Limited proteolysis as a tool to probe the tertiary conformation of dysferlin and structural consequences of patient missense variant L344P

Dysferlin is a large transmembrane protein that plays a key role in cell membrane repair and underlies a recessive form of inherited muscular dystrophy. Dysferlinopathy is characterized by absence or marked reduction of dysferlin protein with 43% of reported pathogenic variants being missense variants that span the length of the dysferlin protein. The unique structure of dysferlin, with seven tandem C2 domains separated by linkers, suggests dysferlin may dynamically associate with phospholipid membranes in response to Ca$^{2+}$ signaling. However, the overall conformation of the dysferlin protein is uncharacterized. To dissect the structural architecture of dysferlin, we have applied the method of limited proteolysis, which allows nonspecific digestion of unfolded peptides by trypsin. Using five antibodies spanning the dysferlin protein, we identified a highly reproducible jigsaw map of dysferlin fragments protected from digestion. Our data infer a modular architecture of four tertiary domains: 1) C2A, which is readily removed as a solo domain; 2) midregion C2B-C2C-Fer-DysF, commonly excised as an intact module, with subdigestion to different fragments suggesting several dynamic folding options; 3) C-terminal four-C2 domain module; and 4) calpain-cleaved mini-dysferlinC72, which is particularly resistant to proteolysis. Importantly, we reveal a patient missense variant, L344P, that largely escapes proteasomal surveillance and shows subtle but clear changes in tertiary conformation. Accompanying evidence from immunohistochemistry and flow cytometry using antibodies with conformationally sensitive epitopes supports proteolysis data. Collectively, we provide insight into the structural topology of dysferlin and show how a single missense mutation within dysferlin can exert local changes in tertiary conformation.

Mutations in the dysferlin (DYSF) gene underlie a recessive form of inherited muscular dystrophy (1, 2) with disease pathogenesis linked to a role for dysferlin in the calcium-triggered vesicle fusion for membrane repair (3). Dysferlinopathies are characterized by absence or marked reduction of the dysferlin protein and can manifest as two distinct clinical presentations: weakness of the proximal muscles in limb–girdle muscular dystrophy 2B or weakness of the distal muscles in Miyoshi myopathy. Dysferlinopathy presents between the second and third decades of life, often in previously asymptomatic patients. Following onset, there is often rapid progression with independent ambulation lost within 5–10 years (4–6). Clinical progression includes muscle weakness and atrophy with elevated serum creatine kinase indicative of muscle damage (7, 8). Inflammatory infiltrate is a common feature (9–11), and subclinical cardiac involvement is also present in some patients (12–14). There is currently no treatment for dysferlinopathies.

Dysferlin is expressed ubiquitously with high expression in skeletal muscle, cardiac muscle, and blood monocytes (15, 16). In skeletal muscle, dysferlin is predominately expressed at the sarcolemma and t-tubule system (17, 18). The DYSF gene contains 55 exons, four of which are alternatively spliced, resulting in 14 different isoforms and a cDNA sequence that encodes a multidomain protein of ~237 kDa (19–21).

Dysferlin belongs to the ferlin family of proteins that possess a short C-terminal extracellular domain, a transmembrane domain (TM), and the rare feature of multiple (five to seven) tandem cytosolic C2 domains. C2 domains are independently folding motifs of about 100–130 amino acids that are organized in an eight-$\beta$-strand structure connected by surface loops (22, 23) and are well characterized as Ca$^{2+}$-regulated, protein–protein-, or protein–lipid–binding domains (22, 24). Dysferlin contains seven C2 domains coupled via long linker regions. The tertiary structure of dysferlin has not been characterized; only the C2A and DysF domains have been solved by crystallography (25, 26). C2A and splice variant C2A(v) possess distinct Ca$^{2+}$

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2 The abbreviations used are: DYSF, dysferlin; TM, transmembrane domain; DYSF40a, dysferlin bearing alternatively spliced exon 40a; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N''-tetraacetic acid; BafA1, bafilomycin A1; EGFP, enhanced green fluorescent protein; HBSS, hanks’ balanced salt solution.
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and phospholipid binding properties. C2A and C2Av1 show features of dynamically folding domains in vitro with one of the lowest free energies of stability reported among purified recombinant C2 domains (0.17 and 0.33 kcal/mol, respectively) (25) compared with other purified C2 domain proteins (~5–15 kcal/mol) (27).

In addition to seven C2 domains, dysferlin contains a DysF domain, found only in ferlins and yeast peroxisomal proteins Pex30p and Pex31p (28). In dysferlin, the DysF domain resembles a duplicated module of the yeast Pex proteins, existing as an unusual nested repeat with inner and outer DysF domains (29). The crystal structure of the inner DysF domain was shown to possess six β-strands connected by loops and stabilized by arginine-tryptophan stacks (26). Recent studies of a recurrent missense variant within DysF, R959W, suggest the DysF domain may fold into an open or closed state with R959W shifting the conformation toward the open state (30). Dysferlin also bears Fer domains, conserved motifs specific to the ferlin family (31) but of unknown function.

The ferlin family of proteins is believed to function as Ca\(^{2+}\)-regulated vesicle fusion proteins (32–34) with dysferlin proposed to play a key role in Ca\(^{2+}\)-triggered vesicle-mediated membrane repair (3, 35). In vitro studies with fragments of purified dysferlin C2 domains provide cumulative evidence for Ca\(^{2+}\)-regulated phospholipid binding (36, 37), interaction with soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins (38), and insertion-based disruption of artificial membranes (39). Our studies have shown that dysferlin can be cleaved enzymatically by activated calpains in response to membrane injury, releasing a C-terminal fragment, “mini-dysferlin\(_{C72}” which bears two highly conserved C2 domains (40, 41). It is therefore plausible that dysferlin is a modular protein with separate functions attributed to its N- and C-terminal domains.

Data from sucrose gradient fractionation, cross-linking assays, and fluorescence resonance energy transfer (FRET) suggest dysferlin may exist as a parallel dimer with intermolecular interactions reported between all C2 domains and transmembrane domain with the exception of C2A, which showed no evidence for self-interaction (42). As the C2A domain shows selectivity for negatively charged phospholipids, it has been suggested that C2A may function as a "sensor" domain with specific affinity for exposed phosphatidylinerine at injury sites (25, 36).

Valuable insight into the normal biology of dysferlin has been gleaned by the dysfunction of dysferlin patient variants (30, 36, 43). Dysferlinopathy is a rare disease with the exact prevalence difficult to estimate. At present, ~500 pathogenic DYSF variants are reported in the Leiden muscular dystrophy database (www.dmd.nl)\(^3\) with ~43% missense mutations and ~57% nonsense mutations or small in-frame insertions or deletions (44). As dysferlinopathy is characterized by deficiency or marked reduction in protein levels, it remains enigmatic how a single amino acid missense variant can result in complete loss of protein. Herein we detail the method of limited proteolysis to study the structural topology of dysferlin in situ as well as a means to probe the functional consequences of patient missense variant L344P on dysferlin tertiary conformation.

Results

Limited proteolysis of dysferlin reveals a highly reproducible fragmentation pattern, suggesting dominant conformations

The tertiary arrangement of the seven cytoplasmic C2 domains of dysferlin has not been characterized but could provide helpful insight as to how dysferlin may respond dynamically to Ca\(^{2+}\) to bind phospholipid membranes and promote vesicle fusion. We applied the technique of limited proteolysis to study the conformation of dysferlin in situ, utilizing nonspecific trypptic digestion of lightly permeabilized cells to identify dysferlin domains that are protected from proteolysis (see “Experimental procedures” and Fig. 1A). In silico analysis of the dysferlin protein reveals 218 predicted tryptic cleavage sites that span the length of dysferlin (Fig. 1B) (www.expasy.org). Thus, any regions of dysferlin existing as linear, unstructured polypeptides may be digested when exposed to trypsin, whereas structured or protein- or lipid-bound domains may be protected from proteolysis.

Limited proteolysis of primary human myotubes and transfected human embryonic kidney (HEK) 293 cells showed similar and highly reproducible patterns of trypsin-resistant fragments (Fig. 2A). Using five anti-dysferlin antibodies spanning the length of the protein (Fig. 2B), we calculated the apparent molecular weight of fragments consistently observed via Western blotting and cross-referenced these fragments (and their recognition by different dysferlin antibodies) to the dysferlin domain structure.

Fig. 2A shows representative Western blots of primary human myotubes (top) and transfected HEK293 cells (bottom). In these experiments, HEK293 cells were transfected with dysferlin bearing alternatively spliced exon 40a, DYSF40a (NCBI reference sequence NM_001130978.1). Parallel experiments performed with the canonical skeletal muscle isoform of dysferlin lacking exon 40a, wild-type dysferlin (WT-DYSF) (NCBI reference sequence NP_001130978.1) showed an identical banding pattern (supplemental Fig. 1A) with the exception of an absence of the mini-dysferlin\(_{C72}” fragment released by calpain cleavage following injury as the calpain cleavage site is encoded by exon 40a (40, 41). The first lane of each gel shows scrape-harvested cells in the absence of trypsin and shows formation of the two fragments of dysferlin resulting from calpain cleavage (Fig. 2B, N\(_{170}\) and C\(_{272}\)). The following lanes are from replicate samples exposed to decreasing concentrations of trypsin. The Hamlet-1 blot shows the mini-dysferlin\(_{C72}” fragment (right, C\(_{272}\)), which is protected at even the highest concentration of trypsin (lane 2, 250 μg/ml trypsin). Conversely, Romeo, Hamlet-2, and C2DE show the N-terminal counter fragment (N\(_{170}\)), which appears less stable and degraded with higher trypsin concentrations.

Collective data from multiple limited proteolysis experiments suggest that dysferlin structurally partitions into four modules. The first module, C2A, is readily excised as a protected module at low trypsin concentrations. Fig. 2C highlights a doublet band detected by Hamlet-1 at ~200–240 kDa for all
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**Figure 1. Schematic representation of limited proteolysis.** A, human myotubes containing endogenous dysferlin or HEK293 cells transfected with dysferlin expression constructs were subjected to a scrape injury. Cell pellets were evenly divided into five reactions and subjected to proteolytic digestion either in the presence of 0.005% saponin only or 0.005% saponin and increasing concentrations of trypsin (see “Experimental procedures”). The inset depicts a hypothetical example of dysferlin within plasma membrane following digestion of available/exposed regions with trypsin (scissors); only protected dysferlin domains remain to be detected via SDS-PAGE. B, in silico determination of trypsin cleavage sites. The schematic of the dysferlin protein (NCBI reference sequence NM_001130978.1) shows the position of all 218 predicted cleavage sites (black lines) within dysferlin (ExPASy).

trypsin concentrations, whereas only the top band of the dou-
blet is recognized by N-terminal antibody Romeo. This cor-
responds to removal of the C2A domain and linker bearing the
Romeo epitope (residues 123–142; see Fig. 2A, Romeo, N25–30).
Evidence showing that C2A is excised as a protected module
was supported using an enhanced green fluorescent protein
(EGFP)-dysferlin fusion protein, which similarly showed
removal of EGFP-C2A module (now ~60 kDa) at very low tryp-
sin concentrations (Fig. 2C, right).

The second module, the midregion of dysferlin, is regularly
excised as a large protected fragment consistent with a module
encompassing C2B-C2C-FerA-DysF domains (M80). This
module may be further digested into fragments most consistent
as a C2B-C2C module (see Hamlet-2, M15) and FerA-DysF frag-
ments that extend to either C2C or C2D (M60) or Fer-DysF only
(Ms0). As M80 and M60 fragments are recognized only by anti-
FerA, the exact boundaries are the most difficult to assign (Fig.
2B). Fragments in **solid lines** are our inferred likely boundaries
(Fig. 2B; based on data derived from missense variant L344P to
be discussed in Fig. 6). Alternative boundaries are indicated with **dotted lines** (Fig. 2B).

The third module, the four C-terminal C2 domains, is com-
monly observed in a single protected fragment resistant to pro-
teolysis (C115; C2D-C2DE-C2E-C2F-TM). C94 corresponds to
subdigestion within the C2D-C2DE linker, producing a C2DE-
C2E-C2F-TM fragment. Finally, the fourth module is the cal-
pain-cleaved mini-dysferlinC72 produced during scrape injury
that shows remarkable resistance to tryptic digestion. Interest-
ingly, anti-C2DE does not recognize any smaller protected frag-
ments (for *e.g.* C2D-C2E-C2F-TM), with C2DE-C2F-TM being
the most resistant to trypsin digestion and release of mini-dysferlinC72, suggesting C2DE may unravel and be proteolyzed during scrape injury. The C2D-C2E-C2F-TM fragment contains the transmembrane domain and short extracellular tail as confirmed using anti-Myc (supplemental Fig. 1B). Importantly, limited pro-
teolysis with chymotrypsin showed a distinct fragmentation
pattern, which confirmed a theme of four modular dysferlin
domains (supplemental Fig. 2).

**Ca^{2+}** has subtle influence on in situ conformation of dysferlin
in cells subject to scrape injury; the C2DE-C2E linker is seen
only by calpain

To determine whether **Ca^{2+}** influences protected dysferlin
domains, we carried out limited proteolysis in the presence or
absence of extracellular **Ca^{2+}**, chelating released **Ca^{2+}** with
EGTA and pretreating with BAPTA-AM (see “Experimental
procedures”). Scrape harvesting with or without **Ca^{2+}** pro-
duced no new fragments or different ratios of fragments. Mini-
dysferlinC72 is detected only when cells are scrape-harvested in
the presence of **Ca^{2+}**, consistent with cleavage by activated cal-
pains (Fig. 3A).

Of note, the C2DE-C2E linker (where exon 40a inserts the
calpain cleavage site) bears 12 predicted tryptic sites. However,
tryptic proteolysis does not release a ~70-kDa mini-dysferlin
fragment (C2E-C2F-TM) for either the canonical or exon 40a-
containing isoform (Fig. 3A). Nonetheless, calpain can readily
access and cleave within the exon 40a-encoded motif (Fig. 3B).
Moreover, addition of recombinant activated calpain increases
levels of cleaved mini-dysferlinC72 product (Fig. 3B). These
results infer that calpains have unchallenged access to the exon
40a-encoded cleavage motif. In contrast, access of trypsin to the
C2DE-C2E linker is precluded.

**Sucrose density ultracentrifugation is consistent with C2A as a
solitary domain with evidence for multimerization of
midregion and mini-dysferlinC72 modules**

We next used sucrose gradient ultracentrifugation to examine
whether protected domains showed evidence for multimer-
ization as reported previously (42). We first examined
sedimentation of endogenous dysferlin and the two calpain
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A. Primary Human Myotubes

Transfected HEK293

B. Romeo Hamlet-2 Fer-A C2DE Hamlet-1

C. Romeo Hamlet-1

HEK EGFP-DYSF

Tryptsin (μg/ml)

N170 N146 N126 M100 M80 M35

N25-30

HEK EGFP-DYSF

Tryptsin (μg/ml)

SAP Only 250 125 62.5 31.2

185 115 C200

Romeo Hamlet-1
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Figure 3. Influence of calcium and the accessibility of the C2DE-C2E linker. A, in situ conformation of dysferlin is unaltered with calcium influx following injury. Primary human myotubes differentiated for 4 days and HEK293 cells transiently transfected with the DYSF40a expression construct were harvested 24 h post-transfection via scrape injury in the presence or absence of calcium (PBS with or without Ca\(^{2+}\) where [Ca\(^{2+}\)] = 0.9 mM). Cells in the absence of extracellular Ca\(^{2+}\) were treated with BAPTA 3 h prior to and during scrape harvesting to chelate free Ca\(^{2+}\). Harvested cells were then subject to tryptic digestions also in the presence or absence of Ca\(^{2+}\) (see "Experimental procedures"). Samples were analyzed via SDS-PAGE, and Western blots were probed with the C-terminal antibody Hamlet-1. No new fragments or different ratios of fragments were detected. B, calpain readily cleaves the motif encoded by exon 40a within the C2DE-C2E linker, whereas trypsin is unable to cleave the 12 tryptic sites within this region. HEK293 cells were transiently transfected with DYSF40a and harvested via scrape injury 24 h post-transfection in the presence of increasing concentrations of recombinant calpain-2. Replicate samples were separated by SDS-PAGE, and Western blots were probed with anti-dysferlin Romeo and Hamlet-1 antibodies, which show increased mini-dysferlin\(_{C72}\) with increasing calpain-2 (black wedge of increasing width). The schematic shows the 12 predicted tryptic cleavage sites within the exon 40a-containing linker region. SAP, saponin.

Figure 2. Limited proteolysis of dysferlin produces a highly reproducible fragmentation pattern, inferring regular adoption of one or more typical conformations. A, top, primary human myotubes, differentiated for 4 days, expressing endogenous dysferlin. Bottom, HEK293 cells transfected with the exon40a-containing dysferlin expression construct DYSF40a (NCBI reference sequence NM_001130978.1). Cultured cells were subjected to a scrape injury and tryptic digestion (see "Experimental procedures" and Fig. 1A). Digested samples were analyzed via SDS-PAGE, and a separate Western blot was probed for each of the dysferlin antibodies: the N-terminal antibody Romeo (red), midregion antibodies Hamlet-2 and FerA (blue), and C-terminal antibodies C2DE and Hamlet-1 (green). B, schematic of dysferlin protein with the position of antibody epitopes and predicted tryptic cleavage fragments. Dysferlin domain boundaries were determined by homology modeling. The epitopes of the five anti-dysferlin antibodies utilized in this study are shown. The molecular weights of prominent dysferlin fragments generated via limited proteolysis were calculated using a standard curve of log molecular weight versus migration for molecular weight markers. Bands repeatedly detected in \( n = 6 \) proteolysis experiments were cross-referenced to the dysferlin domain structure and their detection with multiple dysferlin antibodies. The most likely domain structure of each fragment is depicted based on collective data from WT dysferlin and studies of L344P dysferlin presented in Fig. 6. Dotted lines show alternative domain fragment boundaries for the given molecular weight that are theoretically also possible. These extend from the small vertical dotted line (N-terminal boundary) to the dotted C2D domain (C-terminal boundary). Dysferlin domain fragments are annotated (e.g. N\(_{170}\), M\(_{100}\), and C\(_{72}\)) based on whether they are N-terminal (N), midregion (M), or C-terminal (C) fragments. C, the C2A domain of dysferlin is readily excised from the body of the dysferlin molecule following tryptic digestion. Left, even at the lowest trypsin concentrations (31.2 \( \mu \)g/ml trypsin), full-length dysferlin is detected as a doublet with Hamlet-1 but not Romeo. This represents the removal of a fragment of \( \sim 25–30 \) kDa (see Romeo blot, N\(_{25–30}\)) that necessarily includes the C2A domain and linker region bearing the Romeo epitope. Right, trypsin digest of HEK293 cells transfected with EGFP-DYSF expression construct and probed with Romeo antibody shows removal of EGFP-C2A domain (now \( \sim 60 \) kDa). SAP, saponin.
ments generated via limited proteolysis (Fig. 4B). The C2A fragment migrates as a doublet band in fraction 2 (Romeo, N25–30) with a sedimentation coefficient <4S, similar to sedimentation of syntaxin-4 (34 kDa). The presence of two bands may reflect variable cleavage by trypsin or effects on migration related to secondary structure or post-translational modification. These data are broadly consistent with the sedimentation of C2A as a monomer, although arguably this method is not sufficiently sensitive to exclude a dimer/trimer at this small molecular weight.

Dysferlin midregion fragments detected by Hamlet-2 and/or FerA antibodies show clear sedimentation peaks in fractions 5 and 6, similar to IgG (150–180 kDa; 7S). C-terminal tryptic fragments were reproducibly difficult to detect, often with smeared banding, suggesting that once extracted from membrane bilayers into solution they may be susceptible to proteolysis.

C-terminal fragments detected by Hamlet-1 show a broadly similar profile to WT-DYSF, forming high-density species in fractions 6–9. Interestingly, C-terminal tryptic fragments that show remarkable resistance to tryptic digestion in situ were reproducibly “smeary” and difficult to detect despite Hamlet-1 and anti-C2DE being our most proficient antibodies. This observation anecdotally suggests that resistance of mini-dysferlinC72 to trypsin digestion may be due to lipid binding and once extracted from membrane bilayers into solution is rendered vulnerable to proteolysis.
Patient (p.L344P) dysferlin is absent in muscle although detected in primary myotubes and not obviously targeted by proteasomal surveillance

The Leiden muscular dystrophy variant database (www.dmd.nl) documents 43% of pathogenic DYSF variants as missense mutations with ~57% of reported variants nonsense mutations or small in-frame insertions or deletions (Fig. 5A). We examined the limited proteolysis profile of one particular variant, p.L344P, that occurs within the linker between dysferlin C2B and C2C domains (Fig. 5B) and is invariant among vertebrate dysferlin proteins (Fig. 5C). L344P (chromosome 2: g.71748012T→C, NM_003494.3 c.1031T→C) was identified...
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in two affected siblings (heterozygous c.1031T→C p.L344P and IVS49–2A→G intron 48 essential splice acceptor that causes exon skipping and frameshift) (45).

Fig. 5D shows deficiency of dysferlin via Western blotting (panel i) and immunohistochemistry (panel ii) of Patient 1 compared with age-matched controls. Interestingly, despite dysferlin deficiency in the skeletal muscle biopsy specimen, dysferlin protein was detected (albeit at reduced levels relative to differentiation markers) in primary myotubes from Patient 1 p.L344P (Fig. 5E). To explain the skeletal muscle deficiency of dysferlin protein, we reasoned the dysferlin protein expressed in muscle cells was likely labile due to structural aberrations resulting from the missense mutation. Therefore, we examined levels of dysferlin protein expression following treatment with 1) MG132, a specific inhibitor of the ubiquitin proteasome, and 2) bafilomycin A1 (BafA1), a specific inhibitor of the vacuolar-type H+ -ATPase that prevents vesicular acidification for lysosomal and autophagic degradative pathways. BafA1 inhibition of lysosomal and autophagic degradation had the greater effect, subtly increasing levels of both wild-type and L344P dysferlin (Fig. 5E). These results are consistent with our previous evidence suggesting that dysferlin turnover occurs predominantly via endolysosomal pathways (45). MG132 treatment exerted minimal effect. Overall, we could not find evidence that the missense variant L344P triggers an overt misfolded protein response involving rapid degradation.

Patient missense variant p.L344P shows reduced levels at the plasma membrane and disruption of the C2B-C2C module

Flow cytometric analysis of transfected HEK293 cells revealed that p.L344P dysferlin was capable of transport to the plasma membrane but shows a 40% reduction in plasma membrane expression (Fig. 6A). These data align closely with our previous work that suggests an improper docking mechanism of L344P dysferlin at the plasma membrane in transfected C2C12 mouse myoblasts. L344P dysferlin is endocytosed twice as quickly as wild-type dysferlin, a factor that neatly corresponds to the reduction in plasma membrane expression (45).

We queried the structural impact of the L344P substitution on dysferlin conformation via limited proteolysis (Fig. 6B). Similar fragmentation patterns were observed with Romeo, anti-C2DE, and Hamlet-1 antibodies. However, a notable difference in banding pattern was observed with Hamlet-2, which binds an epitope within the C2B-C2C linker. The L344P substitution results in more frequent digestion of M100 (C2B-C2C-DysF-FerA) to ~M80 (C2C-DysF-FerA) and an absence of the M145 (C2B-C2C) module (Fig. 6B, panel ii). Anti-FerA antibody (Fig. 6B, panel iii) confirmed altered fragmentation with increased digestion of M100 compared with M80 and M60 (Fer-DysF). Closer analysis of migration of M100 shows lower migration (Fig. 6B, panel vi, arrows), most consistent with unfolding and digestion of the C2B domain. Thus, data suggest that the C2B-C2C module ordinarily favors a closed conformation (resistant to trypsin); however, L344P opens the linker, allowing trypsin digestion and resulting in unfolding and digestion of C2B.

Patient missense variant L344P opens the C2B-C2C module that ordinarily favors a closed conformation

To test our hypothesis that the dysferlin C2B-C2C module adopts a closed conformation that is disrupted upon substitution of L344P, we studied the bioaccessibility of Hamlet-2 to its linear epitope within the C2B-C2C linker of WT-DYSF compared with L344P-DYSF. Flow cytometry and confocal microscopy provide supporting evidence that the Hamlet-2 epitope is regularly hidden in cells expressing EGFP-WT-DYSF but is more uniformly available in cells expressing EGFP-L344P-DYSF (Fig. 7).

EGFP fluorescence was used to normalize relative expression levels of EGFP-dysferlin fusion proteins: i.e. as a fusion protein, the level of EGFP fluorescence should increase linearly with antibody binding. As expected, the intensity of Romeo binding increased linearly with increasing EGFP fluorescence for both EGFP-WT-DYSF and EGFP-L344P-DYSF (Fig. 7A). In contrast, Hamlet-2 binding increased linearly with EGFP fluorescence in EGFP-L344P-DYSF but displays a non-linear “dog leg” pattern of binding intensity for EGFP-WT-DYSF. The dog leg pattern suggests an interpretation where a closed C2B-C2C conformation is favored in cells expressing low to moderate levels of EGFP-WT dysferlin, precluding Hamlet-2 binding. However, with increasing levels of dysferlin in high-expressing cells, C2B-C2C is detected in an open conformation by Hamlet-2, likely reflecting saturation of the secretary pathway and forced expression of dysferlin in non-favored membrane compartments.

Quantification of Hamlet-2 binding relative to EGFP biofluorescence in cells moderately expressing dysferlin (black dashed gate) reveals that Hamlet-2 can access its epitope with greater efficiency in L344P dysferlin compared with WT dysferlin. Linear regression in (Fig. 7A, panel ii) demonstrates greater binding for L344P-DYSF (slope, 0.5) versus (slope, 0.17) for WT-DYSF. A histogram and box and whisker plot (Fig. 7A, panel iii) similarly demonstrate greater Hamlet-2 biofluorescence for L344P- compared with WT-DYSF. Masking of the Hamlet-2 epitope in wild-type dysferlin and revelation with the L344P variant was highly reproducible and similarly observed in a polyclonal population of C2C12 cells stably expressing EGFP-dysferlin (supplemental Fig. 3). Interestingly, in both transiently transfected HEK293 cells and polyclonal C2C12 cells, the closed conformation of C2B-C2C bearing the Hamlet-2 epitope is opened with methanol-acetone extraction (a method that extracts lipids), suggesting potential influence of phospholipid binding.

Fluorescence microscopy provides strong supporting evidence for a closed C2B-C2C module (Fig. 7, B and C). Partial masking of the Hamlet-2 epitope in transfected HEK293 cells is illustrated in Fig. 7B (panel i) with a “more green” EGFP signal in the overlay in cells transfected with WT dysferlin compared with cells transfected with L344P dysferlin (quantified in Fig. 7B, panel ii). Complete masking of the Hamlet-2 epitope is convincingly demonstrated in confocal microscopy of HEK293 cells transfected with WT dysferlin whereby Hamlet-2 only recognizes one of two cells clearly positive for EGFP (Fig. 7C, panel i).
Figure 6. Dysferlin variant L344P shows a defect in plasma membrane targeting and influences subtle changes in the tertiary conformation of dysferlin. A, reduced levels of surface expression via flow cytometry for L344P missense variant. Panel i, HEK293 cells were transfected with vector control, WT-DYSF, or L344P-DYSF expression construct. Levels of surface dysferlin expressed in intact cells were detected with anti-Myc-Alexa Fluor 647 (extracellular epitope). Histograms show representative flow cytometry data from duplicate samples within a single experiment (n = 50,000 cells). Nonspecific labeling is shown using vector-only control (gray line). Panel ii, quantification of dysferlin surface expression by flow cytometry. Transfected cells were selectively analyzed whereby specific surface labeling was calculated by subtracting nonspecific anti-Myc-Alexa Fluor 647 binding to untransfected cells from that of transfected cells. Levels of surface dysferlin (anti-Myc-Alexa Fluor 647) versus levels of total EGFP fluorescence were quantified. Scatter plots show that plasma membrane levels of L344P-DYSF are 60% that of WT-DYSF (normalized to 100%). Horizontal and vertical lines represent the mean and S.D., respectively, of four experimental replicates performed in duplicate. B, patient missense mutation L344P results in unstructured C2B-C2C domain. HEK293 cells were transfected with either the DYSF40a or L344P-DYSF expression construct. Cells were then subjected to a scrape injury and tryptic digestion (see "Experimental procedures"). Digested samples were analyzed by Western blotting and probed with anti-dysferlin antibodies. Romeo (panel i), C2DE (panel iv), and Hamlet-1 (panel v) showed no change; however, substitution of L344P disrupts the C2B-C2C module and allows much greater trypsin access compared with dysferlin as seen with Hamlet-2 (panel ii) and Fer-A (panel iii). Panel vi, side-by-side comparison of DYSF40a and L344P-DYSF at 125 μg/ml trypsin for Hamlet-2 and FerA allows closer correlative analysis of the band shift indicated with red arrows. Schematics show dysferlin fragments of interest where dotted lines show alternative domain fragment boundaries for the given molecular weight that are theoretically also possible. These extend from the small vertical dotted line (N-terminal boundary) to the dotted C2D domain (C-terminal boundary). SAP, saponin.
Taken together, immunofluorescence and limited proteolysis data suggest that the C2B-C2C module exists in a closed conformation in WT dysferlin that is opened by the L344P substitution (Fig. 7, panel ii). Moreover, the increased availability of the Hamlet-2 epitope with methanol-acetone extraction (a lipid-extracting method) suggests that

Figure 7. Patient missense variant L344P opens the C2B-C2C module, which ordinarily favors a closed conformation. A, changes in Hamlet-2 antibody accessibility with L344P-DYSF by flow cytometry. HEK293 cells were transfected with WT-DYSF or L344P-DYSF expression construct directly fused to EGFP. Cells were lightly fixed in 1% paraformaldehyde and permeabilized either with saponin or methanol/acetone (1:1). Cells were blocked in PBS with 2% BSA and labeled with dysferlin antibodies Romeo and Hamlet-2. Panel i, representative flow cytometry dot plots for WT-DYSF versus L344P-DYSF. Panel ii, linear regression of Romeo and Hamlet-2 fluorescent labeling of transfected cells moderately overexpressing EGFP-dysferlin (black dashed gate in panel i; n = 5000 cells). The mean fluorescence intensity of Alexa Fluor 647 was plotted relative to EGFP biofluorescence, averaging pooled data from three experiments (error bars represent S.D.). Hamlet-2 showed reduced binding to WT-DYSF (note slope of 0.17) versus L344P-DYSF (slope of 0.50). A more similar correlation between EGFP fluorescence and Romeo avidity for L344P-DYSF may also be influenced by the proximal L344P variant. Panel iii, left, flow cytometry data of low-to-medium transfected cells (black dashed gate in panel i) presented in histogram form. Right, box and whisker plot showing the 90th percentile range of fluorescent intensity data points for the low-to-medium transfected cell gate (10%, 25%, median, 75%, and 90% quartiles). B, changes in antibody accessibility between wild-type dysferlin and patient missense mutation L344P by confocal analysis. Panel i, HEK293 cells were transfected with WT-DYSF or L344P-DYSF expression construct directly fused to EGFP. Cells were fixed, permeabilized with saponin, and stained for Hamlet-2. Bottom, glow-over intensity saturation mode depicting the level of expression. Color scale: red (low) to yellow to white to blue (saturation). Cells marked with an asterisk show Hamlet-2 less intensely labels the cell transfected with WT-DYSF despite a level of EGFP fluorescence similar to that of the cell transfected with L344P-DYSF. Scale bars, 25 μm. Panel ii, quantification of the -fold change of Hamlet-2 staining relative to EGFP, normalized to wild-type dysferlin. The scatter plot represents quantification from n = 100 cells over five fields using ImageJ. *** p < 0.005 (unpaired t test); horizontal and vertical lines represent the mean and S.D., respectively, of five experimental replicates. C, profound masking of Hamlet-2. Panel i, representative example of the profound masking of the Hamlet-2 antibody in one of two cells transfected with EGFP-WT-DYSF. Panel ii, schematic diagram of dysferlin C2 domains and the open versus closed C2B-C2C module. Ctrl, control.
the closed C2B-C2C module may exist as a lipid-binding element.

Discussion

The unique structure of dysferlin, with seven tandem C2 domains separated by linkers, suggests dysferlin may dynamically associate with phospholipid membranes in response to Ca\(^{2+}\) signaling. Studies utilizing a variety of biophysical, spectroscopic, and thermodynamic techniques have revealed the structure and binding properties of individual, isolated dysferlin C2 domains in solution (25, 26, 37). However, despite these significant advances, the overall conformation of the entire dysferlin protein has not been fully elucidated.

Limited proteolysis is a simple biochemical technique used extensively to define boundaries of recombinant protein domains and has been shown to correlate nicely with conformational features obtained from other biophysical and spectroscopic techniques (for a review, see Ref. 46). Therefore, we used limited proteolysis to explore the native conformation of dysferlin in situ. Our study specifically used scrape injury in the presence of saturating Ca\(^{2+}\) such that we may enhance the adoption of one primary conformation of dysferlin. Interestingly, the minus Ca\(^{2+}\) condition was found to have only a subtle influence on the conformational features of dysferlin. However, in the setting of scrape injury and cell permeabilization with saponin, complete chelation of Ca\(^{2+}\) released from intracellular stores may not be achieved.

Although it is important to acknowledge that a protease can readily cleave within an exposed loop of a folded domain, collective data from digestion with three proteases (trypsin, chymotrypsin, and calpain) infer that the dysferlin protein possesses a modular architecture with four tertiary domains: 1) C2A, which is readily removed as a solo domain; 2) midregion C2B-C2C-Fer-DysF, commonly excised as an intact module, with subdigest to several different fragments, suggesting a degree of dynamic folding options for midregion domains; 3) the four C-terminal C2 domains that commonly appear in a module; and 4) calpain-cleaved mini-dysferlin\(_{C72}\), which is particularly resistant to proteolysis.

Interestingly, although our results show linker regions between C2A-C2B and C2D-C2DE are regularly digested (and therefore likely unstructured during some conformational transitions), other linker regions appear resistant to digestion. In particular, the C2DE-C2E linker, where exon 40a sequences are inserted, is readily accessed by calpain but not by trypsin or chymotrypsin. Perhaps this region is masked by a binding partner (or conformation) that is altered dynamically with calpain binding, or indeed the binding partner is calpain itself. The linkers between the last four C2 domains are regularly inacces-sible to trypsin and chymotrypsin, leaving the entire C-terminal domain intact (C\(_{115}\) module).

Sucrose gradient sedimentation confirms a tendency for dysferlin to form high-molecular-weight multimers in solution, consistent with previous studies (42). One must acknowledge that it cannot be determined unambiguously whether multimerization occurs in situ or as a consequence of extraction into solution. From our data, we infer that full-length dysferlin may form a specific oligomer, but these results may be confounded by a tendency to aggregate in solution. Calpain-cleaved mini-dysferlin\(_{C72}\) also forms multimers, in this case showing a more discrete peak and trough than full-length dysferlin. Our most striking finding from sucrose gradient studies relates to the clear sedimentation peak of ~8S for midregion fragments (C2B-C2C-Fer-DysF). Data infer oligomerized midregion domains, differently digested by trypsin, co-migrating to ~8S and broadly consistent as a dimer or trimer.

Disease-causing mutations in dysferlin span the length of the gene with no obvious redundancy or hot spot for pathogenicity. Skeletal muscle dysferlin deficiency resulting from single amino acid variants suggests a high degree of structural constraint and intolerance of amino acid substitutions. Previously identified by Evesson et al. (45), p.L344P was found to be capable of export to the plasma membrane, albeit with enhanced endocytotic turnover and reduced plasma membrane expression. Evolutionary maintenance of L344P and surrounding residues within the C2B-C2C linker among dysferlin orthologues implies a conserved and important function. We could find no clear evidence for proteasome-mediated degradation of L344P dysferlin in either transfected cells or patient primary myotubes. Hence, we queried whether L344P exerted a local folding defect. Limited proteolysis robustly identified a structural aberration induced by L344P that appeared primarily confined to C2B-C2C-Fer-DysF midregion domains.

Detailed analysis of the altered tryptic fragmentation pattern together with accompanying evidence from flow cytometry and confocal analysis provides strong evidence that C2B-C2C typically adopts a closed conformation that precludes access of Hamlet-2 to its linear epitope. Substitution of leucine 344 to proline, an amino acid known to induce a kink in the polypeptide chain, forces C2B-C2C into an open conformation, allowing greater bioaccessibility of Hamlet-2 for its epitope and allowing trypsin to access and digest this linker. Furthermore, our results also suggest that the L344P substitution promotes unfolding of C2B into an unstructured form, reflected by the ability of trypsin to digest C2B from the midregion fragments.

Our collective results from limited proteolysis, density ultracentrifugation, and fluorescence microscopy suggest C2B-C2C may plausibly function as a Ca\(^{2+}\)-regulated phospholipid-binding dimer. C2B is not predicted to bind Ca\(^{2+}\), whereas C2C bears the conserved aspartic acid residues characterized in synaptotagmins to bind Ca\(^{2+}\) (22, 47). Future experiments will experimentally address the migration and lipid binding properties of recombinant C2B-C2C. How the C2B-C2C module interacts in tandem with the Fer-DysF is more difficult to postulate. Our limited proteolysis data suggest Fer and DysF domains may function cooperatively in type 1 ferlins (myoferlin, dysferlin, and Fer1L5), a requirement dispensable in type 2 ferlins (otoferlin and Fer1L6) that lack the DysF domain.

Our recent studies indicate that the DysF domain may play a role in directing whether a ferlin partitions into the plasma membrane upon exocytic delivery (48). Type 1 ferlins (dysferlin and myoferlin with DysF) effectively partition into the plasma membrane and label endolysosomal pathways, whereas Type 2 ferlins (otoferlin and Fer1L6) more briefly transit/appear at the plasma membrane, residing predominantly within vesicular
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and intracellular membrane compartments of the trans-Golgi network and recycling endosome (48).

Fer domains are motifs specific to the ferlin family, and their structure and function are undefined. The crystal structure of the inner DysF domain reveals a β-sheet structure with alternating hydrophilic (arginine) and hydrophobic (tryptophan) faces. The hydrophobic tryptophans may function to mediate lipid binding or perhaps form the core of a multimer. It is interesting to note that another alternately spliced exon, exon 17, encodes residues within the linker connecting the C2B-C2C module with the Fer-DysF module. It is this region that appears differently accessible to trypsin, potentially reflecting a dynamically folding hinge region. We did not study dysferlin isoforms lacking exon 17 as part of this study, although we suspect exon 17 sequences may potentially regulate dynamic interplay between C2B-C2C and Fer-DysF elements.

Conclusion

In summary, this study documents the capacity of limited proteolysis to provide insight into the modular arrangement of dysferlin in situ and demonstrates how a single missense mutation can exert local changes in dysferlin tertiary conformation. As further solved structures for dysferlin domains emerge, limited proteolysis of dysferlin in situ may help us to understand both how patient variants result in absence of dysferlin protein (presumed via degradation) while also providing insight into the dynamic folding of dysferlin in response to different stimuli.

Experimental procedures

Expression constructs and cloning

pcDNA4-EGFP-DysferlinMycHis, containing the cDNA encoding dysferlin isoform 1 (NCBI reference sequence NP_001130978.1), was a generous gift from Kate Bushby (Institute of Human Genetics, International Centre for Life). Dysferlin was subcloned into pIREs2-EGFP and modified to contain exon 40a (DYSF40a; NCBI reference sequence NM_001130978.1) as described previously (41). Patient missense variant L344P was derived as described (45).

Cell culture and transfection

Human myoblast cultures were established as described (40). Myoblasts were cultured in 1:1 DMEM:F-12 (Life Technologies) containing 20% fetal bovine serum (FBS; Life Technologies), 10% AmnioMAX (Life Technologies), and 50 μg/ml gentamicin (Life Technologies). Myoblasts were differentiated up to 4–5 days prior to use. HEK293 cells were cultured in DMEM (Life Technologies) containing 10% FBS and 50 μg/ml gentamicin. HEK293 cells were transfected in 10-cm dishes (BD Falcon) using PEI (Polyethyleneiminé max, Polysciences) as described previously (41). Cells were harvested 24 h post-transfection.

Cell scrape assay

Human myoblast cultures differentiated for 4 days or HEK293 cells 24 h post-transfection were washed twice with phosphate-buffered saline (PBS; Life Technologies) either with or without Ca²⁺ (where [Ca²⁺] = 0.9 mm). Cells were scraped in 500 μl of PBS with a rubber policeman (BD Biosciences) and transferred 30 s post-scraping to an Eppendorf tube. Cells were pelleted at 300 × g for 3 min, and the supernatant was removed. Cells were then subject to limited proteolysis, sucrose gradient centrifugation, or Western blotting.

Limited proteolysis

Cells in the absence of Ca²⁺ were treated with 30 μM BAPTA-AM (Sigma) 3 h prior to and at the time of harvest. Cells harvested by scraping and pelleted were resuspended in 500 μl of PBS (for trypsin digestions) or chymotrypsin buffer (5 × 500 mM Tris-HCl, pH 8.0, 10 mM calcium chloride). 100 μl of the cell suspension was aliquoted into each of five Eppendorf tubes. An equal volume of room temperature digest solution containing 0.005% saponin only or saponin containing increasing amounts of trypsin minus EDTA (Life Technologies 15050057) or chymotrypsin (Thermo Scientific 90056) was added to all samples simultaneously using a multichannel pipette. The 200-μl reaction volumes contained the following final concentrations: trypsin 250, 125, 62.5, or 31.5 μg/ml; chymotrypsin, 5 μg/ml. Trypsin digestes were performed for 1 min at room temperature. Chymotrypsin digestes were performed for 1 min, 20 min, 40 min, and 1 h at 37°C. Reactions were stopped by the addition of 200 μl of ice-cold PBS containing 10% FBS. Cells were pelleted at 300 × g. The cell pellet was solubilized in 200 μl of radioimmune precipitation assay buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 1 mM EDTA, 1:500 protease inhibitor mixture (Sigma)) at 4°C for 45 min. Lysates were spun at 13,000 × g for 10 min at 4°C, and the resultant supernatant was transferred to a fresh Eppendorf tube. Protein content was determined by bicinchoninic acid (BCA) assay (Thermo Fisher Scientific, Waltham, MA). SDS lysis buffer (a fourth of the total reaction volume) was added to each sample (250 mM Tris, 8% SDS, 40% glycerol, 10 mM dithiothreitol, pH 7.4), and the sample was heat-inactivated at 94°C for 3 min prior to being run for Western blotting.

Calculations of apparent molecular weight

Dysferlin domain fragments generated by limited proteolysis were analyzed by SDS-PAGE and Western blotting. The molecular weight of generated fragments was determined via comparison of their position with positions of known protein standards. Migration distance of the standard proteins was measured, and log M₉₀ of molecular weight versus distance migrated was plotted. The average molecular weight for each fragment was calculated from six replicate experiments including both endogenous and transiently expressed dysferlin.

Sucrose gradient ultracentrifugation

Scrape-harvested cells were transferred to an Eppendorf tube on ice containing 50 mM EDTA and 1:500 protease inhibitor mixture (Sigma-Aldrich). Cells were pelleted at 300 × g for 3 min, and the supernatant was removed. Cell pellets were then resuspended in Nonidet P-40 lysis buffer (1% Nonidet P-40, 150 mM sodium chloride, 50 mM Tris, pH 7.4), and ~250 μl was loaded on the top of a 5–20% sucrose gradient. Control human serum lyase was also run on 5–10% gradient. Samples were

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ultracentrifuged at 100,000 × g for 16 h, and 12 fractions (400 µl each) were recovered from the top of the gradient for analysis via SDS-PAGE. For known standards, albumin (45S), IgG (7S), and fibrinogen (9S), SDS-polyacrylamide gels were stained with GelCode Blue according to the manufacturer’s instructions (Thermo Fisher).

**Western blotting**

Heat-inactivated samples were separated by SDS-PAGE on 4–12% sodium dodecyl sulfate-polyacrylamide gels (Life Technologies) with PAGE Ruler (Thermo Fisher Scientific) size marker. Protein was transferred onto polyvinylidene fluoride (PVDF) membrane (Merck Millipore, Billerica, MA) as described previously (41).

**Antibodies**

Antibodies used included Romeo (α-rabbit, 1:1000; Abcam Ab124684), Hamlet-2 (α-mouse, 1:500; Leica Microsystems HAMLET-2-CE), anti-Fer-A (α-rabbit, 1:1000; Abcam HPA021945), anti-C2DE (α-rabbit, 1:500; Sigma-Aldrich SAB2100636), Hamlet-1 (α-mouse, 1:5000; Leica Microsystems HAMLET-CE), anti-Myc 9E10 (α-mouse, 1:1000; Santa Cruz Biotechnology SC-40), anti-Myc (α-rabbit, 1:5000; Abcam Ab9106), anti-GAPDH (α-mouse, 1:10,000; Merck Millipore MAB374), anti-syntaxin-1 (α-rabbit, 1:250; Synaptic Systems 110042), anti-syntaxin-4 (α-rabbit, 1:250; Synaptic Systems 110011), anti-myosin MY32 (1:10,000; Sigma-Aldrich M4276), spectrin (1:100; Leica Biosystems SPEC1-CE), and α-actinin-2 4A3 (1:300,000; a gift from A. Beggs, Children’s Hospital Boston, Boston, MA).

**Flow cytometry**

Transfected cells were trypsinized and replated for 2 h. Cells were then removed from plates with Versene (Life Technologies) and pelleted at 300 × g for 1 min at 4 °C.

**Cell surface anti-Myc labeling**—Live cells were incubated with anti-Myc (α-rabbit, 1:200) in Hanks’ balanced salt solution (HBSS; Life Technologies) and 2% FBS for 1.5 h at 4 °C. Cells were washed with HBSS, pelleted at 300 × g for 1 min, and resuspended in anti-rabbit Alexa Fluor 647 in HBSS and 2% FBS for 1 h at 4 °C. Cells were washed, pelleted, and resuspended in HBSS with propidium iodide (1:200; Life Technologies). Graphs depict the mean fluorescence intensity of surface Myc (labeled with Alexa Fluor 647) in transfected cells. Intact cells were gated based on exclusion of propidium iodide. Transfected cells were identified by positive labeling for EGFP (expressed from the internal ribosome entry site of the expression vector).

**Labeling of permeabilized cells**—Cells were lightly fixed with 1% paraformaldehyde for 10 min at 4 °C and then permeabilized either with 0.15% saponin for 10 min at 4 °C or 1:1 methanol/acetone for 4 min at room temperature. Cells were washed with HBSS and resuspended with Hamlet-2 (1:100) or Romeo (1:500) in HBSS and 2% FBS for 1.5 h at 4 °C. Cells were washed with HBSS, pelleted at 300 × g for 1 min, and resuspended in either anti-rabbit Alexa Fluor 647 or anti-mouse Alexa Fluor 647 (1:200, Life Technologies) in HBSS with 2% FBS for 1 h at 4 °C. Cell fluorescence was then measured using a FACSCanto flow cytometer with FACSDIVA software.

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**Immunocytochemistry and microscopy**

Cells plated on Thermofluor™ coverslips (Nunc) were immunolabeled as described previously (40). Images were captured using a Leica SP5 scanning confocal microscope.

**In silico analysis**

Trypsin cleavage site prediction was determined using ExPaSy (www.expasy.org) and dysferlin protein domain reference schematics constructed using DOG 2.0 (49).

**Image processing and statistical analysis**

Flow cytometry data were analyzed using FACSDIVA software and FlowJo v7.6.5. Graphing and statistical analysis were performed using Prism 6 (GraphPad) and ImageJ (Version 1.48v, Wayne Rasband, National Institutes of Health).

**Author contributions**—N. W. designed and conducted the experiments, performed data analysis and interpretation, and drafted the manuscript. A. B. conducted experiments involving patient muscle sections for Fig. 5. R. A. S. assisted with conducting experiments and data analysis for Fig. 7 as well as editorial support. F. J. E. established the ultracentrifugation assay and provided editorial support for the manuscript. A. L. and B. D. developed the flow cytometry assay to assess epitope availability. R. B. S. performed homology modeling to define dysferlin domain boundaries. S. T. C. conceived the initial study, assisted with data analysis, discussed project direction, and provided editorial support for the manuscript.

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