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Emerging Functional Differences between the Synaptotagmin and Ferlin Calcium Sensor Families

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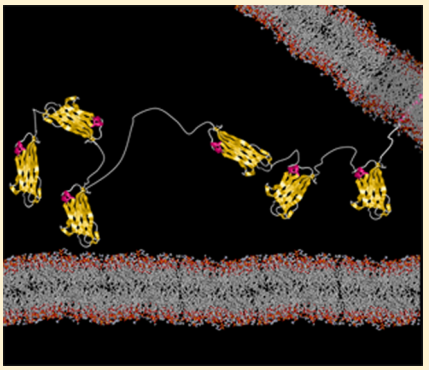
ABSTRACT: The ferlin family proteins have emerged as multi-C2 domain regulators of calcium-triggered membrane fusion and fission events. While initially determined to share many of the features of members of the synaptotagmin family of calcium sensors, ferlins in more recent studies have been found to interact directly with non-neuronal voltage-gated calcium channels and nucleate the assembly of membrane-trafficking protein complexes, functions that distinguish them from the more well studied members of the synaptotagmin family. Here we highlight some of the recent findings that have advanced our understanding of ferlins and their functional differences with the synaptotagmin family.



Calcium-regulated membrane trafficking typically relies on EF hand or C2 domain proteins that couple transient increases in calcium levels to a given process. Among the most well studied of the C2 domain proteins are the members of the synaptotagmin family, which are composed of a transmembrane domain at the N-terminus followed by two closely spaced C2 domains (Figure 1).[1] More than a dozen synaptotagmin genes are encoded in the human genome, which have been implicated in a wide variety of calcium-dependent exocytotic events ranging from presynaptic neurotransmitter release to regulated insulin secretion and lysosome exocytosis.[2,3] Most work on synaptotagmins has focused on synaptotagmin I, the calcium sensor for fast neurotransmitter release at neural synapses, and synaptotagmin 7, which modulates asynchronous neurotransmitter release and lysosomal exocytosis.[1] How these proteins interact with each other and imbibe characteristics to membrane fusion remains an open question, but it is generally accepted that synaptotagmins serve as calcium sensors for exocytosis in neuronal and non-neuronal cells by localizing to the cell membrane and catalyzing SNARE-mediated membrane fusion when their C2 domains bind calcium.[4−8]

More recently, other multi-C2 domain membrane proteins have garnered attention for their apparent activity in calcium sensitive membrane trafficking events. These include the extended synaptotagmins (e-syt), DOC2, the multiple-C2 domain and transmembrane region proteins (mctp), and the ferlins (Figure 1).[9−15] Of these, ferlins have been the focus of considerable interest because of their link to several human pathologies.[16] Ferlins are composed of five to seven C2 domains linked in tandem by long linker regions, with a single-pass transmembrane domain located at the C-terminus (Figure 1).[16] These tail-anchored proteins were first characterized in *Caenorhabditis elegans* where they contribute to calcium-dependent membrane fusion during spermatogenesis.[17] Mam-

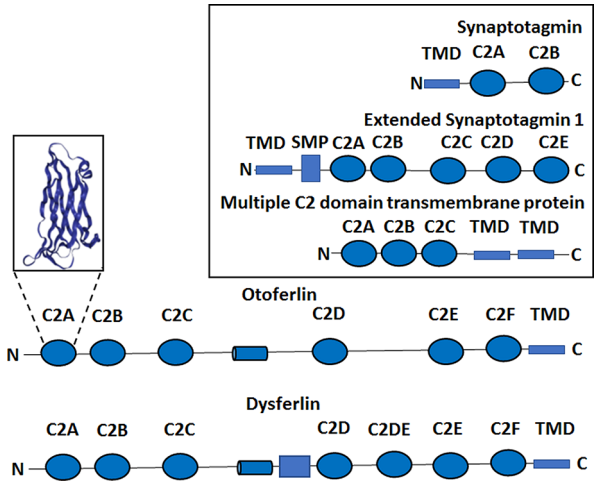


Figure 1. Domain organization for proteins with multiple C2 domains. Structurally, all members of the ferlin family have a single-pass transmembrane domain at the C-terminus (thin rectangle) and between four and seven C2 domains (ovals, denoted C2A−C2F), as shown for representative ferlins otoferlin and dysferlin. A predicted coiled coil domain (cylinder) and dysF domain (thick rectangle) reside between C2C and C2D. The boxed region shows the predicted organization for other proteins with multiple C2 domains. Members of the synaptotagmin family have a single-pass transmembrane domain at the N-terminus (thin rectangle) and two C2 domains (ovals, denoted C2A and C2B). The inset shows the structure of otoferlin C2A (Protein Data Bank entry 3L9B).

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mals harbor six ferlin genes as a result of gene duplication events: otoferlin, dysferlin, myoferlin, Fer1L4, Fer1L5, and Fer1L6.[18] Interestingly, studies of mouse knockout models and human patients afflicted with deafness or muscular dystrophy have concluded that ferlins serve nonredundant physiological functions. For example, otoferlin is found predominantly in hair cells of the inner ear where it regulates the release of the neurotransmitter from the sensory cells during the encoding of sound.[19] By contrast, dysferlin is expressed most prominently in striated muscle and appears to function in calcium signaling and repair of the sarcolemma.[11,20] Unlike otoferlin and dysferlin, myoferlin has been linked to VEGF signaling and tumor growth.[21−23] Due to the presence of C2 domains and their involvement in calcium sensitive cellular processes, it was initially thought that ferlins act as calcium sensors for membrane fusion, not unlike the role played by the synaptotagmins. This has raised questions about the degree of functional overlap between ferlins and synaptotagmins. However, a growing list of functional differences suggest these families fulfill different physiological roles. Below we highlight recent studies of otoferlin and dysferlin that have shed light on the differences between ferlins and synaptotagmins and suggest a common set of functions for ferlins.

OTOFERLIN, SYNAPTOTAGMINS, AND DEAFNESS

Otoferlin is detected predominantly at the presynapse of sensory hair cells that convert sound into neurotransmitter release.[24] Loss of otoferlin activity is associated with a form of recessive profound hearing loss in humans (DFNB9), as well as hearing and balance defects in zebrafish.[19,25−27] Studies of mice have determined that while immature sensory hair cells express synaptotagmin I, the protein does not appear to regulate exocytosis.[28] In mature sound-encoding hair cells, synaptotagmin I and II are not detected, suggesting that these genes are not expressed.[28] In addition, knockout of synaptotagmin I, II, and VII does not affect neurotransmitter release in sensory hair cells. Together, these studies indicate that calcium-regulated exocytosis in hair cells is not mediated by synaptotagmins.[28] This stands in stark contrast to the loss of synaptotagmin I in neurons, which completely abrogates fast exocytosis.[29] Complete loss of neurotransmitter release from hair cells does occur in otoferlin knockout mice, however, despite the accumulation of synaptic vesicles at the presynapse.[24] This finding, in combination with the aforementioned results of studies of *C. elegans*, has led to the proposal that otoferlin functionally replaced synaptotagmin as the calcium sensor for membrane fusion and exocytosis in hair cells. Indeed, the results of recombinant protein measurements determined that five of the six C2 domains of otoferlin bind calcium and membranes and that the C2C and C2F domains interact with PIP2.[13,30,31] Additionally, otoferlin can stimulate liposome fusion in an *in vitro* reconstituted membrane fusion assay.[30] However, electrophysiology studies found that synaptotagmin I could not rescue the otoferlin phenotype, nor could otoferlin rescue the synaptotagmin knockout phenotype. Currently, the reasoning for the distinction between these proteins remains an open question.[32]

DYSFERLIN, SYNAPTOTAGMIN, AND MUSCULAR DYSTROPHY

Unlike otoferlin, dysferlin is expressed predominantly in striated muscle, and loss of dysferlin has been directly linked to limb-girdle muscular dystrophy, Miyoshi myopathy, and cardiomyopathy (collectively known as dysferlinopathies) in part due to the failure to reseal tears in the sarcolemma (muscle cell membrane).[11,33,34] While it is now thought that dysferlin acts as a calcium binding protein directly linked to sarcolemma repair, initial studies had implicated the ubiquitously expressed synaptotagmin 7 as the calcium sensor for repair via regulation of syntaxin 4-mediated lysosomal fusion with the damaged membrane.[35,36] Support for synaptotagmin 7 function in membrane repair came from mouse knockout studies that reported membrane healing deficiencies and an overall phenotype reminiscent of that of muscular dystrophy.[37] However, knockout mice also displayed inflammation, suggestive of autoimmune disease, which has complicated interpretation of the role of synaptotagmin 7 in wound repair.[37] On the basis of human patient and mouse knockout studies, dysferlin has also been linked to membrane repair.[33] Like synaptotagmin 7, dysferlin deficient muscle shows defects in calcium sensitive membrane repair, and recombinant protein studies have determined that the C2 domains of dysferlin bind calcium and negatively charged lipid membranes.[11,38] In addition, results of recent reconstituted membrane fusion studies indicate that dysferlin can promote fusion between liposomes harboring syntaxin 4 in a calcium sensitive manner.[38] Thus, as is the case for otoferlin, the differences in function between dysferlin and synaptotagmin in calcium sensitive membrane trafficking are unclear.

NEW INSIGHT INTO FERLIN FUNCTION

So, what distinguishes synaptotagmins from ferlin proteins, and are there a shared set of functions among the ferlins? Several recent studies of otoferlin and dysferlin have implicated a set of conserved activities that may distinguish ferlins from synaptotagmins. These activities can be broadly categorized into three functions, which involve multiple regions along the length of the ferlin protein.

Ferlins as Vesicle Priming Factors. First, otoferlin and dysferlin may directly contribute to vesicle docking and priming. In the case of otoferlin, analysis of electrophysiology measurements of a missense mutation in the C-terminal C2 domain of the protein found that the rate of conversion of synaptic vesicles into a fusion-competent form was attenuated, while the total number of docked synaptic vesicles and the kinetics of membrane fusion were unaffected.[39] The authors of this study suggested that this mutation may result in a defect in the vesicle priming step upstream of exocytosis (Figure 2). In neurons, docking and priming are not typically associated with synaptotagmin, but rather with CAPS, Munc13, and Munc18 proteins.[40] However, in sensory hair cells, loss of CAPS or Munc13 has no apparent effect on exocytosis, indicating that vesicle priming may occur without Munc13 or CAPS.[41] Thus, it may be that otoferlin fulfills both synaptic vesicle priming and membrane fusion steps in hair cells. Alternatively, other as-yet unidentified protein(s) may act as priming factors in hair cells, either independently or in concert with otoferlin.

Dysferlin has also been implicated in vesicle docking and priming. Quantitative analysis has revealed a 30% reduction in the number of membrane-proximal fusion-ready lysosomes in myoblasts lacking dysferlin, and this reduction was reported to account for the abnormal membrane repair observed in these cells.[42] Re-expression of dysferlin rescued the number of lysosomes tethered to the membrane and membrane repair activity, consistent with a dysferlin-mediated docking/priming event.

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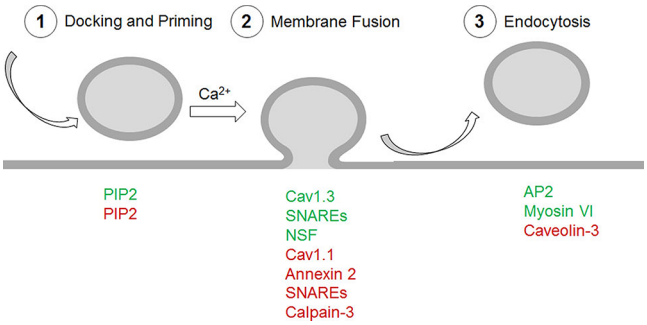

Figure 2. Contribution of otoferlin and dysferlin to the vesicle cycle and associated binding partners. Three stages of the vesicle cycle are depicted, with binding partners for otoferlin (green) and dysferlin (red) depicted for each stage. Vesicle docking/priming (stage 1) via otoferlin and dysferlin may be partly mediated by PIP2 lipids residing at the cell membrane. Ferlin-mediated membrane fusion (stage 2) is regulated by the influx of calcium through Cav1.1 or Cav1.3 channels and is associated with a variety of additional proteins that form an exocytotic complex. Postfusion (stage 3), otoferlin is endocytosed via an adaptor protein 2 (AP2) pathway, while interaction of dysferlin with caveolin-3 influences retention or endocytosis from the membrane.

Ferlins as L-Type Calcium Channel Binding Proteins. Second, there is a growing body of evidence that both dysferlin and otoferlin interact with L-type voltage-gated calcium channels. Specifically, yeast two-hybrid screens and surface plasmon resonance measurements support a direct association between a cytoplasmic loop (loop II–III) of Cav1.3 and the C2 domains of otoferlin.[43] In addition, a single-molecule fluorescence co-localization microscopy study reported that otoferlin, but not synaptotagmin I, bound directly to Cav1.3.[44] Otoferlin co-localizes with Cav1.3 in sensory hair cells, and this interaction could serve to place the calcium sensor for exocytosis proximal to the channel that gates the entry of calcium into the cell. This tight coupling between otoferlin and Cav1.3 would be necessary for exocytosis in sync with the transient changes in calcium concentrations during the encoding of sound. Although synaptotagmin I was incapable of interacting with Cav1.3, the authors of this study reported that it did interact with the loop II–III region of N-type calcium channel Cav2.2, commonly found in neurons.[44,45] This distinction is noteworthy in that it may be one reason why synaptotagmin cannot rescue the otoferlin knockout phenotype. In addition to binding, the presence or absence of otoferlin alters the ratio of Cav1.3 splice isoforms with fast and slow inactivation, which in turn effects the calcium dynamics of the sensory hair cell.[46] Thus, in addition to serving as a tethering point, interaction between otoferlin and Cav1.3 may influence calcium flux through the hair cell membrane.

Like otoferlin, dysferlin has also been linked to an L-type calcium channel, namely Cav1.1 (DHPR). Dysferlin co-immunoprecipitates with Cav1.1 and co-localizes to the muscle transverse tubule where Cav1.1 resides.[47,48] Strikingly, loss of dysferlin activity has been reported to result in abnormalities in both calcium channel activity and calcium dynamics in cells, which may be a factor contributing to dysferlinopathies.[49] In summary, ferlins appear to both directly bind and influence L-type calcium channels.

Ferlins as Multivalent Scaffolding Proteins. Third, both dysferlin and otoferlin act as multivalent scaffolding proteins capable of nucleating the assembly of membrane-trafficking complexes. This stands in contrast to synaptotagmins, which are not commonly thought to be necessary for the assembly of membrane-trafficking complexes in neurons. Because synaptotagmins are smaller and have fewer C2 domains, they may not be capable of the large and varied number of binding partners with which ferlins interact. For example, immunoprecipitation, GST pull-down, and reconstitution experiments have linked otoferlin to many members of both endo- and exocytotic complexes, including AP2, Cav1.3, SNAREs, NSF, myosin VI, and rab 8b.[30,43,44,50−53] It is worth noting that using a three-color single-molecule fluorescence technique (termed smCoBRA), otoferlin was determined to interact with five Cav1.3 channels and five SNARE proteins simultaneously, indicating that otoferlin can act as a multivalent recruitment factor capable of tethering multiple exocytotic proteins.[44] The stoichiometry was reported to be sensitive to calcium, suggesting that the number and type of binding partners may be coupled to the influx of calcium into the cell. Interestingly, an in vivo study using zebrafish found that truncated forms of otoferlin could rescue the phenotype associated with knockdown of endogenous otoferlin, suggesting that the domains within the protein may be partially redundant with respect to binding partners and function.[23]

Dysferlin has also been reported to interact with SNAREs, as well as Cav1.1, suggesting a similar role in assembling membrane fusion proteins and calcium channels into a complex.[38,48] However, dysferlin also recruits MG53 and caveolin-3 and directly interacts with annexins A1 and A2 in a calcium sensitive manner.[54−57] In zebrafish, dysferlin recruits the annexins in a temporally precise manner during the membrane repair process, indicating that dysferlin controls the order of assembly of wound healing proteins at sites of muscle injury.[54]

■ FUTURE DIRECTIONS AND OUTLOOK

It is now apparent that while both ferlins and synaptotagmins play a role in calcium-regulated membrane trafficking events, distinctions between the capabilities of the two families are now becoming evident. Less well understood are the mechanisms underlying ferlin function.

Chief among the shortcomings in our mechanistic understanding are how ferlins mediate the priming and fusion processes and if the mechanism shares common principles with neuronal presynaptic exocytosis. While a common assumption among proposed mechanistic models of ferlin function evokes the full-length protein, there is evidence that calpain may proteolytically cleave certain splice variants of dysferlin in a calcium-dependent manner, yielding a truncated mini-ferlin that participates in the membrane repair process.[58] This shortened variant of dysferlin is composed of the last two C-terminal C2 domains and the transmembrane domain that mimics the layout of the synaptotagmins. Interestingly, a truncated otoferlin lacking all but the last 612 amino acids was found to be sufficient to rescue balance and hearing in zebrafish depleted of endogenous otoferlin. This shortened form encoded the transmembrane and last two C2 domains and may mimic the calpain cleavage product.[23] However, although the calpain cleavage site is conserved in certain exons of all ferlins, it remains to be determined whether calpain plays an active role in modulating ferlin activity.

Finally, while much emphasis has focused on the role of ferlins in calcium-regulated exo- and endocytosis, there is reason to believe ferlins also modulate enzymatic activity. For instance, several studies have linked dysferlin to histone deacetylase (HDAC) either through direct interaction or as part of a three-protein complex with fam65b.[59,60] Interaction between dysferlin

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and HDAC has been implicated in modulating the acetylation of α-tubulin. In turn, otoferlin has been reported to interact with glutamate decarboxylase, an enzyme responsible for converting glutamate to the neurotransmitter GABA.[61] Future studies are needed to elucidate the role, if any, of otoferlin in the biosynthesis of neurotransmitters. That ferlins may play a role in enzymatic activity and post-translational modification represents yet another area that could distinguish ferlins from synaptotagmins.

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ABBREVIATIONS

SNARE, soluble NSF attachment protein receptor *N*-ethylmaleimide sensitive fusion proteins; AP2, adapter protein 2; CAV, voltage-gated calcium channel; DHPR, dihydropyridine receptor; PIP2, phosphatidylinositol 4,5-bisphosphate.

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