The American Journal of Pathology, Vol. 184, No. 1, January 2014

ELSEVIER

The American Journal of
PATHOLOGY
ajp.amjpathol.org

MOLECULAR PATHOGENESIS OF GENETIC AND INHERITED DISEASES

# Dysferlin and Myoferlin Regulate Transverse Tubule Formation and Glycerol Sensitivity

Alexis R. Demonbreun,* Ann E. Rossi,* Manuel G. Alvarez,† Kaitlin E. Swanson,† H. Kieran Deveaux,* Judy U. Earley,* Michele Hadhazy,* Ravneet Vohra,‡ Glenn A. Walter,‡ Peter Pytel,† and Elizabeth M. McNally*§

*From the Departments of Medicine,* Pathology,† and Human Genetics,§ The University of Chicago, Chicago, Illinois; and the Department of Physiology and Functional Genomics,‡ University of Florida, Gainesville, Florida*

Accepted for publication September 16, 2013.

Address correspondence to Elizabeth M. McNally, M.D., Ph.D., The University of Chicago, 5841 S Maryland, MC6088, Chicago, IL 60637. E-mail: emcnally@uchicago.edu.

Dysferlin is a membrane-associated protein implicated in muscular dystrophy and vesicle movement and function in muscles. The precise role of dysferlin has been debated, partly because of the mild phenotype in dysferlin-null mice (*Dysf*). We bred *Dysf* mice to mice lacking myoferlin (*MKO*) to generate mice lacking both myoferlin and dysferlin (FER). FER animals displayed progressive muscle damage with myofiber necrosis, internalized nuclei, and, at older ages, chronic remodeling and increasing creatine kinase levels. These changes were most prominent in proximal limb and trunk muscles and were more severe than in *Dysf* mice. Consistently, FER animals had reduced ad libitum activity. Ultrastructural studies uncovered progressive dilation of the sarcoplasmic reticulum and ectopic and misaligned transverse tubules in FER skeletal muscle. FER muscle, and *Dysf*- and *MKO*-null muscle, exuded lipid, and serum glycerol levels were elevated in FER and *Dysf* mice. Glycerol injection into muscle is known to induce myopathy, and glycerol exposure promotes detachment of transverse tubules from the sarcoplasmic reticulum. Dysf, *MKO*, and FER muscles were highly susceptible to glycerol exposure *in vitro*, demonstrating a dysfunctional sarcotubule system, and *in vivo* glycerol exposure induced severe muscular dystrophy, especially in FER muscle. Together, these findings demonstrate the importance of dysferlin and myoferlin for transverse tubule function and in the genesis of muscular dystrophy. *(Am J Pathol 2014, 184: 248–259; http://dx.doi.org/10.1016/j.ajpath.2013.09.009)*

The muscular dystrophies are a heterogeneous group of genetic disorders characterized by progressive muscle loss and weakness. The mechanisms that underlie muscular dystrophy are diverse, including defective regeneration, plasma membrane instability, and defective membrane repair. Dysferlin (DYSF) has been implicated in all of these processes.[1,2] Autosomal recessive loss-of-function mutations in dysferlin cause three different forms of muscular dystrophy: limb-girdle muscular dystrophy type 2B, Miyoshi myopathy, and distal anterior compartment myopathy.[3–5] Mutations in dysferlin become clinically evident in the second to third decade or later, with muscle weakness. An early characteristic feature of dysferlin mutations is massively elevated serum creatine kinase levels. A spectrum of myopathic changes can be seen in muscle biopsy specimens from humans with dysferlin mutations, including dystrophic features, such as fibrofatty replacement and inflammatory infiltrates.

Dysferlin is a 230-kDa membrane-inserted protein that contains at least six cytoplasmic C2 domains. C2 domains mediate protein-protein interactions and, in some cases, directly bind phospholipids and calcium. The C2 domains of dysferlin are highly related to those found in the membrane trafficking and fusion protein synaptotagmins.[6] Dysferlin is highly expressed in adult skeletal muscle, whereas it is expressed at lower levels in muscle precursor cells, myoblasts.[1,7,8] On sarcolemma damage, dysferlin is found at the sites of membrane disruption and has been specifically implicated in resealing the sarcolemma.[2] Electron microscopy of skeletal muscle biopsy specimens from human dysferlin-mutant patients confirms discontinuity of the

Supported by the NIH through the National Institute of Neurological Diseases and Stroke grant R01 NS047726, the National Institute for Arthritis and Musculoskeletal and Skin Diseases grant U54 AR052646, and the Muscular Dystrophy Association (E.M.M.).


http://dx.doi.org/10.1016/j.ajpath.2013.09.009

sarcolemma and reveals vesicles underneath the basal lamina, suggesting dysferlin plays an active role in vesicle fusion at the membrane lesion.[9] Dysferlin also has been shown to interact with a variety of cytosolic and membrane-associated binding partners, including MG53, caveolin-3, AHNAK, and annexins A1 and A2.[10–13] Similar to dysferlin, MG53, caveolin-3, and the annexins have been implicated in membrane resealing, suggesting a large complex may act coordinately to seal the disrupted plasma membrane in a calcium-dependent manner.[13,14]

An increasing body of evidence suggests that dysferlin's membrane-associated roles are not restricted to the sarcolemma. Dysferlin has been implicated in the development and maintenance of the transverse (T-) tubule, a muscle-specific membrane system essential for electromechanical coupling. The T-tubule is a membrane inversion of the sarcolemma that flanks the Z band of muscle, the anchor for sarcomeric proteins. Dysferlin associates with the T-tubule–like system in differentiated C2C12 myotubes,[15] and dysferlin-null mouse muscle contains malformed T-tubules consistent with a role for dysferlin in the biogenesis and maintenance of the T-tubule system.[16] In mature muscle damaged by stretch, dysferlin localizes to T-tubules, suggesting a reparative function for dysferlin at the T-tubule.[17]

Dysferlin belongs to a family of proteins, the ferlins, that contains six family members. Myoferlin is a dysferlin homologue, which is 76% identical at the amino acid level.[18] Such as dysferlin, myoferlin also contains at least six calcium-sensitive C2 domains, a carboxy-terminal transmembrane domain, an Fer domain, and a DysF domain.[17,19] Myoferlin is highly expressed in myoblasts and is markedly up-regulated in adult skeletal muscle on muscle damage.[20] Myoferlin, such as dysferlin, is required for normal myoblast fusion and muscle growth through regulating steps of vesicle trafficking and endocytic recycling.[1,20,21] Myoferlin, such as dysferlin, is required for the proper trafficking of and response to the insulin-like growth factor-1 receptor in muscle.[22] Myoferlin interacts with endocytic recycling proteins EHD1 and EHD2, as well as AHNAK.[21,23,24] To date, no human forms of muscular dystrophy resulting from myoferlin mutations have been reported. However, mice lacking myoferlin show defects in muscle regeneration, establishing a role for myoferlin in muscle repair.[20]

We generated ferlin (FER) mice that carry both the dysferlin- and myoferlin-null loss of function mutations. We determined that FER mice have a more severe muscular dystrophy than dysferlin-null mice. In addition, FER muscle displays disorganization of the T-tubule system, dilated sarcoplasmic reticulum, and increased levels of serum glycerol. We revealed an enhanced sensitivity of *Dysf*, *MKO*, and especially FER myofibers to glycerol exposure, resulting in T-tubule vacuolation and disrupted membrane potential. Intramuscular glycerol injections into young FER muscle recapitulated the dystrophic phenotype characteristic of old FER muscle. Our data establish a role for both myoferlin and dysferlin in the biogenesis and remodeling of the sarcotubule system and suggest glycerol as a mediator of muscular dystrophy in dysferlin mutations.

## Materials and Methods

### Generation of FER Mice

The naturally occurring dysferlin-null mice from the A/J strain were backcrossed for six generations to the 129/SV emst/J myoferlin mouse line, to generate FER mice with both the *Dysf* and *MKO* alleles.[20,25] Mice were housed in a specific pathogen-free facility in accordance with the University of Chicago (Chicago, IL) Institutional Animal Care and Use Committee regulations.

### Muscle Fiber Analysis

Muscles from 24-week-old mice were dissected and frozen in liquid nitrogen–cooled isopentane. Muscle sections were stained with H&E or anti-dystrophin (Ab15277; Abcam, Cambridge, MA), diluted 1:200. By using ImageJ (NIH, Bethesda, MD) particle analysis, the mean area was determined from >275 fibers from at least five fields from three different animals per genotype. The percentage of fibers with central nuclei was calculated from the number of fibers containing internalized nuclei in each image/the total number of fibers counted per image, standardized as a percentage. At least 2000 fibers per genotype were analyzed ($n = 3$ from each genotype). Statistical analysis was performed using Prism version 4 (Graphpad, La Jolla, CA). Images were captured using a Zeiss Axiophot microscope.

### Muscle Analysis

Quadricep, tricep, abdominal, paraspinal, gluteus/hamstring, and gastrocnemius/soleus muscles from age-matched, wild-type (WT), *Dysf*, mice lacking myoferlin (*MKO*), and FER animals were dissected and frozen in liquid nitrogen–cooled isopentane ($n \geq 3$ animals per genotype per age). Muscle sections were stained with H&E. Images were captured using a Zeiss Axiophot microscope.

### Creatine Kinase and Metabolite Assays

Serum was collected from age-matched, WT, *Dysf,* MKO, and FER animals from eye bleeds using heparinized capillary tubes (Fisher, Pittsburgh, PA) into serum separator tubes (Becton Dickinson, Franklin Lakes, NJ) and centrifuged for 10 minutes at 8000 × *g*. The plasma fractions were frozen and stored at −80°C and then assayed later using the EnzyChrom CK Assay kit (ECPK-100; BioAssay Systems, Hayward, CA). Serum glycerol was determined with the Cayman Chemical Assay kits (number 10010755; Cayman Chemical, Ann Arbor, MI). Activity was measured in the FluoStar Optima plate reader (BMG Labtech, Cary, NC).