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# Dysferlin is essential for endocytosis in the sea star oocyte

Nathalie Oulhen, Thomas M. Onorato [1], Isabela Ramos [2], Gary M. Wessel *

*Department of Molecular and Cell Biology and Biochemistry, Brown University, Providence RI 02912, USA*

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ABSTRACT

Dysferlin is a calcium-binding transmembrane protein involved in membrane fusion and membrane repair. In humans, mutations in the dysferlin gene are associated with muscular dystrophy. In this study, we isolated plasma membrane-enriched fractions from full-grown immature oocytes of the sea star, and identified dysferlin by mass spectrometry analysis. The full-length dysferlin sequence is highly conserved between human and the sea star. We learned that in the sea star *Patiria miniata*, *dysferlin* RNA and protein are expressed from oogenesis to gastrulation. Interestingly, the protein is highly enriched in the plasma membrane of oocytes. Injection of a morpholino against dysferlin leads to a decrease of endocytosis in oocytes, and to a developmental arrest during gastrulation. These results suggest that dysferlin is critical for normal endocytosis during oogenesis and for embryogenesis in the sea star and that this animal may be a useful model for studying the relationship of dysferlin structure as it relates to its function.



## Introduction

Dysferlin is a transmembrane protein associated with rare forms of muscular dystrophies, such as limb-girdle muscular dystrophy type 2B (Bashir et al., 1998), Miyoshi myopathy (Liu et al., 1998), and distal myopathy (Illa et al., 2001). Loss of dysferlin from the plasma membrane of muscle fibers leads to abnormalities in vesicular trafficking and membrane repair (Kobayashi et al., 2012). Moreover, freshly isolated monocytes of dysferlinopathy patients show deregulated expression of fibronectin and fibronectin-binding integrins which is recapitulated by transient knockdown of dysferlin in the human monocytic cell line, THP1. Dysferlin regulates cellular interactions by forming a complex with integrins at the cell membrane (de Morree et al., 2013). In humans, the *dysferlin* transcript is highly expressed in skeletal muscle, heart, placenta, and more weakly in liver, lung, kidney and pancreas (Bashir et al., 1998). More recently, it was also found in cultured vascular endothelial cells (Leung et al., 2011; Sharma et al., 2010). Dysferlin is a member of the ferlin family of proteins, based on the similarity to the *C. elegans* protein Fer-1 (Fertilization defective-1). In *C. elegans*, the full-length dysferlin homolog Fer-1 (230 kDa) is present early in spermatogenesis and becomes less abundant in spermatids, perhaps because it is proteolytically processed into smaller isoforms (195 and 180 kDa) (Washington and Ward, 2006). Fer-1 is required for the fusion of specialized vesicles, called membranous organelles, with the plasma membrane during spermatogenesis (Achanzar and Ward, 1997; Washington and Ward, 2006).

Two other ferlin proteins have been identified in humans, myoferlin and otoferlin. Myoferlin was first considered as a muscle specific protein and its deletion results in impaired mouse myoblast fusion into mature skeletal myotubes (Doherty et al., 2005). However, myoferlin has been shown to be critical for endocytosis in endothelial cells (Bernatchez et al., 2009). Myoferlin is also abundantly expressed in invasive breast tumor cells and remarkably, its depletion stalls the invasion of these cells (Li et al., 2012). Otoferlin is essential for hearing and pathogenic mutations are associated with nonsynchronic autosomal recessive deafness (Yasunaga et al., 1999). Otoferlin is required in the priming and fusion of synaptic vesicles during sound encoding, which occurs at synapses between cochlear inner hair cells and the auditory nerve (Pangrsic et al., 2012).

Ferlin proteins function in various developmental and reproductive processes in several organisms. While dysferlin and myoferlin are co-expressed in the human placenta, only dysferlin expression is positively correlated to cell fusion in trophoblastic cells (Robinson et al., 2009). In *Drosophila melanogaster*, only one ferlin gene, called *misfire* (*mfr*), has been found. The transcript is expressed in the testis and ovaries of adult flies. In males, mfr expression is required for efficient breakdown of the sperm plasma membrane and completion of sperm activation during fertilization. In females, it is required for egg patterning by limiting the spread of proteins such as Gurken. Mutations in misfire delay embryogenesis (Ohsako et al., 2003; Smith and Wakimoto, 2007).

* Correspondence to: 185 Meeting Street, Box G, Brown University, Providence, RI 02912, USA.
*E-mail address:* rhet@brown.edu (G.M. Wessel).
[1] Current address: Department of Natural Sciences, LaGuardia Community College, Long Island City, NY 11101.
[2] Current address: Instituto de Bioquímica Médica, Universidade Federal do Rio de Janeiro, RJ 21940-590, Brazil.

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In zebrafish, the *dysferlin* transcript is first detected at 11 h post-fertilization, remains detectable until 96 h, and is expressed in different tissues including muscle, brain and eye (Kawahara et al., 2011). Reduction of dysferlin expression in this animal by morpholino injection causes abnormal formation of muscle structures (Kawahara et al., 2011). The *dysferlin* gene is also present in the sea urchin genome (Sodergren et al., 2006) and this animal was used to test the role of dysferlin in membrane healing (Covian-Nares et al., 2010). Laser wounding of one cell in a 4-cell stage embryo triggered calcium spikes in neighboring cells through voltage-gated calcium channels whereas embryos injected with a dysferlin morpholino lost their calcium spike activity in neighboring cells after wounding. The authors conclude that the dysferlin-depleted embryos have lost an ability to receive a signal, possibly a nucleotide, released by the wounded cell.

Here, we found that dysferlin is enriched in the plasma membranes of oocytes and embryos from the sea star, an animal with oocytes and embryos tractable for studying cellular dynamics (Fresques et al., in press; Hinman et al., 2003a; Wessel et al., 2010). Sea stars are invertebrate deuterostomes, a sister group to chordates. Oocytes, eggs and embryos of the species used here, *Patiria miniata*, are optically clear, enabling excellent observations of development. Developing oocytes are plentiful, manipulatable, are arrested in prophase of first meiotic division and meiotic reinitiation is induced by the hormone 1-methyladenine (Kanatani et al., 1969). After fertilization, embryos develop quickly, reach the blastula stage in one day, and gastrulate in two days. In this study, we show that in the starfish *Patiria miniata* (*Pm*), knockdown of the dysferlin protein results in reduced endocytosis during oogenesis and significant developmental defects in embryos.

## Materials and methods

### *Animals*

*P. miniata* and *A. forbesi* were housed in aquaria with artificial seawater (ASW) at 16 °C (Coral Life Scientific Grade Marine Salt; Carson, CA). Gametes were acquired by opening up the animals. Oocytes were collected in filtered seawater and sperm was collected dry. To obtain mature oocytes, the full-grown immature oocytes were incubated for an hour in filtered sea water containing 2 μM 1-methyladenine. After addition of sperm, fertilized eggs were cultured in filtered seawater at 16 °C (Foltz et al., 2004; Wessel et al., 2010).

### *Isolation of plasma membrane-enriched fractions*

Full-grown immature or mature oocytes were isolated in Calcium-Free Sea Water (CFSW), resuspended in buffer A (Sucrose 0.36 M, 50 mM Tris pH8, 5 mM EDTA) and homogenized on ice by five up and down strokes of a loose-fitting glass pestle in a 40 mL glass dounce tissue grinder. Following a 15-min incubation on ice, the sample was homogenized with an additional five strokes and the homogenate was then incubated at 4 °C overnight to settle the plasma membrane-enriched fraction (PMEF) by gravity. This fraction was washed three times with buffer A and the membranes were collected for analysis, or pelleted in a microfuge at 14 000 g for 5 min. For SDS-PAGE, the pellet was resuspended in Laemmli Sample Buffer (LSB) containing 5 mM DTT, and the samples were boiled for 5 min at 100 °C.

### *Rhodamine-B-Isothiocynate (RITC) labeling of oocyte surface proteins*

Full-grown immature oocytes were isolated in CFSW and all labeling steps were performed in CFSW. Oocytes were pelleted by gentle centrifugation and rotated at 16 °C for about 90 min in a 15 ml conical centrifuge tube containing RITC at 0.5 μg/ml in CFSW. Oocytes were then rinsed with filtered SW from the Marine Biological Laboratories (MBL; Woods Hole, MA) three times. An aliquot (200 μl) of RITC-labeled whole oocytes was pelleted and suspended in 200 μl of 2 × LSB containing 5 mM DTT for SDS-PAGE analysis. PMEFs were isolated from these labeled oocytes using the protocol described above. Images were taken with a fluorescence dissecting stereoscope (Olympus SZX16) connected to an Olympus X cite 120 lamp and to a camera (Nikon D90), managed by Pro 2 software. Samples were electrophoresed through a 4%–12% SDS-PAGE, stained with Coomassie Brilliant Blue for 1 h and the RITC signal was detected using a Typhoon Imager (GE Healthcare, Typhoon 9410).

### *Mass spectrometry and protein analysis*

PMEF was loaded on a SDS-PAGE for Coomassie Brilliant Blue staining. The two main high molecular weight proteins were obtained and processed for *in gel* digestion using the *In gel* tryptic digestion kit (Pierce). Proteins were digested overnight at 37 °C in presence of 10 ng/μl trypsin. Samples were analyzed using a Thermo-Finnigan LTQ linear ion trap mass spectrometer. Tryptic peptides were fractionated on a reversed phase column and introduced directly onto an LTQ mass spectrometer via electrospray ionization. *Pm*-dysferlin was identified via database matching with the program SEQUEST (Eng et al., 1994), using the *Pm* ovary transcriptome (Adrian Reich and Gary Wessel, unpublished data) which has been entered at NCBI (KJ081282).

EMBOSS Needle was used to obtain protein identities and similarities between the species (EMBL-EBI). SOSUI was used to identify the transmembrane domains (Mitaku et al., 2002). Conserved Domain Database (CDD) was used to identify the protein domains (Marchler-Bauer et al., 2011). The DysF domains of the human dysferlin protein were also defined according to a previous analysis (Lek et al., 2010). YinOYang (Gupta and Brunak, 2002), NetPhos (Blom et al., 1999), Sulfinator (Monigatti et al., 2002), and ProP (Duckert et al., 2004) were used to analyze the potential post-translational modifications of the proteins.

### *Antibody production*

Rabbit polyclonal antibody against the peptide sequence ALPRPKFSDSTGKI of dysferlin from *P. miniata* was custom developed and affinity purified by GenScript (Piscataway, NJ). This peptide, specific to dysferlin, is located in the DysFN domain (amino-acids 889–902).

### *Western blot*

Western blot analysis was performed following electrophoretic transfer of proteins from SDS-PAGE onto 0.22 μm nitrocellulose membranes (Towbin et al., 1979). Membranes were incubated with antibodies directed against *Pm*-dysferlin (1/5000) in 20 mM Tris–HCl (pH7.6), 3% BSA, and 0.1% Tween-20, overnight at 4 °C. The antigen–antibody complex was measured by chemiluminescence using horseradish peroxidase-coupled secondary antibodies according to the manufacturer's instruction (ECL; GE Healthcare Biosciences, Pittsburgh, PA, USA).

### *Real-time quantitative PCR (QPCR)*

RNA was extracted from the different developmental stages using the RNeasy Micro Kit (Qiagen; Valencia, CA). cDNA was prepared using the TaqMan® Reverse Transcription Reagents kit (Applied Biosystems; Foster City, CA). QPCR was performed on a 7300 Real-Time PCR system (Applied Biosystems; Foster City, CA) with the FastStart Universal