesicle exocytosis in neurons and other cell types [39]. Several lines of evidence support that SNAREs are involved in membrane repair. Membrane resealing in both sea urchin eggs and fibroblasts is inhibited by injections of botulinum and tetanus toxins [40], which cleave various SNARE proteins and are known to inhibit Ca^{2+}-mediated synaptic vesicle exocytosis. Also, antibodies to syntaxin-1 and Syt-1 inhibit resealing in crayfish giant axons [41], and antibody-based or peptide-based inhibition of Syt-VII blocked Ca^{2+}-dependent exocytosis of lysosomes in permeablized fibroblasts [42]. Moreover, Syt-VII has been implicated in the plasma membrane repair of skeletal muscle, since Syt-VII-deficient mice developed inflammatory myopathy with extensive fibrosis, high serum creatine kinase levels, and progressive muscle weakness [43]. Furthermore, otoferlin has recently been shown to bind SNAP25, and syntaxin-1 to trigger synaptic vesicle exocytosis in inner hair cells, which do not express Syt-I [25••]. Since dysferlin shares high homology to otoferlin, it is reasonable to hypothesize that dysferlin might also work through SNAREs to regulate vesicle fusion in membrane repair.

Recent work provides insights into the mechanism of synaptotagmin-potentiated membrane fusion. In response to Ca^{2+}, the C2A and C2B domains of synaptotagmin-1 insert into the target membranes [44,45], induce high positive curvature of the membranes, and thus lower the activation energy barrier for membrane fusion [46••]. As mentioned earlier, dysferlin and other ferlin proteins also contain C2 domains and at least some of these C2 domains bind phospholipids in response to Ca^{2+}. It is very intriguing to examine whether dysferlin triggers membrane fusion by the same mechanism.

Dysferlin interactors that may be involved in membrane repair

Recent studies of dysferlin interacting proteins have provided new mechanistic insights into dysferlin function. Dysferlin normally associates with annexins A1 and A2 in a Ca^{2+}-dependent and membrane injury dependent manner [10•]. In dysferlinopathy patients, expression of annexins A1 and A2 is elevated compared to controls, and annexin expression levels are significantly correlated with clinical severity scores [47]. These data suggest that annexins A1 and A2 play a role in dysferlin-mediated membrane resealing in skeletal muscle. Indeed, a requirement for annexin A1 in membrane repair was recently confirmed in HeLa cells [48•]. Although the mechanism of annexin action in membrane repair is currently unclear, the findings that annexins firstly, bind phospholipids in a Ca^{2+}-dependent manner, secondly, initiate vesicle aggregation *in vitro*, and thirdly, interact with the actin cytoskeleton [49] suggest an involvement in vesicle–vesicle fusion for 'patch' formation and vesicle movement (Figure 3). It would be interesting to examine model systems in which endogenous annexins A1 and A2 are deleted. One might expect that the loss of these proteins would lead to defective membrane repair in skeletal muscle and to muscular dystrophy, or that it would exacerbate the phenotype of dysferlin deficiency.

Caveolin-3 and calpain-3 are both muscle-specific proteins that are responsible for distinct forms of muscular dystrophy (LGMD1C and LGMD2A, respectively) and both have also been found to interact with dysferlin [50,51]. Whether these interactions are important in the membrane repair pathway remains unclear. However, patients deficient in caveolin-3 have been reported to exhibit a reduction or mislocalization of dysferlin in caveolinopathy patients [50,52], and patients deficient in dysferlin have reduced levels of calpain-3 [51]. Thus, these diseases may well involve overlapping or associated pathogenic mechanisms.

Dysferlin also binds to AHNAK [53], a previously reported marker of enlargeosomes [37]. Again, it is not known whether the association of AHNAK and dysferlin is relevant for membrane repair. In fact, both proteins redistribute to the cytosol during skeletal muscle regeneration [53], suggesting that they may act together in membrane fusion events that are necessary during regeneration rather than in events crucial to membrane repair.

Affixin, an integrin-linked kinase focal adhesion protein, has also been reported to localize to the sarcolemma and to coimmunoprecipitate with dysferlin [54]. Affixin immu-

noreactivity is reduced at the sarcolemma of MM and LGMD2B muscles, and also in other muscle diseases including LGMD1C. In all these cases, affixin and dysferlin show quite similar changes in their expression patterns, including a reduction in sarcolemmal staining (with or without cytoplasmic accumulations, depending on the specific disease forms) [54]. Given what is known about affixin, such an interaction could potentially coordinate cytoskeletal reorganization required for efficient vesicle trafficking during the resealing process. However, such a role has not been established, nor has the functional significance of this interaction been addressed.

Defective membrane repair and disease

As described above, defective dysferlin-mediated membrane repair is responsible for three clinically distinct forms of muscular dystrophy in humans. The cause of the observed disease heterogeneity remains unclear. Interestingly, the same mutations in dysferlin gene have been found to cause different disease manifestation even within the same family [55,56], suggesting that other genetic factors may affect the clinical symptoms in the patients. But these distinct symptoms share overlapping features: age of onset, slow disease progression, and early, marked elevation of serum CK.

Recent work has shown that dysferlin deficiency greatly reduces the membrane repair efficiency of cardiac muscle, with the aged dysferlin-null mice manifesting hallmarks of cardiomyopathy, for example, elevated serum cardiac troponin T levels, cardiac necrosis, and cardiac fibrosis [57••]. Although echocardiography recordings from ∼1-year-old dysferlin-null mice failed to detect abnormalities in cardiac physiology, mechanical stress disturbed ventricular function sufficiently to unmask the cardiac phenotype of these mice [57••]. The importance of maintaining an efficient dysferlin-mediated membrane repair system for cardiac muscle is further supported by the fact that dysferlin deficiency greatly accelerates the progression of cardiomyopathy in mdx mice (which carry a point mutation in the dystrophin gene), where skeletal and cardiac muscle are highly susceptible to membrane damage [57••]. Although cardiomyopathy is not commonly reported in human dysferlinopathy patients, a 57-year-old woman with dysferlin deficiency presented with cardiomyopathy after more than 20 years of progressive muscle wasting [58], suggesting that dysferlinopathy patients are indeed prone to develop cardiomyopathy but with a late onset. A systematic examination of cardiac involvement in dysferlinopathy patients has been initiated, and an early report has shown that a significant proportion (9 out of 11) of these patients (ages 19–48) have elevated serum cardiac troponin T levels (S Yilmazer *et al.*, abstract in *Neuromuscul Disord* 2006, 16:S110). Similarly, dysferlin-null mice show an increase in serum cardiac troponin T levels at early ages (e.g. 30 weeks old), although they do not present clear cardiac muscle abnormality, by either histological or echocardio-graphy analyses. Further examination of cardiomyopathy in older dysferlin-deficient patients is therefore necessary.

Conclusions

Membrane resealing is an emergency response that is highly conserved among different species and cell types. It is mediated by rapid Ca^{2+}-triggered exocytosis of intracellular vesicles in animal cells, which form a patch at the disruption site (Figure 3). Skeletal and cardiac muscles are mechanically active tissues that are often subjected to injury; thus, they require a robust membrane resealing mechanism and may therefore have developed a specialized mechanism for this purpose. Dysferlin plays a crucial role in the membrane repair of both skeletal and cardiac myocytes. The dysferlin-mediated resealing response in muscle is likely to involve the SNARE family of proteins and may also require the participation of other Ca^{2+}-activated proteins such as calpains and annexins (Figure 3). At this time, it is not clear specifically which SNARE proteins might be involved. Although both Syt-VII-regulated membrane repair and dysferlin-mediated membrane repair seem to be active in skeletal muscle, it remains unclear whether they regulate fusion of the same vesicle pools in the same step or different steps or different vesicle pools. Future experiments addressing these questions will significantly advance our understanding of the detailed mechanism of muscle membrane repair.

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