HHS Public Access

Author manuscript

Curr Top Dev Biol. Author manuscript; available in PMC 2015 June 13.

Published in final edited form as:

Curr Top Dev Biol. 2011 ; 96: 203–230. doi:10.1016/B978-0-12-385940-2.00008-5.

Ferlin proteins in myoblast fusion and muscle growth

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Abstract

Myoblast fusion contributes to muscle growth in development and during regeneration of mature muscle. Myoblasts fuse to each other as well as to multinucleate myotubes to enlarge the myofiber. The molecular mechanisms of myoblast fusion are incompletely understood. Adhesion, apposition, and membrane fusion are accompanied by cytoskeletal rearrangements. The ferlin family of proteins is implicated in human muscle disease and has been implicated in fusion events in muscle, including myoblast fusion, vesicle trafficking and membrane repair. Dysferlin was the first mammalian ferlin identified and it is now know that there are six different ferlins. Loss of function mutations in the dysferlin gene lead to limb girdle muscular dystrophy and the milder disorder Miyoshi myopathy. Dysferlin is a membrane-associated protein that has been implicated in resealing disruptions in the muscle plasma membrane. Newer data supports a broader role for dysferlin in intracellular vesicular movement, a process also important for resealing. Myoferlin is highly expressed in myoblasts that undergoing fusion, and the absence of myoferlin leads to impaired myoblast fusion. Myoferlin also regulates intracellular trafficking events, including endocytic recycling, a process where internalized vesicles are returned to the plasma membrane. The trafficking role of ferlin proteins is reviewed herein with a specific focus as to how this machinery alters myogenesis and muscle growth.

Keywords

myoblast; myogenesis; ferlin proteins; membrane fusion; muscle

1. INTRODUCTION

Muscle development and regeneration require the fusion of myoblast to the multinucleate syncytium. In many different genetic forms of muscular dystrophy, muscle damage or dysfunction is caused by a specific gene mutation. In the face of enhanced degeneration, there is often a concomitant increase in muscle regeneration, although typically the increase in regeneration is insufficient to match the pace of degeneration. With the imbalance favoring muscle degeneration, there is a loss of functioning myofibers and replacement of

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the muscle by fibrosis and fat. This replacement process, referred to as dystrophy, also creates an environment that is thought to further reduce effective myogenesis since the normal matrix cues that promote myoblast differentiation and support myoblast fusion are destroyed or otherwise rendered defective.

Limb girdle muscular dystrophy (LGMD) type 2B is caused by mutations in the gene encoding dysferlin. Loss of function mutations in the dysferlin gene lead to this autosomal recessive form of LGMD as well as the milder disorder Miyoshi Myopathy (Bashir *et al.*, 1998; Liu *et al.*, 1998). The identification of dysferlin as a disease gene has led to the identification of the ferlin family of proteins and their role in muscle repair and regeneration. In the mammalian genome, there are six ferlin proteins. To date, three have been characterized as protein products. Dysferlin is a membrane associated protein and like all members of the ferlin family, dysferlin contains multiple C2 domains. The ferlin family is unique in that members have as few as four and as many as seven C2 domains, whereas all other C2 domain containing proteins have one or two of these domains. C2 domains are found in at least 100 membrane-associated proteins where they mediate protein-lipid, protein-calcium, and protein-protein interactions. Ferlin proteins are found in non-muscle cell types and have been associated with additional disease phenotypes. For example, otoferlin mutations cause nonsyndromic deafness suggesting that the ferlin proteins are critical for basic functions such as intracellular trafficking (Yasunaga *et al.*, 1999). Dysferlin and myoferlin are two members of the ferlin proteins that are highly expressed in myoblasts and myofibers. This review will focus on what is known about ferlin proteins in muscle regeneration and myogenesis.

1.1. *C. elegans Fer-1*, the ferlin prototype

The ferlin family of proteins is defined by their sequence similarity to the *C. elegans Fer-1*, and *Fer-1* is considered the prototypical ferlin proteins. *Fer-1* was originally identified from genetic screens for fertility defects in *C. elegans*. In the normal maturation process of the spermatozoa, multiple large membranous organelle must fuse to the plasma membrane. *Fer-1* mutants were infertile and the maturing spermatozoa displayed a characteristic abnormal intracellular retention of membranous organelles (Ward *et al.*, 1981). This intracellular accumulation impaired normal motility of the spermatids leading to infertility (Achanzar and Ward, 1997). The resultant sperm cannot adhere to uterine walls and are defective in oocyte fertilization. Ten different mutations have been cataloged within *Fer-1*; five of these fall within C2 domains and two generate premature stop codons (Achanzar and Ward, 1997; Washington and Ward, 2006). These mutations disrupt calcium sensitive fusion. Fer-1 protein localizes to the membranous organelle. It was initially believed that *Fer-1* was expressed only in the testis; however recent reports counter the original observation and suggest that *Fer-1* is also expressed in *C. elegans muscle* where it alters muscle gene expression (Krajacic *et al.*, 2009).

The *Fer-1* gene encodes a 235 kDa protein that contains a carboxy-terminal transmembrane domain similar to viral fusion proteins and at least four C2 domains. C2 domains are independently folding domains formed from approximately 130 amino acids. The domains assemble as eight parallel β-strands that fold into a β sandwich. At one end is a cluster of

aspartate residues formed from several different loops to generate a Ca^{2+} binding domain (Sudhof and Rizo, 1996). C2 domains mediate lipid-binding and protein-protein interactions in Ca^{2+}-dependent and Ca^{2+}-independent fashion (Davletov and Sudhof, 1993). Among the best studied C2-domain-containing proteins are the synaptotagmins; synaptotagmins are mediators of Ca^{2+}-dependent membrane fusion including the fast exocytosis that occurs at nerve terminals (Brose *et al.*, 1992; Sudhof and Rizo, 1996). By analogy, it can be expected that exocytosis events such as those related to vesicle fusion and membranous organelle fusion are mediated by ferlin proteins.

1.2. C2 domain-containing proteins, including the ferlins, are involved in lipid binding and fusion

The synaptotagmins are a family of membrane-trafficking proteins that contain a transmembrane domain and two carboxy-terminal C2 domains, C2A and C2B. Synaptotagmin I facilitates synaptic vesicle membrane fusion with the presynaptic membrane, a function that shares striking similarity to Fer-1 function (Brose *et al.*, 1992). Synaptotagmin I is located in the synaptic vesicles and interacts with syntaxin, found on the plasma membrane (Chapman *et al.*, 1995). In the presence of calcium, the C2A domain of synaptotagmin I binds syntaxin with a high affinity and also binds negatively charged phospholipids with increased affinity (Brose *et al.*, 1992; Perin *et al.*, 1990). The Ca^{2+} binding loops of the C2A and C2B domains of synaptotagmin I insert into the lipid bilayer (Bai *et al.*, 2002). Synaptotagmin I has the spatial opportunity to mediate vesicular-plasma membrane fusion while bound to the vesicular membrane and interacting with syntaxin in the presence of calcium. Thus, synaptotagmin I is thought to regulate timing of membrane fusion through its calcium-sensing ability and its ability to promote membrane fusion through Ca^{2+} loop insertion into the membrane, causing a local disruption (Peuvot *et al.*, 1999). One theory of membrane coalescence suggests that the disruption of the lipid bilayer by the angled insertion of calcium-sensing proteins induces membrane fusion (Peuvot *et al.*, 1999).

Ca^{2+} binding by C2 domains is cooperative and synaptotagmin C2 domains bind at least three Ca^{2+} molecules. A similar series of in vitro analyses were conducted with dysferlin and myoferlin C2 domains (Davis *et al.*, 2002; Therrien *et al.*, 2009). In these studies, the individual C2 domains were purified from bacteria as fusion proteins. The C2A domains of both myoferlin and dysferlin bind negatively charged phospholipids, particularly phosphotidylserine, but only in the presence of Ca^{2+} (Davis *et al.*, 2002). Under these same conditions, the remaining C2 domains of myoferlin were not found to bind any of the phospholipid combinations tested. A similar approach demonstrated that only the first C2 domain of dysferlin could bind phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate in response to Ca^{2+} (Therrien *et al.*, 2009). The concentration of phosphotidylserine required for binding was rather high at 50%, but such concentrations may be important in the inner surface of the membrane where myoblast fusion occurs (Davis *et al.*, 2002). The Ca^{2+} concentration required to elicit lipid binding differed slightly for myoferlin C2A compared to dysferlin C2A. The half maximal lipid binding for myoferlin C2A was 1 μM while it was 4.5 μM for dysferlin C2A (Davis *et al.*, 2002). Although synaptotagmin C2 domains bind three Ca^{2+} ions, the ferlin C2A domains may bind more.

Localized Ca^{2+} concentrations and the specific composition at the inner plasma membrane surface are likely to be critical for triggering appropriate and regulated fusion events (Corbin *et al.*, 2007). A V67D mutation in the C2A domain of dysferlin that causes both Limb-Girdle Muscular Dystrophy Type 2B and Miyoshi myopathy (Illarioshkin *et al.*, 2000) specifically disrupts Ca^{2+} induced lipid binding and results in the loss of cooperativity of binding (Davis *et al.*, 2002). A similar mutation, I67D, in the C2A domain of myoferlin also completely abolishes Ca^{2+}-dependent phospholipid binding (Doherty *et al.*, 2005).

1.3. The Mammalian Ferlin Family

Humans and mice have six Fer-1-like genes that form the ferlin family: dysferlin (Fer1L1), otoferlin (Fer1L2), myoferlin (Fer1L3), Fer1L4, Fer1L5, and Fer1L6 (Achanzar and Ward, 1997; Bashir *et al.*, 1998; Britton *et al.*, 2000; Jimenez and Bashir, 2007; Liu *et al.*, 1998; Smith and Wakimoto, 2007; Yasunaga *et al.*, 1999) (Figure 1). The ferlins share similarity in protein structure. In common to all family members is a single pass transmembrane domain localized at the carboxy-terminus (Jimenez and Bashir, 2007; Perin *et al.*, 1990). The ferlins are type II transmembrane proteins containing a series of positively charged residues at the beginning of the transmembrane domain, and these residues are thought to be critical for anchoring the transmembrane domain within the membrane. This topology, whether at the plasma membrane or embedded in cytoplasmic vesicles, places the carboxy-terminus anchored in the membrane and the remainder of the protein, containing the C2 domains within the cytoplasm. The ferlin C2 domains are named in order from amino- to carboxy-terminus as C2AC2F. Across ferlin family members, specific C2 domains are most homologous to each other. That is, C2A of dysferlin is most like C2A of myoferlin and less like the remaining C2 domains within dysferlin and myoferlin. Only the first C2 domain, C2A, binds to lipids and the position of the C2A domain at the very amino terminus may allow this domain to stretch some physical distance from the membrane surface itself where it could be positioned to mediate fusion of opposing membranes. These opposing membranes could be between two vesicles or between a vesicle and the plasma membrane.

1.3.1. Dysferlin—Dysferlin was originally identified through positional cloning as the gene responsible for both LGMD2B and Miyoshi Myopathy (Bashir *et al.*, 1998; Liu *et al.*, 1998). The LGMDs are a collection of genetically diverse disorders that include both dominant and recessive gene mutations (Urtizberea *et al.*, 2008). The clinical presentation of LGMD2B is characterized by onset in the second decade, variable progression, and predominant wasting of the proximal muscle groups surrounding the pelvic girdle with mild shoulder girdle weakening over time. LGMD2B is inherited autosomal recessively. Miyoshi Myopathy also has an onset in the late teens and early adulthood but predominantly affects the distal muscle groups, particularly the gastrocnemius muscle. Weakness can occur in the proximal muscles over time. Miyoshi Myopathy is also inherited autosomally recessive. Linkage analysis showed that both LGMD2B and Miyoshi Myopathy were associated with a region on chromosome 2 and two groups independently identified dysferlin as the gene defect for these disorders (Bashir *et al.*, 1998; Liu *et al.*, 1998).

The dysferlin gene encodes a 230 kDa protein with at least six C2 domains. Some algorithms predict seven C2 domains and with an additional domain positioned between

C2E and C2F. Because of this the nomenclature for C2E, C2F and C2G may be shifted (Therrien *et al.*, 2009; Washington and Ward, 2006). Antibodies raised to dysferlin were used to show that dysferlin is highly expressed in adult skeletal muscle as well as in the heart. The predominant pattern for dysferlin immunoreactivity in adult skeletal muscle is at the cell membrane and this is well visualized in cross sectional analysis of muscle (Anderson *et al.*, 1999). In cell culture, dysferlin is highly expressed in differentiated myotubes, although it is still detectable in myoblasts (Davis *et al.*, 2002; Doherty *et al.*, 2005) (Figure 2B). Muscle biopsies from humans with dysferlin mutations reveal a prominent accumulation of small subsarcolemmal vesicles and empty, swollen cisternae in the Golgi apparatus (Cenacchi *et al.*, 2005; Piccolo *et al.*, 2000; Selcen *et al.*, 2001). The finding of accumulation of intracellular vesicles is reminiscent of what was seen with *C. elegans Fer-1* mutations.

Dysferlin and *C. elegans Fer-1* each contain a DYSF domain. In dysferlin, this domain is found positioned between C2C and C2D. In *C. elegans Fer-1*, the DYSF domain is similarly positioned, but this ferlin lacks C2A and C2B. This domain is found in other proteins, notably in the yeast peroxisomal proteins Pex30p and Pex31p, and is the hypothesized binding site of caveolin-3 within dysferlin (Patel *et al.*, 2008; Yan *et al.*, 2008).

1.3.2. Otoferlin—Otoferlin is responsible for the autosomal recessive deafness disorder DFNB9 (Yasunaga *et al.*, 1999). Although otoferlin is expressed in a wide range of tissues, including skeletal muscle, it is highly expressed in the cochlea of the ear. Otoferlin has been implicated in vesicle trafficking and secretion through interaction with the endosomal protein EEA1, the Golgi marker GM130, and the GTPase Rab8a (Heidrych *et al.*, 2008; Schug *et al.*, 2006). Recent evidence suggests that otoferlin interacts with myosin VI at the inner hair cell synapse and that the interaction is involved in the recycling of synaptic vesicles (Roux *et al.*, 2009). Structurally, otoferlin is 226 kDa in size and contains either five or six C2 domains, depending on the algorithm used. Otoferlin is most like Fer1L4 and Fer1L6 and is less related to dysferlin, myoferlin and Fer1L5. Of interest, otoferlin lacks the C2A domain suggesting that this protein may mediate different events given the lack of a lipid-binding domain.

1.3.3. Myoferlin—Myoferlin has more similarity to dysferlin than any other ferlin protein (Davis *et al.*, 2000) (Figure 2A). Myoferlin is also a 230 kDa protein that contains one carboxy-terminal transmembrane domain and seven C2 domains. Like dysferlin, myoferlin has a DYSF domain. Myoferlin is highly expressed in myoblasts, especially those myoblasts that have begun to differentiate (Davis *et al.*, 2000; Doherty *et al.*, 2005) (Figure 2B). In their proliferative stage, myoblasts can appear flattened and more fibroblast-like in appearance. When induced to differentiate, myoblasts undergo a series of morphological changes in that they become more refractile and undergo a cell shape change. Just before fusion, myoblasts are no longer fibroblast shaped but rather are elongated with a centrally position nucleus and a long axis. These prefusion myoblasts express the highest levels of myoferlin. After myoblasts fuse to form myotubes in culture, myoferlin expression is reduced. The mature myofiber expresses some myoferlin and, like dysferlin, it is found at the plasma membrane (Davis *et al.*, 2000). There is also a population of myoferlin seen at

the nuclear membrane, but the role of this perinuclear myoferlin is not known. It may represent myoferlin undergoing trafficking to the plasma membrane or to intracellular vesicles. During fusion, myoferlin is enriched at sites where cells are contacted (Doherty *et al.*, 2005). Upon muscle injury, myoferlin levels are dramatically increased (Doherty *et al.*, 2005). A recent characterization of the myoferlin promoter shows that it is regulated by the NFAT transcription factors (Demonbreun *et al.*, 2010a). With muscle injury, myoferlin expression is increased in both myoblast and myotubes (Demonbreun *et al.*, 2010a).

1.3.4. Fer1L4, Fer1L5, Fer1L6—Based on database analysis, there are three other ferlin genes in the mammalian genome. The three additional ferlins family members are: Fer1L4, Fer1L5, and Fer1L6. Fer1L4 and Fer1L6 are 201 kDa 209 kDa, respectively, and both contain five C2 domains. Fer1L5 is a 241 kDa protein that contains six C2 domains, including the C2A domain which Fer1L4 and Fer1L6 lack. Sequence analysis suggests that Fer1L5 is highly similar to myoferlin, Fer1L6 is highly similar to otoferlin, and Fer1L4 is similar to otoferlin but to a lesser degree than Fer1L6. A detailed characterization of the proteins produced from these genes is lacking. It is worthwhile to note that cDNAs in the electronic databases suggest that alternative splicing may occur in these mRNAs leading to a range of proteins. The role of Fer1L4, Fer1L5 and Fer1L6 in myogenesis, if any, remains to be determined.

2. FERLIN EXPRESSION AND LOCALIZATION & INTERACTING PROTEINS IN MUSCLE

Dysferlin is strongly expressed in heart, brain, placenta, and skeletal muscle (Liu *et al.*, 1998). In mature skeletal muscle, dysferlin is normally localized to the plasma membrane (Anderson *et al.*, 1999). In C2C12 myotubes, dysferlin colocalizes with the marker Bin1 in T-tubules and translocates to the plasma membrane upon damage to the myotubes (Klinge *et al.*, 2007). In a rat model of muscle regeneration, dysferlin is localized to the T-tubules and dysferlin-deficient muscle shows abnormal T-tubule development suggesting that dysferlin is required for T-tubules formation (Klinge *et al.*). Also in C2C12 myotubes, dysferlin colocalizes to microtubules and immunoprecipitates with tubulin suggesting that dysferlin-containing intracellular vesicles may translocate to the plasma membrane via microtubules (Azakir *et al.*).

Dysferlin immunoprecipitates with membrane specific proteins, including caveolin-3, from normal human skeletal muscle (Matsuda *et al.*, 2001). Caveolin-3 is a small molecular mass protein that anchors into membranes leaving its amino and carboxy domains in the cytoplasm. Mutations in the gene encoding caveolin-3 have been described in patients with inherited forms of muscular dystrophy and clinically milder disorders associated with elevated serum creatine kinase and comparatively less muscle weakness. LGMD1C is a dominantly inherited muscular dystrophy that affects the proximal muscle groups and it is associated with caveolin-3 gene mutations. Patients with LGMD1C exhibit abnormally localized dysferlin. Similarly, dysferlin is aberrantly localized due to caveolin-3 mutations in a dominant-negative fashion (Cai *et al.*, 2009). Dysferlin also interacts with mitsugumin 53 (MG53), a muscle-specific TRIM (tripartite motif, consisting of a RING finger domain, a B-

box zinc finger domain, and a coiled-coil region) protein involved in intracellular vesicle trafficking during muscle repair. Along with MG53, dysferlin and caveolin-3 represent an essential muscle membrane repair complex and disrupting of either component affects the localization and membrane repair function of the other complex components.

Other dysferlin-interacting proteins have been identified by coimmunoprecipitation and mass spectrometry. One of these proteins includes AHNAK, a large (approximately 700kDa) protein primarily located near the nucleus that translocates to the plasma membrane and associates with annexin 2 and actin for stability during the membrane repair process (Benaud *et al.*, 2004; Hohaus *et al.*, 2002; Huang *et al.*, 2007). AHNAK interacts with the C2A domain of dysferlin, the same domain responsible for phospholipid binding. Down-regulation of AHNAK with small-interfering RNA was found to disrupt cortical actin organization, suggesting that AHNAK participates in cytoskeletal rearrangements (Benaud *et al.*, 2004). Dysferlin also interacts with annexin A1 and A2, Ca^{2+} and phospholipid-binding proteins involved in the aggregation of lipid rafts at the plasma membrane (Lennon *et al.*, 2003). Annexin A1 and A2 localization is disrupted in dysferlin mutant muscle. Also present in the dysferlin protein complex is calpain-3, encoded by *CAPN3*. Calpain-3 is a skeletal-muscle specific nonlysosomal, Ca^{2+}-dependent cysteine protease involved in sarcomere reorganization (Huang *et al.*, 2005). Mutations in *CAPN3* cause a recessive form of muscular dystrophy called LGMD2A. Calpain-3 cleaves AHNAK into smaller fragments, and this cleavage specifically disrupts AHNAK binding to dysferlin (Huang *et al.*, 2008). Muscle from *CAPN3* mutant patients exhibits increased levels of AHNAK. These results highlight a shared property of defective sarcomeric and subsarcolemmal reorganization associated with mutations that affect either calpain-3 or dysferlin.

Myoferlin is expressed in heart and skeletal muscle, as well as at low levels in the lung and most other tissues and cell types (Davis *et al.*, 2000). In C2C12 myoblasts undergoing fusion, myoferlin is found in discrete structures at the cell periphery (Doherty *et al.*, 2008). During myoblast fusion, myoferlin is enriched at sites on the plasma membrane of cell-cell merger (Doherty *et al.*, 2005). Myoferlin directly interacts with the endocytic recycling protein EHD2, a member of the EH-domain-containing (EHD) family of proteins, arguably members of the dynamin superfamily; EHD2 binds an asparagine-proline-phenylalanine (NPF) motif in the myoferlin C2B domain (Doherty *et al.*, 2008). Similarly, the insulin growth factor receptor-1 (IGFR-1) immunoprecipitates and colocalizes with myoferlin in proliferating myoblasts (Demonbreun *et al.*, 2010b). Myoferlin, like dysferlin, also interacts with AHNAK through its C2A domain (Benaud *et al.*, 2004).

Otoferlin is expressed in the cochlear sensory hair cells (Yasunaga *et al.*, 1999). Otoferlin colocalizes with the endosomal markers EEA1 and Golgi proteins like GM130. Also, otoferlin immunoprecipitates with the Rab8b GTPase as well as myosin VI (Heidrych *et al.*, 2008; Heidrych *et al.*, 2009). Myosin VI and otoferlin interact at the inner hair cell ribbon synapse, providing a possible mechanism for the transport of intracellular vesicles from the trans-Golgi network and the recycling endosome to the plasma membrane (Roux *et al.*, 2009). These interactions may also be present with other ferlins. For instance, the Rab8a GTPase interacts with myosin Vb and localizes to endosomal tubules containing EHD2 homologs EHD1 and EHD3 (Roland *et al.*, 2007). The Rab family of interacting proteins

(Rab-FIPs) contains NPF motifs, similar to the NPF motif found in myoferlin, suggesting possible binding sites for EHD interaction. Thus, the homologs of Rab GTPase and myosins may vary by tissue and cell type, but it is plausible that this interaction links the recycling endosome to intracellular trafficking via microtubules to the plasma membrane.

While dysferlin and myoferlin are strongly expressed in heart and muscle tissue, they are also expressed in other cell types. Dysferlin is present in secretory and plasma membrane vesicles of polymorphic neutrophils (Jethwaney *et al.*, 2007). In fact, neutrophil recruitment is attenuated in dysferlinopathy, suggesting that dysferlin plays a role in the immune response to muscle damage (Chiu *et al.*, 2009). This aspect of dysferlin function in the regeneration of muscle damage will be explored below. Like dysferlin, myoferlin is also expressed outside of myoblasts and myofibers. Myoferlin is also expressed in infiltrating immune cells, including Mac-1 antigen-positive cells, in regenerating muscle (Demonbreun *et al.*, 2010a). In endothelial cells, myoferlin silencing decreases clathrin- and caveolae-dependent endocytosis and overexpression of myoferlin increases endocytosis (Bernatchez *et al.*, 2009). Additionally, myoferlin has been shown to regulate the recycling of vascular endothelial growth factor receptor-2 (VEGFR-2) and exists in a complex with VEGFR-2, caveolin-1, and dynamin-2 which functions similar to EHD2 (Bernatchez *et al.*, 2007; Bernatchez *et al.*, 2009).

In common to all the ferlin family members described to date is a role in protein interactions important for trafficking vesicles within cells. For *C. elegans* Fer-1, this trafficking is critical for mobilizing the membranous organelles required for normal spermatogenesis but more recently it has been suggested to also be important for muscle function in the worm (Krajacic *et al.*, 2009). In otoferlin, intracellular vesicle movement and docking is critical for the fusion of synapse vesicles at the inner hair cell ribbon synapse. In muscle function, vesicle docking and fusion are required to repair disrupted membranes. In myogenesis, vesicle trafficking is not only important for providing membrane lipid components to a fusing myoblast but also for the protein cargo it carries to the sites of fusion and potentially for intercellular protein movement. We suggest that the ferlins share a general role in the trafficking of intracellular vesicles in multiple cell types for the exocytosis of intracompartmental contents and the membrane replacement of recycled signaling receptors. A role in endocytosis cannot be excluded, but this process is emerging as one that is an essential step in myoblast fusion, and myoblast fusion is tightly linked to differentiation.

3. CYTOSKELETAL REARRANGEMENT IN MYOGENESIS

Throughout myogenesis, singly nucleated myoblasts fuse to other singly nucleated myoblasts to form syncytial multinucleated myofibers. These fusion events are important during embryogenesis for muscle development and also during adulthood as damaged muscle undergoes regeneration. The mechanism of cell-cell fusion has previously been poorly understood, however, genetic and cell biology studies in *Drosophila* and mammalian cell culture have provided *in vivo* and *in vitro* evidence for the involvement of several molecules in myoblast fusion and revealed information about the sequential events of the fusion process.

The first step of myoblast-myoblast fusion is the recognition of opposing myoblasts. In Drosophila, two separate populations of myoblasts exist in the somatic mesoderm: muscle founder cells and fusion-competent cells. Founder cells are the anchors for future myofibers and are specified through Notch-mediated lateral inhibition (Haralalka and Abmayr, 2010). Fusion-compentent myoblasts (FCMs) derive from the same population of somatic mesoderm myoblasts but are not included in the founder cell selection. FCMs are attracted to and fuse to the founder cells (Chen and Olson, 2005). This attraction is thought to occur through either the random extensions of FCM filopodia or the directed extentions of FCM filopodia towards a concentrated founder cell attractant (Chen and Olson, 2004). Such an attractant has yet to be described.

Founder cells and FCMs express immunoglobulin (Ig)-domain-containing transmembrane receptors on the cell surface that are responsible for cell adhesion (Haralalka and Abmayr, 2010). In founder cells, those receptors are Dumbfounded (*Duf*) and its redundant paralog Roughest (*Rst*), while in FCMs, they are Sticks and stones (*Sns*) and its paralog Hibris (*Hbs*) (Bour *et al.*, 2000; Dworak *et al.*, 2001; Ruiz-Gomez *et al.*, 2000; Strunkelnberg *et al.*, 2001). Deletion of *Duf* and *Rst* causes a complete block in myoblast fusion, but the fusion defect can be rescued with one copy of either paralog (Strunkelnberg *et al.*, 2001). Loss of *Sns* also results in a lack of fusion, while only minor fusion defects are observed with the loss of *Hbs* (Artero *et al.*, 2001). Ectopic expression of *Duf* and *Sns* in *Drosophila* S2 cells demonstrates a direct interaction between the two molecules as *Duf*-expressing cells aggregate with *Sns*-expressing cells (Galletta *et al.*, 2004). Coincidently, *Duf* and *Sns* display a specific subcellular localization and colocalize with F-actin foci at sites of myoblast fusion (Kesper *et al.*, 2007). Taken together, these results suggest that the Ig cell adhesion molecules *Duf/Rst* and *Sns/Hbs* are responsible for the attachment, and possibly attraction, of founder cells and FCMs.

The second step of myoblast fusion, where the fusion machinery is recruited and there is cytoskeletal rearrangement to accommodate fusion, is less well understood. Electron micrographs of several steps preceding myoblast fusion have provided insight into the organelle arrangement associated with myogenesis (Doberstein *et al.*, 1997; Kalderon *et al.*, 1977). One of the early steps after cell-cell adhesion is the juxtaposition of “prefusion complexes” which consists of dense core vesicles aligned on the cytoplasmic side of both cell membranes (Doberstein *et al.*, 1997). These prefusion vesicles are observed to derive from a subcellular location near the Golgi, associate with microtubules, and are transported to actin-rich foci at the plasma membrane prior to myoblast fusion (Kim *et al.*, 2007). Thus, the membrane fusion step of myoblast fusion involves the intracellular signaling that transmits the cell-cell attachment signal to the molecules responsible for actin cytoskeleton rearrangement as well as actin foci enrichment that allows for translocation of the prefusion vesicles from exocytic origins to the plasma membrane (Figure 3).

The primary signal is transmitted from the cell-adhesion molecules Duf and Sns. Truncation of the intracellular domain of Duf severely compromises myoblast fusion efficiency (Bulchand *et al.*, 2010). The cytoplasmic region of Sns is also essential for myoblast fusion (Galletta *et al.*, 2004). In founder cells, the intracellular domain of Duf interacts with two secondary messengers: Antisocial/Rolling pebbles (*Ants/Rols7*) and Loner (Bulchand *et al.*,

2010). Ants/Rols7 and Loner are localized in specific subcellular foci and can be independently recruited to fusion sites at the plasma membrane by Duf and Rst. Subcellular localization of Ants/Rols7 and Loner is *Duf/Rst*-dependent; *duf rst* double mutants show Ants/Rols7 and Loner distributed throughout the cytoplasm (Chen and Olson, 2001; Chen *et al.*, 2003).

After receiving signals from Duf/Rst, Ants/Rols7 physically interacts with a member of the CDM (CED-5, Dock180, Myoblast city) family of proteins, Myoblast city (Mbc) (Chen and Olson, 2001). Mbc is the Drosophila homolog of the mammalian Dock180, which is responsible for modulation of the small GTPase Rac (Nolan *et al.*, 1998). Mbc forms a bipartite guanine nucleotide exchange factor with dELMO, and when overexpressed together, produce a phenotypic defect reminiscent of Drac1 overexpression, suggesting that Mbc and dELMO act in concert to activate Drac1 (Geisbrecht *et al.*, 2008). Drac1, Drac2, and Mtl are Rac GTPases with overlapping function (Hakeda-Suzuki *et al.*, 2002). Mutants of each Rac exhibit a myoblast fusion defect and a dominant negative Drac1 demonstrates unregulated actin polymerization (Hakeda-Suzuki *et al.*, 2002; Luo *et al.*, 1994). In a murine model, Rac1 mutant myoblasts fail to recruit actin fibers to myoblast contact sites (Vasyutina *et al.*, 2009). Rac1 is responsible for activation of the actin nucleation factor Arp2/3, which induces actin cytoskeletal rearrangements (Ten Klooster *et al.*, 2006). Taken together, these results suggest that Rac1 is involved in dynamic actin reorganization.

The other secondary messenger, Loner, displays guanine nucleotide exchange activity on the GTPase ARF6 (Chen *et al.*, 2003). Dominant-negative ARF6 mutations exhibit a myoblast fusion defect in the Drosophila embryo as well as in mammalian cell cultures, similar to the defect observed for loss-of-function *loner* mutations. ARF6 has also been implicated in the control of the subcellular localization of Rac1 (Radhakrishna *et al.*, 1999). In mammalian cells, ARF6 associates in a multiprotein complex with M-cadherin, Rac1, and the guanine nucleotide exchange factor Trio, and ARF6 activates phospholipase D and phosphatidylinositol 4,5-bis-phosphate production (Bach *et al.*, 2010). Silencing of ARF6 inhibits Trio and Rac1 association with M-cadherin. ARF6 may provide a link between Loner and regulation of cytoskeletal rearrangements.

Other actin binding and polymerizing factors with roles in myoblast fusion have also been identified. WASp is a nucleation-promoting factor that activates Arp2/3 in restricted developmental contexts (Zallen *et al.*, 2002). WASp is required for myoblast fusion and the WASp-interacting protein (WIP) Solitary (*Sltr*) recruits WASp to sites of fusion occupied by Duf and Sns. The WASp/Stlr/Arp2/3 complex is responsible for actin polymerization at fusion sites, necessary for the recruitment of the pre-fusion vesicles (Kim *et al.*, 2007). It remains unclear when actin polymerization would preferably proceed from downstream signaling of Ants/Rols7 or Loner.

The last step of myoblast fusion was also observed through electron micrographs. After the alignment of paired vesicles at the plasma membrane, the paired vesicles fuse to the plasma membrane and a resultant electron-dense plaque remains (Doberstein *et al.*, 1997). The Drosophila gene Singles Bar (*Sing*) is required for progression past the prefusion complexes; *sing* mutant myoblasts contain more pre-fusion complexes than wildtype myoblasts *Sing*

may be required for the fusion of the vesicles to the plasma membrane (Estrada *et al.*, 2007). The function of *Sing* is not well understood. After the pre-fusion complexes coalesce with the plasma membrane, fusion pores develop between the juxtaposed membranes allowing union of cytoplasmic material. Transmission electron micrographs of WASp and WIP mutants demonstrate that WASp and WIP are required at a late state of myoblast fusion, shortly after the initial fusion pores form (Massarwa *et al.*, 2007). Finally, the detached membrane forms vesicles sacs of the previous extracellular contents as the nascent bilayer fusion is resolved (Kalderon and Gilula, 1979). Efficient rearrangement of the actin cytoskeleton is also critical during this step of myoblast fusion in order to allow the paired vesicle-plasma membrane fusion and the vesiculation of detached plasma membrane.

Drosophila have one ferlin gene, *misfire*. As with the initial reports of the C. elegans Fer-1, misfire has been reported to be involved in spermatogenesis and not expressed in the musculature of the organism (Smith and Wakimoto, 2007). However, the characterization of misfire did not include a developmental analysis, as only adult flies were observed. A general role for ferlin involvement in intracellular trafficking is emerging and it is quite plausible that this role is conserved in *Drosophila*.

Drosophila has provided a wealth of information about the myoblast fusion process through the use of genetic models. The field has not advanced as fast in mammalian systems. Some mammalian homologs of Drosophila myogenesis genes are not expressed in the developing mesoderm, such as the cell-adhesion receptors Duf, Rst, Sns, and Hbs, and an ortholog of the secondary messenger Ants/Rols7, Tanc1. Cell-adhesion in mammalian cells may involve N-cadherin and the Ig receptor CDO (Krauss, 2010). Downstream activation of Rac1 can be modulated by M-cadherin. While some molecular functions are not conserved between mammals and insects, others are conserved. ARF6 can modulate Rac1 activity (Chen *et al.*, 2003). Dock180 interacts with ELMO to activate Rac1 as well (Komander *et al.*, 2008). Because of the importance of actin polymerization throughout the developing embryo for cell motility, cell-cell fusion, and organelle transport, developing genetic models is difficult since many mutants are lethal. The cre recombinase system could potentially provide an excellent way to study the effects of these genes in myoblast fusion, but many of the cre models that currently exist to study muscle are not expressed specifically in the myoblast or not expressed in the myoblast at all.

4. FERLIN PROTEINS IN CYTOSKELETAL REARRANGEMENTS DURING MYOGENESIS

Myoferlin is highly expressed in proliferating and early differentiating myoblasts and at sites of myoblast-myoblast or myoblast-myotube fusion (Davis *et al.*, 2002; Doherty *et al.*, 2005). Myoferlin directly interacts with the endosomal recycling protein EHD2, and, based on homology, it is conceivable that myoferlin may interact with other members of the EHD family (Doherty *et al.*, 2008). This interaction suggests that myoferlin participates in the intracellular trafficking of recycling vesicles (Figure 4). Loss of myoferlin results is known to produce defective endocytic recycling in primary myoblasts (Doherty *et al.*, 2008). Like dysferlin-null muscle biopsies, myoferlin-null muscle shows an accumulation of small subsarcolemmal vesicles, suggesting that myoferlin is also required for fusion of these

vesicles to the plasma membrane (Demonbreun *et al.*, 2010b). The primary assay for recycling relies on internalization and recycling of transferrin. It has been observed that more transferrin accumulates in myoferlin-null myoblasts than wild type myoblasts at baseline, and using pulse-chase experiments, that the recycling of the accumulated transferrin is also delayed (Doherty *et al.*, 2008).

The EHD family of proteins consists of four highly homologous members, each containing an amino-terminal nucleotide-binding domain, a carboxy-terminal EH domain, and a central coiled-coil domain. EHDs have been implicated in the trafficking of many cell signaling and metabolic molecules, including IGF1R, EGFR, GLUT4, LDL receptor, and the transferrin receptor (Austin *et al.*, 2010; Demonbreun *et al.*, 2010b; Naslavsky *et al.*, 2007; Rotem-Yehudar *et al.*, 2001). A direct interaction between EHD1 and SNAP29, a SNARE protein, suggests that EHD1 may have a role in membrane fusion (Rotem-Yehudar *et al.*, 2001). The *C. elegans* EHD homolog, *RME-1*, was identified in a genetic screen for mutants defective in receptor-mediated endocytosis (Grant and Hirsh, 1999). *rme-1* mutants accumulated RME-2 yolk receptors within recycling endosomes and not on the cell surface, indicating that RME-1 functions in the endocytic recycling and not internalization (Grant *et al.*, 2001). Dominant negative substitutions within RME-1, G81R and G429R, failed to recycle yolk receptors as well. The Drosophila EHD homolog, Past1, has also been implicated in endocytosis (Olswang-Kutz *et al.*, 2009). Garland cells from *Past1* mutants demonstrated a reduced ability to endocytose fluorescently labeled avidin. A genetic interaction between Past1 and the Notch signaling pathway was inferred from a wingvein phenotype, suggesting that Past1 may be responsible for the recycling of Notch receptor.

While the EHDs are highly homologous molecules, they are not completely redundant. siRNA reduction of EHD1–4 in mammalian cells demonstrated that loss of EHD1 and EHD3 function severely reduces transferrin exit from the endocytic recycling compartment (George *et al.*, 2007). However, transferrin recycling was only mildly affected by loss of EHD4, and EHD2 reduction had no affect on transferrin recycling. Deletion of the EH domain in EHD1 and EHD3 resulted in a perinuclear clustering of the cytoplasmic Rab11, while EHD2 and EHD4 deletions had no affect. Reduction of EHD protein levels demonstrated that EHD1 and EHD2 function in the exit from the recycling endosome, while EHD3 and EHD4 function in the transport from the early endosome to the endosomal-recycling compartment. EHD1 and EHD4 null mice both exhibit defects in spermatogenesis and decreased male fertility (George *et al.*, 2010; Rainey *et al.*, 2010). siRNA reduction of EHD3 in cardiomyocytes indicated that EHD3 has a role in the trafficking of the cardiac ankyrin-B membrane protein (Gudmundsson *et al.*, 2010). In addition, EHD3 and EHD4 were sufficiently elevated following myocardial infarction, suggesting that these molecules may have a role in cardiomyocyte damage response. The necessity for these molecules throughout myogenesis has yet to be determined. Yet if we posit that those pre- fusion complexes that align themselves adjacent to the apposed membranes at sites of fusion are of endocytic recycling origin, the EHDs are candidates for the transport from the exit of the recycling endosome to the plasma membrane. This argument is strengthened by the interaction of EHDs and the EH-domain binding protein, EHBP1, which contains a novel amino-terminal C2 domain version coupled with a calponin-homology domain (Zhang and

Aravind, 2010). Loss of EHBP1 disrupts recycling of GLUT4 in cultured adipocytes, and overexpression of EHBP1 disrupts transferring uptake and induces actin rearrangements, suggesting that EHBP1 regulates actin polymerization (Guilherme *et al.*, 2004a; Guilherme *et al.*, 2004b).

Defective endocytic recycling prevents signaling receptors from being properly returned to the plasma membrane for subsequent signaling. In myoferlin-null primary myoblasts, the insulin growth factor (IGF) receptor-1 is abnormally internalized and is shuttled for lysosomal degradation (Demonbreun *et al.*, 2010b). IGF1 mediates cell growth and is responsible for skeletal muscle hypertrophy (Barton, 2006). Mice lacking IGF1 are smaller in size, while mice overexpressing IGF1 have increased skeletal muscle fiber number and increased fiber cross-sectional area (Liao *et al.*, 2006; Liu *et al.*, 1993; Powell-Braxton *et al.*, 1993). While wild type myoblasts hypertrophy upon IGF stimulation, myoferlin-null myoblasts do not respond to stimulation (Demonbreun *et al.*, 2010b). Although IGF1 signaling has been shown to be important for myogenesis, there are other signaling pathways required as well. Myoferlin may be required for the intracellular trafficking of these receptors also. In addition to those receptors required for myogenesis, myoferlin also participates in the endocytosis of the transferrin receptor, the cholera toxin-B receptor, and the vascular endothelial growth factor receptor-2 (VEGFR-2) (Bernatchez *et al.*, 2007; Bernatchez *et al.*, 2009). Myoferlin deficiency results in a reduction in VEGFR-2 protein levels; myoferlin prevents the ubiquination and degradation of VEGFR-2 (Bernatchez *et al.*, 2007).

As a result of myoferlin-null defective endocytic recycling, myoferlin-null mice have decreased body mass and muscle mass, and *in vivo* muscle analysis reveals myoferlin-null muscle fibers are decreased in area and size, similar to IGF-null mice (Doherty *et al.*, 2005; Liu *et al.*, 1993; Powell-Braxton *et al.*, 1993). Primary myoblasts from myoferlin-null mice form smaller myotubes due to defective myoblast fusion. Similarly, primary myoblasts from transgenic mice overexpressing a dominant negative IGF-1R also demonstrate impaired myoblast fusion (Heron-Milhavet *et al.*, 2010).

Defective myoblast fusion is observed with dysferlin deficiency. Primary myoblasts from dysferlin mutant patients begin to fuse later than control myoblasts and demonstrate an incomplete myotube differentiation, as majority of myotubes are binucleated (de Luna *et al.*, 2006). Myogenin is decreased in dysferlin mutant myotubes, suggesting that dysferlin may influence myogenesis by increasing *myogenin* gene expression. Correspondingly, dysferlin-deficient C2C12 cells demonstrate a reduction in long myotubes and a reduction of muscle differentiation proteins, including myosin heavy chain (Belanto *et al.*, 2010).

5. FERLIN PROTEINS PARTICIPATE IN MUSCLE DAMAGE REPAIR

There is evidence that the ferlins participate in the repair of damaged adult muscle fibers. Dysferlin-deficient muscle demonstrates a pathological progressive pattern of fiber necrosis, fibrotic and immune infiltrate, and increased sarcolemmal membrane disruption and regenerating fibers (Bansal *et al.*, 2003). Loss of dysferlin is also characterized by a poor repair response to muscle damage. Three-fold overexpression of a skeletal-muscle specific

dysferlin transgene rescues all histological defects as well as increases the recovery of muscle function after contraction injury, suggesting that the defects observed in dysferlin-deficiency are initially caused by the loss of dysferlin in skeletal muscle (Millay *et al.*, 2009).

Bansal et al. provided the quintessential study demonstrating that dysferlin was involved in the repair of damaged myofibers. Control and dysferlin-deficient myofibers were injured using a laser in the presence of a membrane impermeable fluorescent dye (Bansal *et al.*, 2003). In the absence of Ca^{2+}, neither wildtype nor dysferlin-deficient myofibers were able to repair the membrane disruptions induced by the laser, demonstrating the Ca^{2+}-dependence of muscle membrane resealing. However, in the presence of Ca^{2+}, wildtype fibers efficiently prevented dye entry by repairing the damaged membrane while dysferlin-null myofibers were unable to do so. Electron micrographs of wildtype and dysferlin-null skeletal muscle also showed an accumulation of subsarcolemmal vesicles in dysferlin-null but not wildtype muscle (Piccolo *et al.*, 2000; Selcen *et al.*, 2001). Recent studies showed that antisense dysferlin morpholinos inhibit intercellular wound-triggered Ca^{2+} signaling in neighboring cells of sea urchin embryos, but not Ca^{2+} spikes in the wounded cell itself (Covian-Nares *et al.*, 2010). Taken together, these results suggest that dysferlin responds to the Ca^{2+} influx after damage and influences the fusion of intracellular and exocytic vesicles to the plasma membrane. These exocytic vesicles provide additional phospholipids to seal the damaged area through vesicular-plasma membrane fusion and also release molecules into the extracellular environment for cell-cell signaling.

Roche et al. injected wildtype and dysferlin-null mice intraperitoneally with fluorescein dextran (FDx) and induced large-strain injury in the ankle dorsifelxors (Roche *et al.*, 2008). FDx is a fluorescent dye, similar to that used in the Bansal et al. laser-wounding study, that diffuses into the myofiber through the damaged sarcolemmal membrane and is trapped inside the cell as the membrane is resealed. Wildtype tibialis anterior muscle retained the FDx fourteen days post-injury, dysferlin-deficient TA muscle retains FDx three days post-injury but demonstrated a significant reduction in FDx retention seven days post-injury (Roche *et al.*, 2008; Roche *et al.*, 2010). Three days post injury, there was a significant increase in mononuclear cells, mostly CD68+ macrophages, in dysferlin-null TA muscle, but not wildtype TA muscle (Roche *et al.*, 2010). Dysferlin-null TA muscle also showed a significant increase in centrally nucleated fibers and developmental myosin heavy chain positive (dMHC+) fibers fourteen days post-injury. Central nuclei and dMHC expression are markers of regenerating myofibers. Interestingly, inhibition of satellite cell activation by hindlimb irradiation prior to injury prevented the increase in centrally nucleated and dMHC + fibers in dysferlin-null muscle, which suggests that central nuclei and dMHC expression rely on satellite cell activation and myogenesis. The single localized 25 Gy dose of radiation did not inhibit macrophage infiltration; instead, inflammation was increased in both wildtype and dysferlin-null muscle following a longer large-strain injury protocol. These results are interesting because they demonstrate that satellite cell activation and myogenesis is increased with dysferlin-deficiency. However, wildtype muscle did not show an increase in central nuclei and only showed a modest increase in dMHC+ fibers, indicating that the repair of the sarcolemmal membrane may occur independent of myogenesis. Supporting

these findings, dysferlin is expressed in a subset of c-met+ satellite cells in normal muscle and satellite cells in pathological muscles (De Luna *et al.*, 2004). All activated (MyoD+) satellite cells also express dysferlin. Thus, dysferlin has an apparent role in satellite cells, possibly through the regulation of satellite cell activation.

Myoferlin is normally expressed throughout myogenesis in myoblasts and nascent myotubes (Davis *et al.*, 2000; Doherty *et al.*, 2005). Myoferlin protein levels are normally low in adult skeletal muscle and nearly absent in healthy myofibers. One plausible mechanism for the increase in myogenesis in dysferlin-null muscle is that an increase in satellite cell activation leads to an accumulation of mononuclear myoferlin-positive myoblasts that anxiously wait to repair damaged myofibers; irradiation could destroy these myoblasts and prevent the myogenesis observed by Roche et al. Conversely, an alternate mechanism is that an increase in myoferlin expression, due to dysferlin deficiency, could directly stimulate satellite cell activation. Studies have demonstrated that alterations in dysferlin expression lead to changes in the expression levels of myogenic transcription factors. Increased induction of dysferlin with dexamethasone treatment in C2C12 muscle cells increases the expression of muscle differentiation proteins, including myosin heavy chain (Belanto *et al.*, 2010). Dysferlin deficiency causes a sharp reduction in myogenin, a myogenic transcription factor expressed in activated satellite cells, so this alternative mechanism is plausible (de Luna *et al.*, 2006).

In disease and injury models, myoferlin appears to have a role in the repair of the damaged muscle. Myoferlin mRNA is upregulated 7.3 fold in quadriceps biopsies from Duchenne Muscular Dystrophy patients (Haslett *et al.*, 2002). Myoferlin mRNA, as well as IGF-1 mRNA, was significantly upregulated in the hindlimb muscles of resistance exercise trained rats (Adams *et al.*, 2007). At the protein level, myoferlin is focally upregulated at the membrane in *mdx* skeletal muscle, suggesting that it is important during muscle regeneration (Davis *et al.*, 2002; Demonbreun *et al.*, 2010a). When *Sgcg*-null mice are injected intraperitoneally with Evan's blue dye (EBD), a marker sequestered in myofibers with disrupted sarcolemma, myoferlin is abundantly expressed in dye-positive but not dye-negative fibers (Demonbreun *et al.*, 2010a). *In vitro*, myoferlin is increased in myotubes after cardiotoxin-induced injury, while nearly myoferlin is absent in uninjured myotubes. A GFP-reporter for the myoferlin promoter replicates these findings. The myoferlin GFP reporter is normally expressed in myoblasts but not mature myotubes. However, upon injury, when endogenous myoferlin is normally upregulated, GFP is expressed. These GFP-positive myofibers accumulate EBD as well as increased intracellular Ca^{2+}, suggesting that myoferlin is upregulated in myofibers with damaged membranes. Myoferlin-null mice also demonstrate delayed muscle repair after injury, highlighting that myoblast fusion is not only required during embryogenesis but also during damage repair and muscle regeneration (Demonbreun *et al.*, 2010b; Doherty *et al.*, 2005).

6. CONCLUDING REMARKS

The discovery of the ferlin family of proteins, specifically dysferlin and myoferlin, has led to a new appreciation for the importance of vesicle trafficking and its importance to normal muscle growth and repair. The ferlin proteins harbor the capacity to bind directly to negatively charged phospholipids and additionally scaffold a number of distinct proteins via

their C2 domains. Future studies will focus on identifying binding partners and understanding the dynamic intercellular changes that occur for these proteins. The role of dysferlin in disease likely arises from both defects in the mature myofiber and the myoblast, reinforcing the importance of vesicular trafficking for these processes. The ferlins interact with other trafficking proteins during damage repair to transport internal membrane structures to the site of damage and seal the breach in sarcolemma. These interacting molecules include those covered earlier with roles in exocytic vesicle transportation, membrane docking, and actin cytoskeletal rearrangement.

Acknowledgments

Supported by NIH R01 NS47726, T32 HL 7381 and the Muscular Dystrophy Association.

Abbreviations

EHD	Eps homology domain
FCM	fusion competent myoblast
Fdx	fluorescein dextran
IGF	insulin like growth factor
LGMD	limb girdle muscular dystrophy
TRIM	tripartite motif

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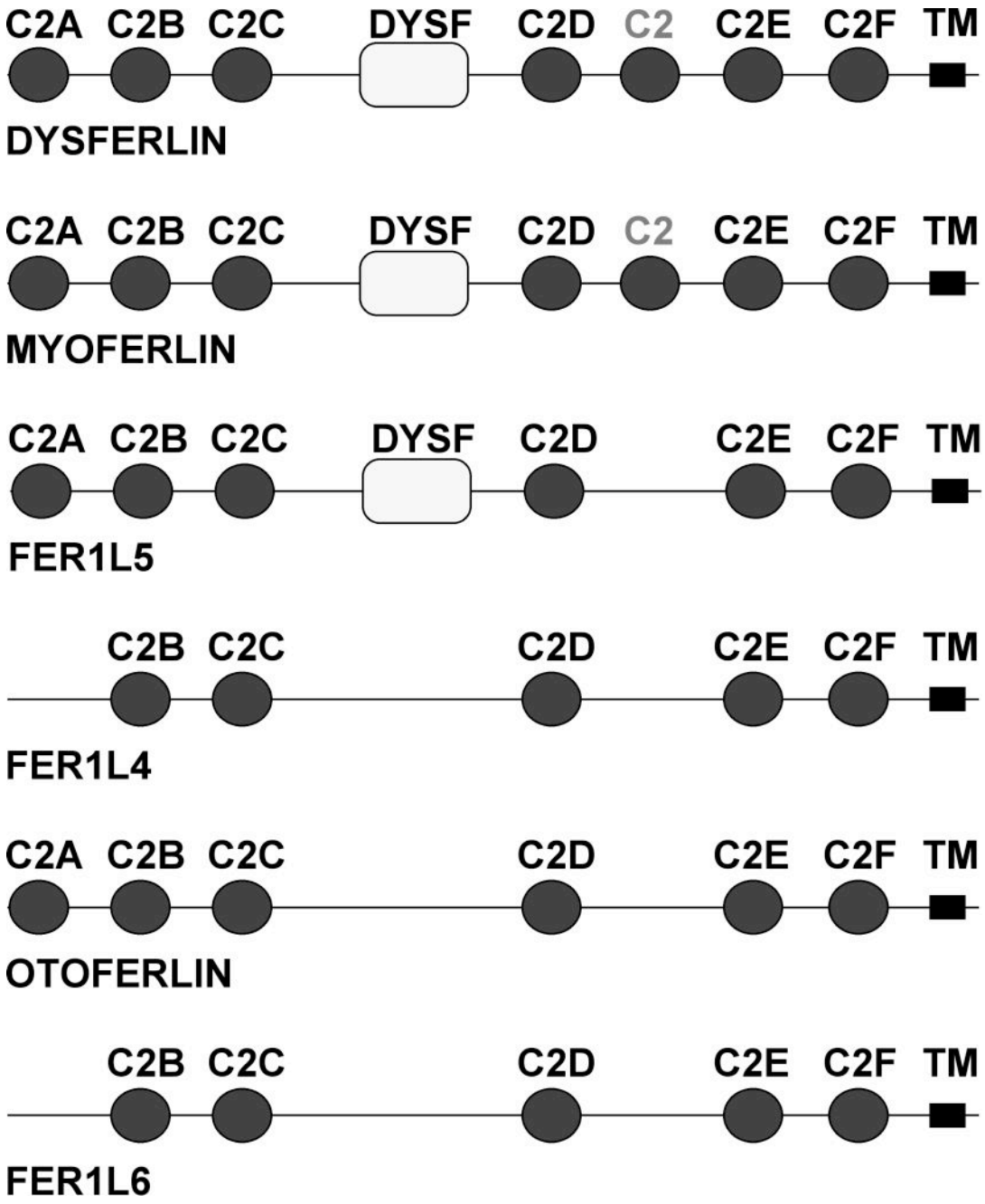

Figure 1.

Schematic of the mammalian ferlin family members. The ferlins share similar domain architecture: a carboxy-terminal transmembrane domain and multiple amino-terminal C2 domains, including a C2A domain that has been shown to bind negatively charged phospholipids in dysferlin and myoferlin. The DYSF domain is the putative binding site of caveolin-3 and is found within dysferlin, myoferlin, and fer1L5.

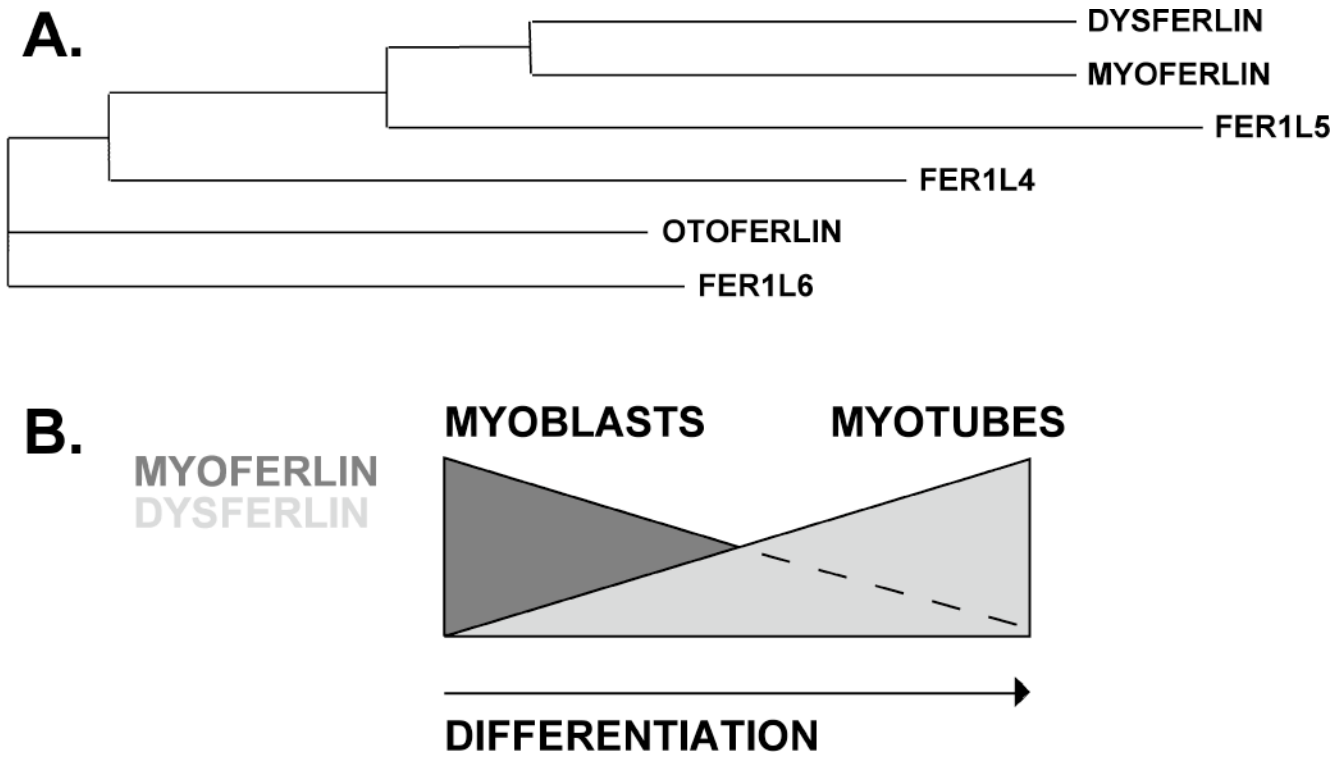

Figure 2.

A Phylogram of the mammalian ferlin family. Dysferlin and myoferlin are closely related. Fer1L5 also shares close relation to dysferlin and myoferlin, while fer1L4, otoferlin, and fer1L6 are distantly related. *B* Throughout myoblast differentiation, myoferlin (dark grey) is highly expressed in proliferating myoblasts and nascent myotubes, while dysferlin (light grey) is highly expressed in mature myotubes.

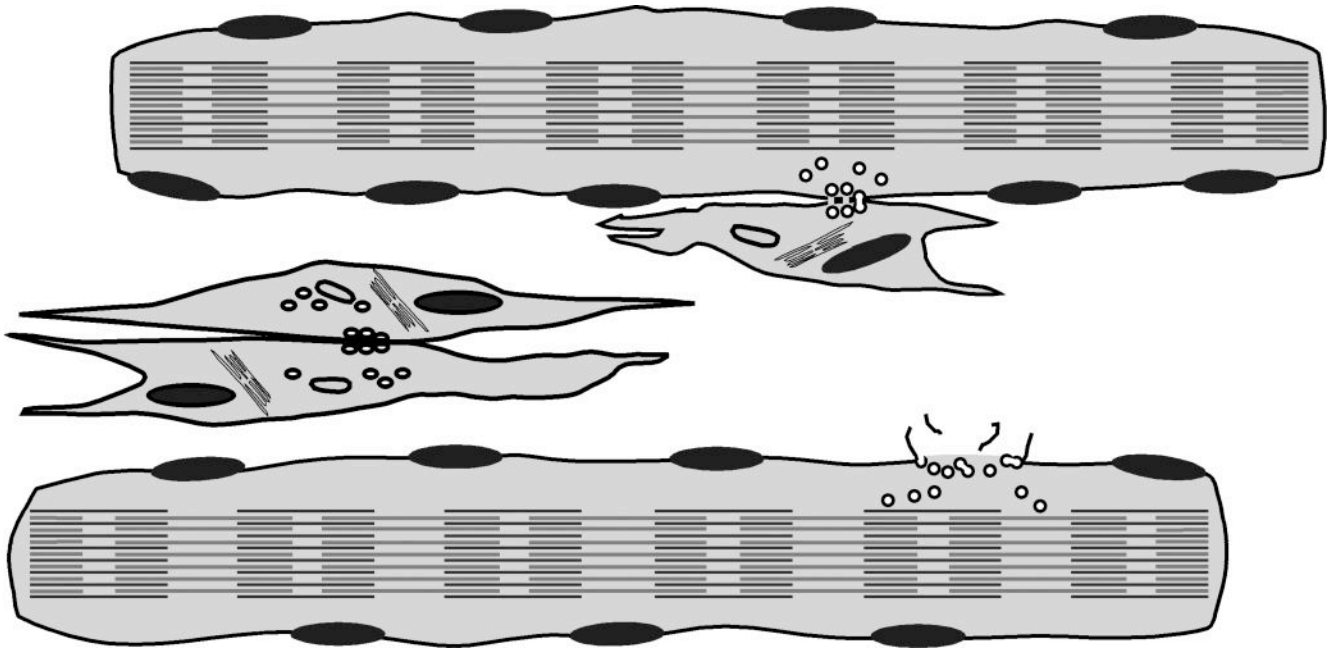

Figure 3.

Membrane fusion events in muscle repair. Shown is a site of muscle injury. At the top is myoblast to myofiber fusion. The middle portion reflects myoblast to myoblast fusion. The lower portion of the picture depicts plasma membrane disruption undergoing resealing. Ferlin family members, particularly, dysferlin and myoferlin, are poised to mediate these events.

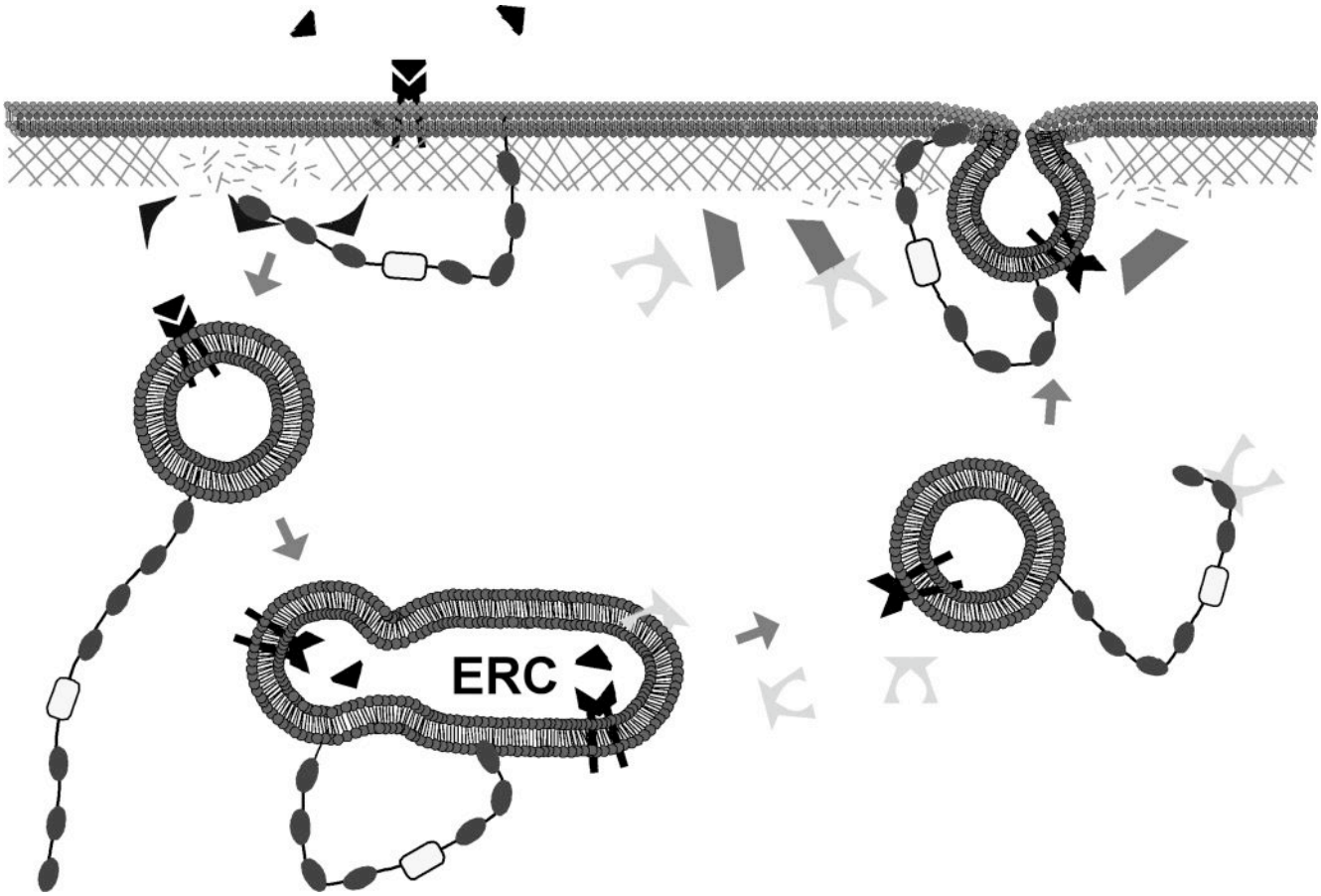

Figure 4.

Model of ferlin-mediated endocytic recycling that contributes to myoblast-myoblast fusion, myoblast-myotube fusion, and membrane damage repair. Ferlin-interacting actinbinding proteins (ferlins – string of beads; AHNAK – boomerang shape) remodel the actin cytoskeleton, allowing endocytosis of ligand-bound receptors. Ferlins mediate fusion of endocytosed vesicles with the endocytic recycling compartment (ERC) where the ligand is removed. EHD proteins (wishbone shape) participate in the scission of recycling vesicles from the ERC. EHD proteins interact with the ferlins for transport back to the plasma membrane. EHD proteins also interact with EHBP1 (trapezoid) for actin cytoskeletal rearrangement, and ferlin proteins assist in fusion of recycling vesicles to the sarcolemma.