DYSFERLIN'S C2A DOMAIN, Ca^{2+} BINDING AND PKCα SUPPRESS RYR1-MEDIATED Ca^{2+} LEAK INTO THE TRIAD JUNCTION

Robert J. Bloch, Valeriy Lukyanenko, Joaquin Muriel, Daniel Garman

Department of Pharmacology and Physiology, School of Medicine, University of Maryland Baltimore

ABSTRACT

Dysferlin is an integral membrane protein of the triad junction (TJ). It absence in skeletal muscle leads to Limb Girdle Muscular Dystrophy R2, an autosomal recessive disease. Our studies of dysferlin-null mouse myofibers *in vitro* show the following. (i) Mild injury by hypoosmotic shock decreases the amplitude of the voltage-induced Ca^{2+} transient and generates Ca^{2+} waves via Ca^{2+}-induced Ca^{2+} release (CICR). (ii) These effects are suppressed by drugs that stabilize the DHPR-RyR1 couplon or inhibit RyR1 Ca^{2+} leak. (iii) They are also suppressed by low concentrations (10nM) of BAPTA-AM in the bathing medium; (iv) BAPTA-AM also increases the voltage-induced Ca^{2+} transient amplitude in dysferlin-null fibers, bringing them to control levels. (v) Dysferlin's C2A domain binds Ca^{2+}. A chimera in which C2A is replaced with GCaMP6fu, which binds Ca^{2+} rapidly, targets TJs normally and suppresses abnormal Ca^{2+} signaling. (vi) Expression of dysferlin's C2A domain via transfection also rescues Ca^{2+} signaling. These results suggest that dysferlin -- specifically its C2A domain -- stabilizes DHPR -RyR1 coupling to suppress Ca^{2+} leak and CICR, which is likely pathogenic.

We also found a role for PKCα. (vii) PKCα concentrates at TJs. (viii) Dysferlin and PKCα co-immunoprecipitate. (ix) Targeting dysferlin's C2A domain to TJs as a chimera with the C2 domain of PKCα increases its ability to rescue Ca^{2+} signaling. (x) Activation of PKCα with PMA suppresses abnormal Ca^{2+} signaling in injured dysferlin-null fibers. (xi) Inhibition of PKCα with Gö6976 induces abnormal Ca^{2+} signaling, including Ca^{2+} waves, in injured wild type fibers.

These results suggest that dysferlin stabilizes DHPR-RyR1 coupling and suppresses Ca^{2+} leak by facilitating targeted phosphorylation by PKCα, but that activation of PKCα can compensate for dysferlin's absence to shut down RyR1-mediated leak.

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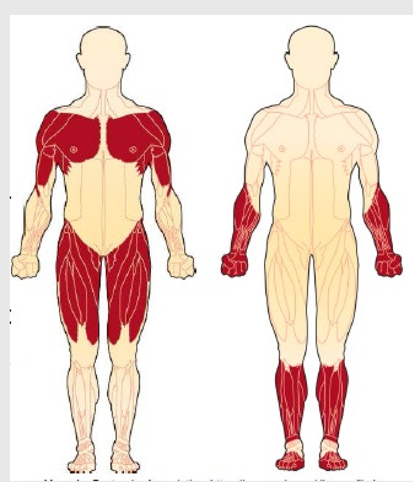
Muscular Dystrophies Linked to Dysferlin: Limb Girdle Muscular Dystrophy Type R2



Limb Girdle Musc. Dystrophy Type 2B (LGMD2B)

Miyoshi Myopathy (MMD1)

Earlier called MMD1 (distal), LGMD2B (proximal), or distal myopathy of the anterior tibialis (DMAT), but differences minimal; now called LGMD R2

Autosomal recessive

Encoded on chromosome 2p13.2

Gene symbol: *DYSF*

Prevalence: ~1:100,000 or lower

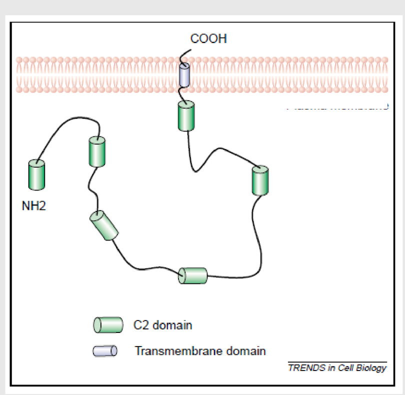
Young adult onset

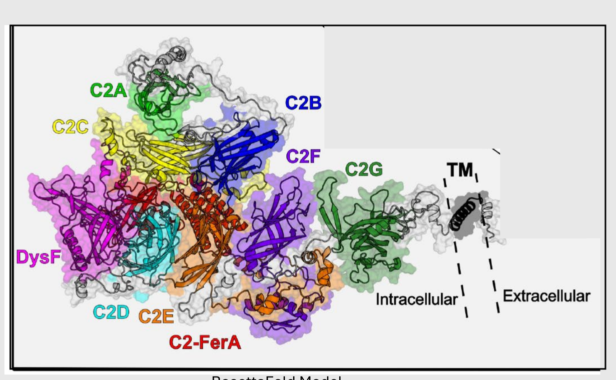
Wheelchair use within ~20 years of diagnosis

Little cardiac or respiratory involvement

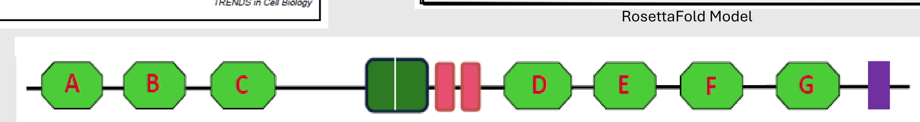
No treatment (gene therapy trial in Phase 1)

Dysferlin Protein





RosettaFold Model



2080 amino acid (6240 bp) tail anchored integral membrane protein

7-8 C2 domains; distinctive Fer and DysF domains

Binds calcium, phospholipid, and several proteins

Major functions: membrane repair; stabilization of Ca^{2+} signaling

(Bansal and Campbell, Trends Cell Biol., 2004; Glover and Brown, Traffic, 2007 Dominguez et al., PLoS One 17"e0270188)

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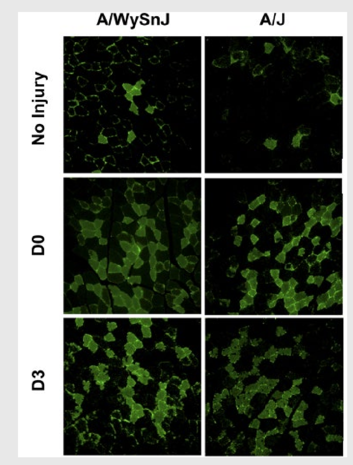
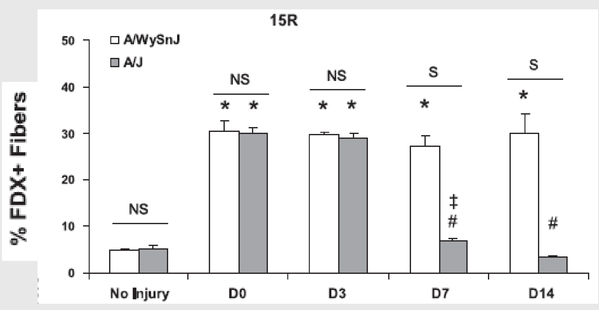
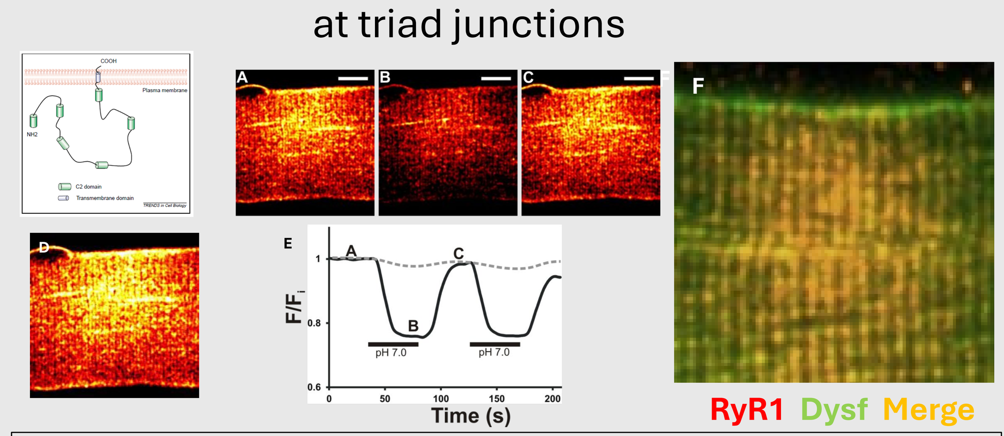
Dysferlin is not required for membrane repair after a physiological injury in vitro





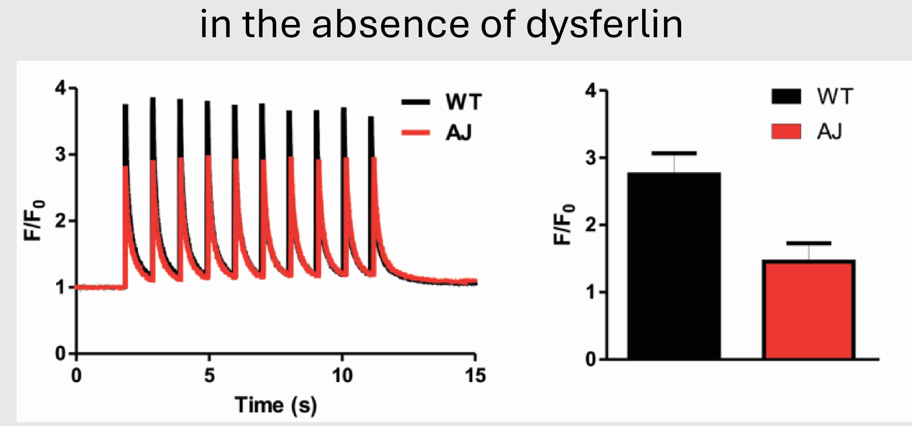
Control (A/WySnJ) and dysferlin-null (A/J) mice were injected IP with fluorescein dextran, then injured by repeated eccentric contractions. The number of labeled fibers was identical in both sets of samples for the first 3 days after the injury.

Dysferlin concentrates in the t-tubules at triad junctions



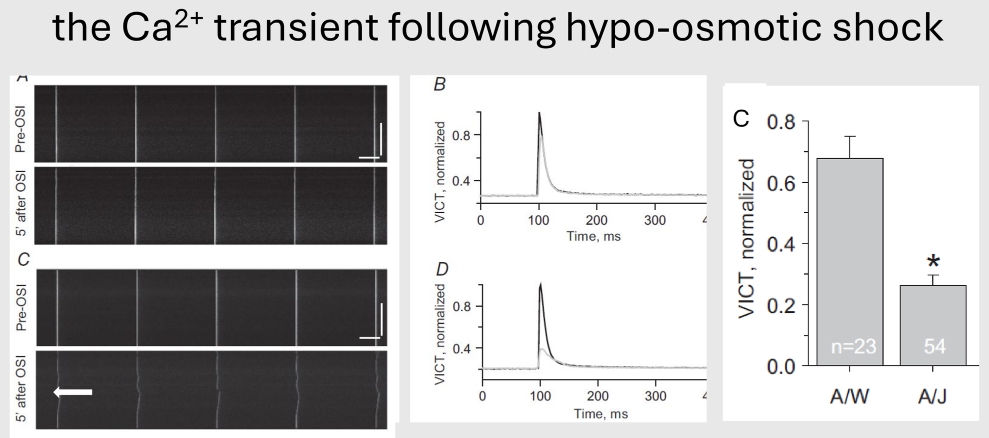
Upper Left: Cartoon of dysferlin with pH-sensitive GFP (pHluorin: pHl) at its C-terminus, showing its orientation in the t-tubule; **A:** localization and fluorescence of Dysf-pHl at pH7.4; **B:** similarly at pH6.5; **C:** similarly after returning to pH7.4; **D:** localization and fluorescence of pHl-Dysf (pHl at the N-terminus) at pH7.4; **E:** fluorescence as pH is changed (solid line; Dysf-pHl; dotted line: pHl-Dysf; *Right*: **F:** colabeling of dysferlin and RyR1 at triad junctions.

The amplitude of the Ca^{2+} transient is lower in the absence of dysferlin



Left: Examples of a series of Ca^{2+} transients monitored with Fluo-4, a fluorescent Ca^{2+} indicator; Black, wild type; red, dysferlin-null. Note that the overall kinetics of the transient are not significantly altered in the dysferlin-null; *Right*: Data from several experiments. The difference is significant.

Dysferlin protects against the loss of amplitude of the Ca^{2+} transient following hypo-osmotic shock

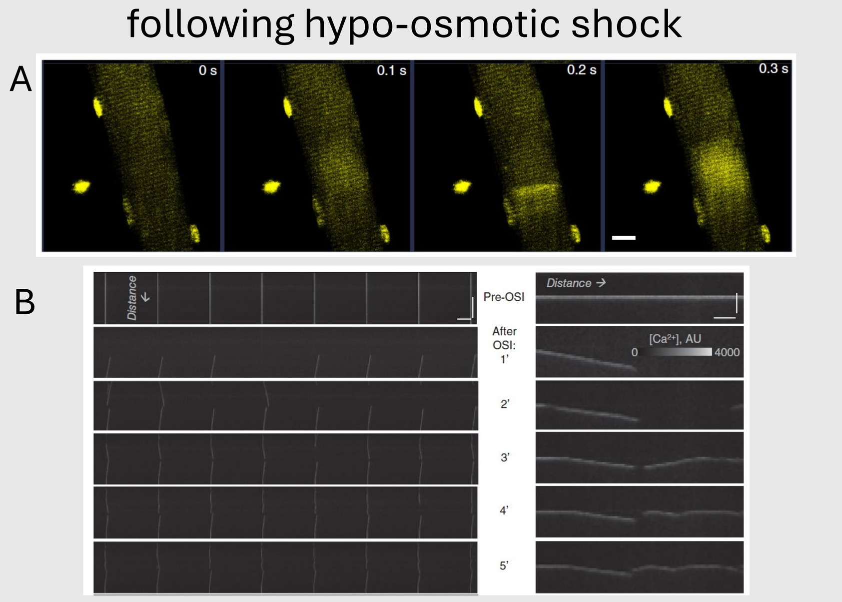


A. Line-scan images of Ca^{2+} transients in WT (upper pair) and A/J (lower pair) myofibers before and after hypo-osmotic shock injury (OSI), visualized with Rhod2. **B.** Data from several experiments. **C.** Averaged data. The difference is significant (P<.005). Note the appearance of a small Ca^{2+} wave in the A/J fiber after OSI (arrow).

Visualization of Ca^{2+} waves in dysferlin-null fibers following hypo-osmotic shock

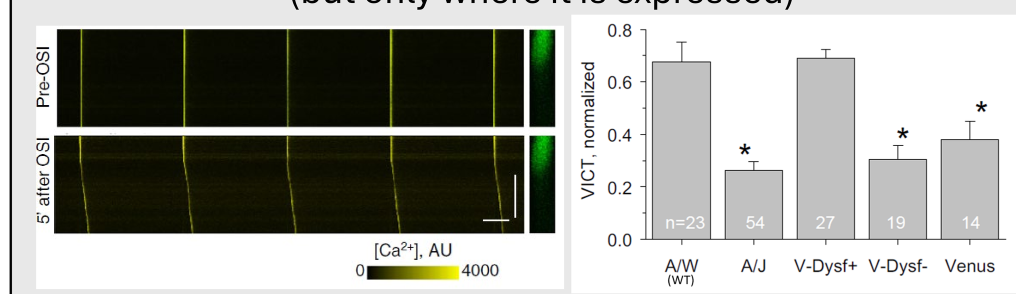
A

B



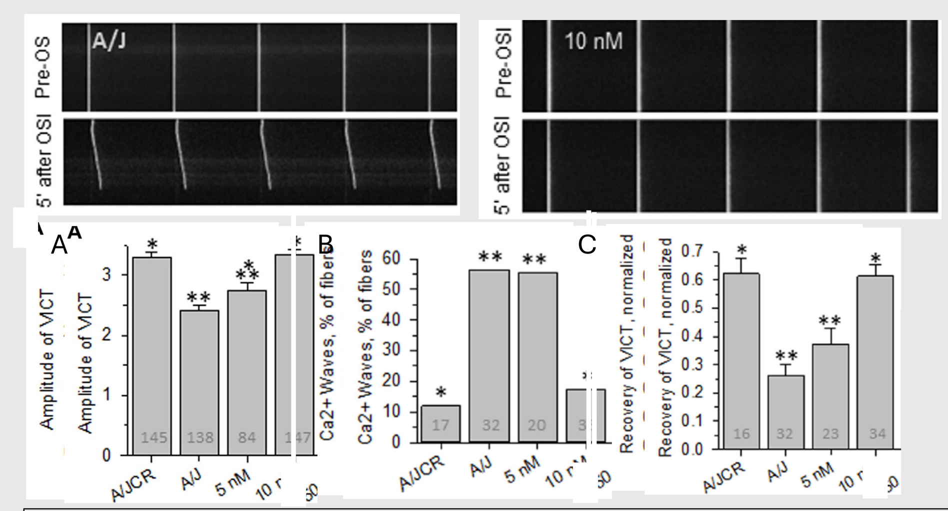
Images of Ca^{2+} waves, collected over short (A: 0 to 3 sec) and longer (B: 0 to 5 min) in two A/J fibers after OSI, visualized with Rhod2.

Dysferlin suppresses Ca^{2+} waves and protects Ca^{2+} transients after hypo-osmotic shock (but only where it is expressed)



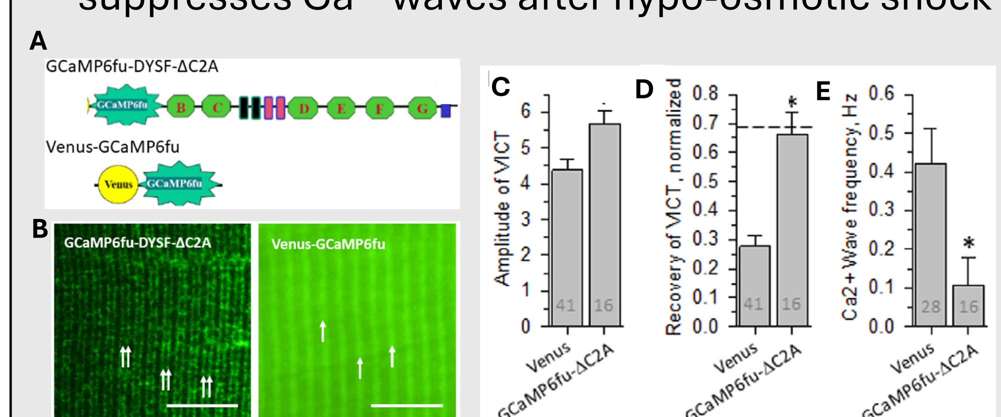
Left: A/J fibers were electroporated to express Venus-dysferlin, loaded with Rhod2-AM and subjected to OSI. Ca^{2+} waves appeared only where Venus-dysferlin was absent. *Right*: Quantitation of Ca^{2+} transient amplitudes in WT (A/W), and A/J fibers that were not electroporated (A/J), electroporated with Venus (Venus), or electroporated with Venus-dysferlin. V-Dysf+ and V-Dysf- indicate transients in regions that expressed the transgene (+), or not (-).

BAPTA protects Ca^{2+} transients and suppresses Ca^{2+} waves after hypo-osmotic shock



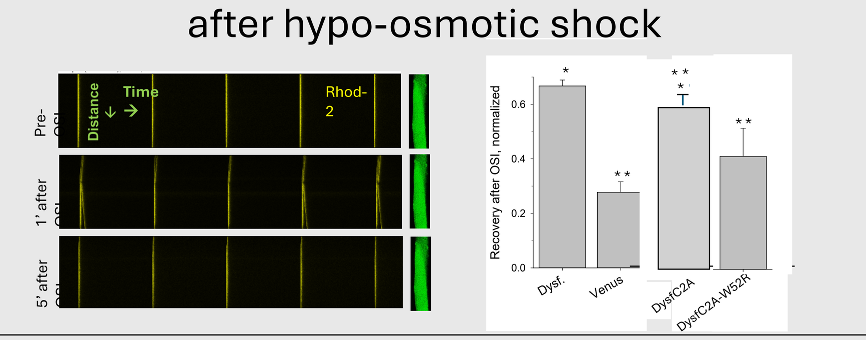
Top: Examples of Ca^{2+} transients in A/J fibers before and after OSI, pre-incubated with (left) or without (right) 10 nM BAPTA-AM. *Bottom:* Amplitudes of voltage-Induced Ca^{2+} transients (VICT) in controls (A/JCr), A/J and A/J fibers with 5 or 10 nM BAPTA-AM. **A**, Ca^{2+} transient amplitudes before OSI; **B.** Ca^{2+} waves after OSI; **C.** Ca^{2+} transient amplitudes after OSI. *, significantly different from A/J; ** significantly different from A/JCr control.

DysfΔC2A-GCaMP6fu protects Ca^{2+} transients and suppresses Ca^{2+} waves after hypo-osmotic shock



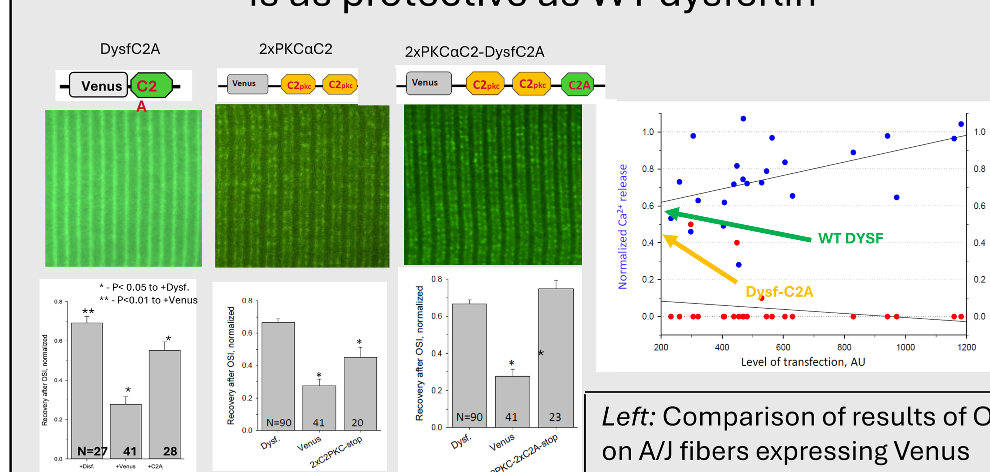
A. We deleted the C2A domain of dysferlin, which binds Ca^{2+} , and replaced it with GCaMP6fu, which has a high affinity Ca^{2+}. We tagged it, as well as GCaMP6fu, with Venus, and expressed Them in A/J fibers. **B** shows that the chimeric protein, but not GCaMP6fu, targets the triad Junctions. **C**,**D** and **E** show that the chimera increases the amplitude of Ca^{2+} transients before OSI, and prevents the loss of transient amplitude and appearance of waves after OSI.

Dysferlin's C2A domain specifically protects Ca^{2+} transients and suppresses Ca^{2+} waves after hypo-osmotic shock



A/J myofibers expressing DysfC2A resist damage by OSI. Recovery of the transient amplitude, while statistically different from WT dysferlin (**), is significantly better than Venus alone (*), and only 13% of fibers show Ca^{2+} waves. That this was specific is shown by the pathogenic point mutant, DysfC2A-W52R, which fails to recover and shows 33% fibers with waves.

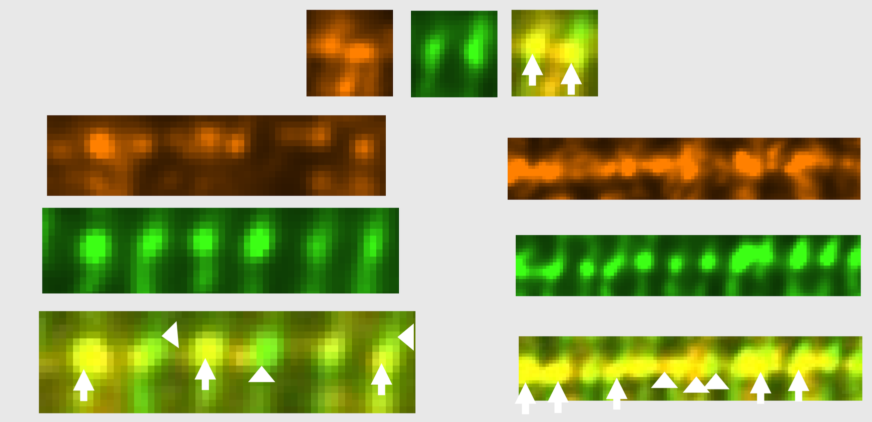
Coupling of dysferlin's C2A domain to the C2 domain of PKCα targets it to the triad junction and is as protective as WT dysferlin



13% waves 57% waves 15% waves

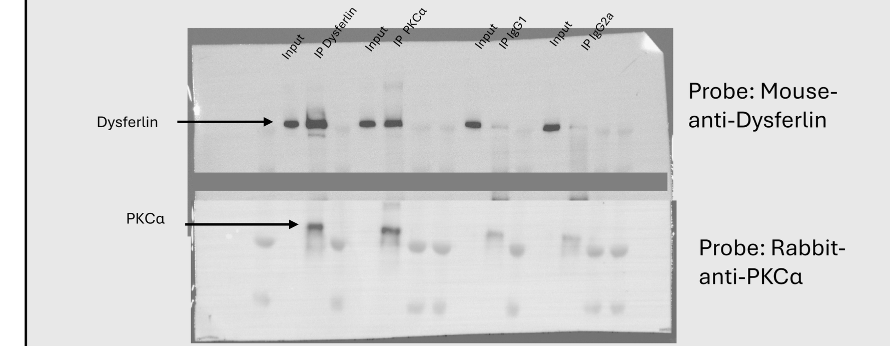
Left: Comparison of results of OSI on A/J fibers expressing Venus chimeras of DysfC2A, PKCαC2-PKCαC2 and PKCαC2-PKCαC2-DysfC2A. *Right:* Graph of the normalized transient amplitude and frequency of Ca^{2+} waves vs the level of transfection of the Venus- PKCαC2-PKCαC2-DysfC2A construct, read as Venus fluorescence. The Y-intercept is that of WT dysferlin and ~25% higher than DysfC2A alone.

PKCα concentrates at some triad junctions



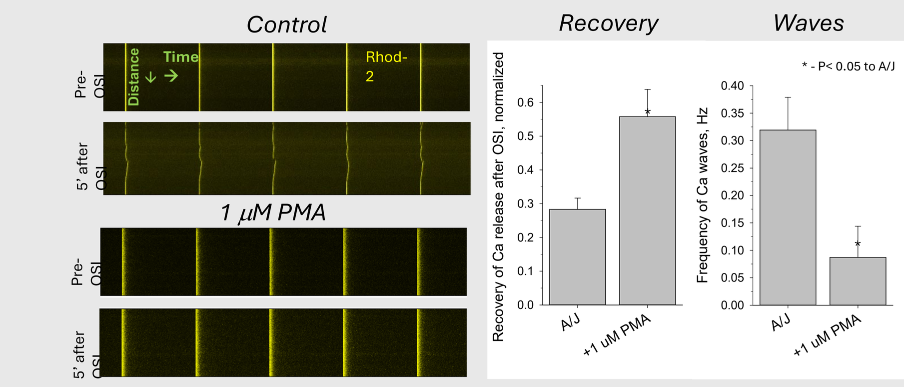
A/J muscles were electroporated to express Venus-dysferlin, cultured, and immunolabeled with antibodies to PKCα followed by Alexa568-labeled anti-antibody. Some (arrows) triad Junctions (but not all: arrowheads) colabeled (merged images with overlap in yellow).

PKCα co-immunoprecipitates with dysferlin



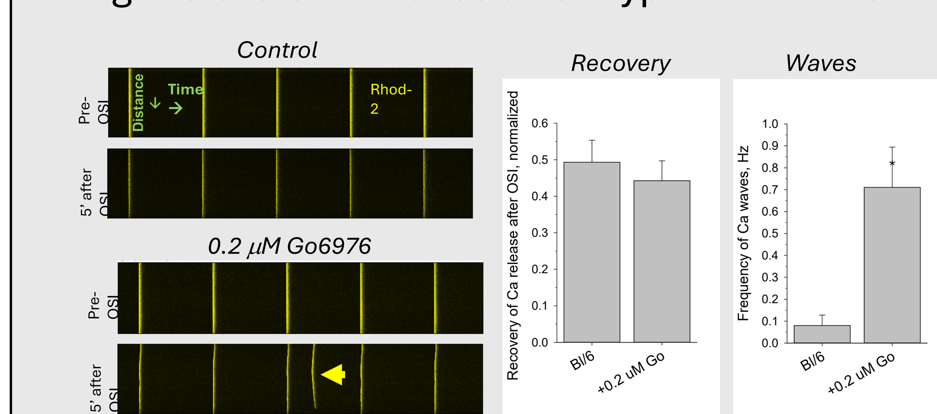
COS7 cells were co-transfected to express WT dysferlin and PKCα. After solubilization in RIPA buffer, immunoprecipitates were generated with anti-dysferlin, with anti-PKCα, or with a non-immune IgG. Immunoprecipitates were analyzed by SDS-PAGE and immunoblotting with antibodies to dysferlin (*top panel*) or PKCα (*bottom panel*).

Activation of PKC with PMA protects A/J fibers from damage by hypo-osmotic shock



A/J myofibers were injured by OSI in the presence or absence of 1 μM phorbol myristyl acetate (PMA), an activator of PKC, and recovery and suppression of waves after injury were measured. (NB: the A/J scans to the left were also used in an earlier figure of this poster)

Inactivation of PKCα with Gö6976 causes WT fibers to generate Ca^{2+} waves after hypo-osmotic shock



C57Bl/6 myofibers were injured by OSI in the presence or absence of 0.2 μM Gö6976. a specific inhibitor of the α and β isoforms of PKC, and recovery and suppression of waves after injury were measured.

Conclusions

1. Dysferlin stabilizes Ca^{2+} release but is not essential for membrane repair.
2. Defects in Ca^{2+} release in dysferlin-null fibers are likely due to increased Ca^{2+} through the RyR1.
3. Dysferlin suppresses Ca^{2+} leak by binding Ca^{2+} in the cleft of the triad junction.
4. Dysferlin's C2A domain is the domain responsible for binding Ca^{2+}.
5. Dysferlin's C2A domain protects the Ca^{2+} transients of dysferlin-null fibers against damage, but does not accumulate in the triad junction. The homologous C2 domain of PKCα accumulates in the junction but does not protect.
6. Dysferlin's C2A domain targeted to the triad junction as a PKCαC2 chimera is as effective as WT dysferlin in preserving normal Ca^{2+} release.
7. PKCα associates with dysferlin and may concentrate at or near triad junctions.
8. Activation or inhibition of PKCα profoundly affects the mechanisms of Ca^{2+} release