Identification of Proteins Interacting with Dysferlin Using the Tandem Affinity Purification Method

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Abstract: Mutations of DYSF, the gene encoding dysferlin, cause two types of muscular dystrophies: limb-girdle muscular dystrophy type 2B and Miyoshi myopathy. Recent work suggests a role of dysferlin in membrane repair and demonstrates that defective membrane repair is a novel mechanism of muscle degeneration. We used the tandem affinity purification method for the purification of proteins interacting with dysferlin. Three interacting partners were identified by this method (striatin, adaptin alpha, utrophin) and were confirmed by co-immunoprecipitations. All three proteins play a role in vesicle trafficking. Knowing the interacting partners of dysferlin will help to understand how muscle cells repair tears in the sarcolemma and will give a deeper insight into this very important cell function. At the same time the identified proteins could serve as potential candidates for other muscular dystrophies and muscle-related diseases with unknown aetiology.

INTRODUCTION

Dysferlin is a member of a mammalian gene family sharing homology with the *Caenorhabditis elegans* spermatogenesis factor *fer-1* gene, which mediates vesicle fusion to the plasma membrane in spermatids [1].

The human dysferlin gene is located on chromosome 2p13 [2]. Mutations in dysferlin are linked to two clinically autosomal recessive forms of muscle disorders, limb-girdle muscular dystrophy type 2B (LGMD2B) and Miyoshi myopathy (MM) [3,4]. Although the onset of these two diseases is generally in the late teens, they are clinically distinct because they differ in the muscle groups that initially show the onset of the disease. LGMD2B is a predominantly proximal muscular dystrophy. By contrast, MM is a predominantly distal muscular dystrophy. Both diseases progress slowly, and high levels of creatine kinase are detected in the serum of the patients [5,6].

The muscle fibres of dysferlin-null mice maintain a functional dystrophin-glycoprotein complex and a stable structure of the plasma membrane [7,8]. However, these mice develop a progressive muscular dystrophy caused by inefficient resealing of muscle cell plasma membrane [7]. These results suggest that dysferlin has a role in the process of membrane repair in muscle fibres and highlight the importance of this basic cellular function.

Dysferlin is the first identified member of the membrane repair machinery in skeletal muscle. It is highly probable that the protein-binding partners of dysferlin are also members of this machinery. These proteins could serve as potential candidate for other muscular dystrophies and muscle-diseases with unknown aetiology. Therefore, we attempted to identify the interacting partners of dysferlin and used the tandem affinity purification (TAP) method for this purpose.

MATERIAL AND METHODS

Cloning of Mouse Dysferlin

mRNA was prepared from C57BL/6 mouse skeletal muscle according to the manufacturer's instruction (Qiagen). cDNA was synthesized with oligo(dT) primer. Dysferlin cDNA was amplified with PCR. The PCR products were inserted into *Eag*I-*Kpn*I site of the vector pBluescript II KS(+) (Stratagene) and transformed into XL1-Blue competent cells (Stratagene). Plasmid DNA was isolated according to the manufacturer's instruction (Qiagen) and the insert was fully sequence-verified.

Construction of TAP-Tagged Dysferlin

The C-terminal TAP-tagged dysferlin was constructed in two steps. First, the TAP tag was inserted into the *Sma*I-*Xho*I site of the vector pSFV. The TAP tag consists of two IgG binding domains of *Staphylococcus aureus* protein A (ProtA) and a calmodulin binding peptide (CBP) separated by a tobacco etch virus (TEV) protease cleavage site. Second, the entire open reading frame of the mouse dysferlin was inserted into the *BssH*II-*Sma*I site of pSFV to generate pSFV-dysferlin-TAP.

Preparation of Recombinant Virus Particles

To produce recombinant Semliki Forest virus (SFV) particles, RNA from *in vitro* transcribed pSFV-dysferlin-TAP was introduced with RNA from pSFV-helper into BHK cells by electroporation [9]. The electroporated cells were incu-

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bated at 31°C. Recombinant virus particles were harvested after 24 h.

Transfection with Recombinant Virus Particles

For checking the activity of the recombinant virus particles mouse myoblast cell line [10] was maintained in 6-well plates and grown in Ham's F-10 medium (Sigma) supplemented with 15% horse serum (Sigma). The differentiation of myoblasts into myotubes was initiated by reducing the serum content to 2%. 72 h after the induction of myogenesis, cells were infected by adding recombinant virus particles to the cell culture medium. Prior to the transfection, the recombinant SFV particles were activated with chymotrypsin [9]. Cells were harvested 24 h after the transfection and the expression of the tagged dysferlin was checked by Western blot using a peroxidase-antiperoxidase complex (PAP, Sigma) that detects ProtA.

Extract Preparation and TAP Tag Purification

For the TAP tag purification 25 15 cm plates were transfected with recombinant SFV particles as described above. After 24 h cells were scraped, pelleted, lysed in lysis buffer (20 mM HEPES buffer, pH 7.4, 1% Triton X-100) supplemented with protease inhibitor cocktail (Roche) and centrifuged at 10 000 x g for 20 min at 4°C. The supernatant was added to paramagnetic beads coated with a monoclonal human anti-mouse IgG antibody (Dynal Biotech), rotated for 3 h at 4°C, washed 3x with TEV buffer (50 mM Tris, pH 7.4, 100 mM NaCl, 0.1% Tween 20, 1 mM DTT, 5 mM EDTA), resuspended in TEV buffer with TEV protease (Invitrogen) and rotated overnight at 4°C. Eluate obtained after the TEV cleavage was further incubated with calmodulin beads (Stratagene) for 2 h at 4°C. After washing the beads 3x with calmodulin binding buffer (50 mM Tris ph 8.0, 100 mM NaCl, 0.1% Tween 20, 1 mM DTT, 100 mM imidazole, 2 mM $CaCl_2$, 1 mM magnesium acetate), proteins bound to the beads were eluted in the presence of EGTA (25 mM).

Sample Preparation for Mass Spectrometry

The material recovered from TAP tag purification was precipitated with TCA [11], resuspended with LDS sample buffer (Invitrogen) and separated on a 10% SDS-PAGE gel (Invitrogen). After Coomassie staining (Invitrogen), bands were cut out and analyzed by mass spectrometry.

Mass Spectrometric Analysis

The resulting peptides from each gel piece were analyzed by liquid chromatography coupled to a LTQ ion trap mass spectrometer (MS, Thermo Electronics) equipped with a nano-LC electrospray ionization source. Peptides were dissolved in 1% formic acid (buffer A: 0.5% acetic acid, 0.012% heptafluorobutyric acid) and concentrated and desalted online on a C18 PepMap 100 micro precolumn (5 µm particle size, 300 µm x 1 mm; Dionex Corporation) that was coupled to a self packed (7 cm, 3µm; ProntoSil C18-ACE-EPS, Bischoff Chromatography) and pulled (P-2000 laser puller, Sutter Instrument) fused silica capillary (100 µm i.d. × 365 µm o.d.). The chromatographic separation was then performed by a 100 minute nonlinear gradient from 5 to 55% buffer B (80% CAN, 0.5% acetic acid, 0.012% HFBA) with a constant flow rate of 0.2 µl/min. The mass spectrometric data acquisition was performed with a survey scan followed by 7 data dependent MS2 scans with a repeat count of 2. The collision energy was set to 35%. SEQUEST (Thermo Electronics) was used to search the MouseGP database for peptide sequence and protein identification. MouseGP is an in-house protein sequence database that is derived by assembling in sequences the results of Blast searches against the mouse chromosomes of a non-redundant protein set from Swissprot and Trembl. Search parameters included differential mass modification to methionine due to possible oxidation and static mass modification to cysteine due to alkylation by iodoacetamide. Furthermore, one missed cleavage of trypsin was accepted. Peptides identified by SEQUEST may have three different charge states (+1, +2, or +3), each of which results in a unique spectrum for the same peptide. Except in rare instances, an accepted SEQUEST result had to have a ΔCn score of at least 0.1 (regardless of charge state). Peptides with a +1 charge state were accepted if they were fully tryptic or the C-terminus of a protein and had a cross correlation (Xcorr) of at least 1.8. Peptides with a +2 charge state were accepted if they were fully tryptic or partially tryptic between the Xcorr ranges of at least 2.3 and higher. Finally, +3 peptides were only accepted if they were fully or partially tryptic and had an Xcorr > 2.8. Only proteins identified with at least 2 different peptides, with a pepcount (number of identified peptides) larger than 7 were taken into account.

Immunoprecipitaion (IP)

Cells were harvested 96 h after the induction of myogenesis and lysed in the same lysis buffer used for the TAP tag purification. After centrifugation at 10 000 x g for 20 min at 4°C, the supernatant was transferred to a fresh tube and pre-cleared with paramagnetic beads coated with Protein G (Dynal Biotech) for 1 h at 4°C. The beads were separated with a magnet and the supernatant was transferred to a fresh tube. For co-IP mouse monoclonal antibodies were added to the pre-cleared protein extracts: anti-dysferlin (NCL-Hamlet, Novocastra), anti-adaptin alpha (BD Biosciences), anti-striatin (BD Biosciences), anti-utrophin (BD Biosciences). No antibody was added to the control. Samples were incubated overnight at 4°C. After 3 h of incubation with Protein G coupled paramagnetic beads (Dynal Biotech) at 4°C, the protein complex was precipitated with a magnet and the pellet was washed 3x with TEV buffer. Proteins bound to the beads were eluted with LDS sample buffer (Invitrogen) and loaded on a SDS-PAGE gel (Invitrogen). For the Western blots the same antibodies like for the IP were used as primary antibodies and blots were developed with HRP conjugated goat anti-mouse IgG antibody (SouthernBiotech).

RESULTS

To allow purification of proteins associated with dysferlin, we constructed an alphaviral TAP vector. As shown in Fig. (**1**), the TAP cassette, which contains two copies of the IgG binding domains of ProtA of *Staphylococcus aureus*, the TEV protease cleavage site, and a CBP tag, was fused to the C-terminus of murine dysferlin and was inserted into the pSFV vector.

To asses whether these tandem affinity tags are in a correct fusion format and to determine the activity of the virus, mouse myoblast cell line was maintained in 6-well plates and transfected with increasing amounts of SFV (10 µl to

500 µl). The expression of the tagged dysferlin in the cell lysates was checked by Western blot. For the detection, we used a complex of PAP which binds to ProtA and allows us to distinguish between endogenous and tagged dysferlin. A specific band of about 230 kDa was visualized in the Western blot (Fig. **2**). The use of higher amount of the virus results at first in an increase and then in a decrease of the expression yield of the tagged dysferlin. This can be explained due to the cytotoxicity of SFV for the host cell. Therefore, the amount in lane e was defined as optimum and was upscaled for transfection in 15 cm plates.

Fig. (1). Schematic representation of dysferlin with the C-terminal TAP tag. The IgG binding domain of *Staphylococcus aureus* protein A (ProtA) is separated by a tobacco etch virus protease cleavage site (TEV) from the calmodulin binding peptide (CBP).

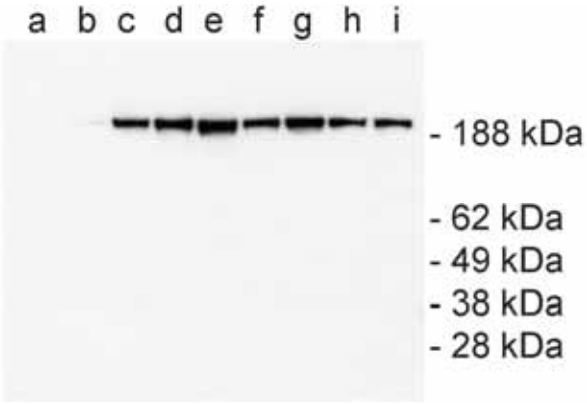

Fig. (2). Western blot analysis of dysferlin-TAP expression using a complex of PAP. Equal amounts of total cell lysates were loaded in each lane. Endogenous dysferlin is not recognized by PAP (lane a), and only the recombinant dysferlin-TAP tag was detected (lane b-i). Increasing amounts of the virus have been used for the transfection of the cells in lane b to i: b: 10 µl, c: 25 µl, d: 50 µl, e: 100 µl, f: 200 µl, g: 300 µl, h: 400 µl, i: 500 µl.

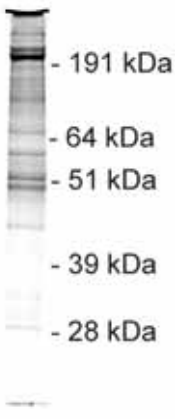

Fig. (3). TAP purification of dysferlin-TAP tag and its associated proteins. After both purification steps, the proteins were separated using a 10% SDS-PAGE gel and visualized by Coomassie staining.

Table 1. List of Proteins Identified with TAP Tag Technique with the Corresponding Accession Number of the UniProt Database.

AAA-ATPase TOB3	Q925I1
Aconitase 2	Q505P4
Actin	P60710
Actinin alpha	Q8K3Q4
Adaptin alpha	P17426
Adaptin beta	Q9DBG3
ADP,ATP carrier protein	P48962
Afadin	Q9QZQ1
A-kinase anchor protein 1	O08715
Ankyrin repeat domain 25	Q923B9
Annexin A2	P07356
Arginyl-tRNA synthetase	Q9D0I9
ATP synthase	P00848
B-cell receptor-associated protein 37	O35127
Bcl 2 associated transcription factor 1	Q8BNZ0
Calcium/calmodulin-dependent protein kinase II	P28652
Calcium-binding transporter	Q7TPC2
Caldesmon 1	Q8VCQ8
Calmodulin	O82018
Calsequestrin 1	Q6P3C3
Cardiomyopathy associated 1	O70373
Cardiomyopathy associated 4	Q8CGY6
Caseinolytic protease X	Q8BRZ3
Catenin delta 1	P30999
Chaperonin subunit 7	Q3TET0
Citrin	Q9QXX4
Clathrin heavy chain	Q68FD5
Coatomer protein complex, alpha subunit	Q3TAU7
Coatomer protein complex, epsilon subunit	Q9D1J2
Cytoskeleton-associated protein 4	Q8BMK4
DEAD box protein 3	Q62167
Dedicator of cytokinesis 7	Q8R1A4
Desmin	P31001
Dihydrolipoamide S-succinyltransferase	Q3UEA0
Drebrin 1	Q9QXS6
Dynactin 1	O08788
Dystonin	Q91ZU6

(Table 1). Contd…..

Electron transferring flavoprotein	Q921G7
Protein enabled homolog	Q03173
Epithelial protein lost in neoplasm	Q9ERG0
Eukaryotic translation initiation factor 1	P48024
Fatty acid synthase	P19096
Filamin A	Q8BTM8
Protein flightless-1 homolog	Q9JJ28
FMRP interacting protein 1	Q9QXX8
FYVE and coiled-coil domain containing 1	Q8VDC1
Glutamyl-prolyl-tRNA synthetase	Q3UFJ2
Glyceraldehyde-3-phosphate dehydrogenase	P16858
Golgi apparatus protein 1	Q61543
Golgi autoantigen	Q3UZV1
GPI-anchored membrane protein 1	Q60865
Grb10 interacting GYF protein 2	Q6Y7W8
GRIP and coiled coil domain containing protein 2	Q8CHG3
Guanine nucleotide binding protein	P68040
Heat shock cognate 71 kDa protein	P63017
Heterogenous nuclear ribonucleoprotein U	Q9R205
Hexokinase 1	P17710
Hexokinase 2	O08528
High density lipoprotein binding protein	Q8VDJ3
HLA-B associated transcript 2	Q7TSC1
Inositol polyphosphate phosphatase-like 1	Q6P549
Insulin-like growth factor binding protein 3	P47878
Internexin neuronal intermediate filament protein alpha	Q3UMG4
IQ motif containing GTPase activating protein 1	Q80UW7
Isoleucine-tRNA synthetase	Q3UWS7
Junction plakoglobin	Q02257
Kinectin	Q61595
Kinesin heavy chain	P33175
Kinesin light chain	O88447
Lactate dehydrogenase	P00342
Large GTP binding protein	Q80V36
Leucine-rich PPR motif-containing protein	Q6PB66
Leucyl-tRNA synthetase	Q8BMJ2
Liprin beta 1	Q8C8U0
Microtubule-actin crosslinking factor 1	Q3UPG9
DNA replication licensing factor MCM7	Q61881

(Table 1). Contd…..

Mitochondrial inner membrane protein	Q8CAQ8
Mitochondrial Tu translation elongation factor	Q497E7
Myc binding protein 2	Q7TPH6
Myoferlin	Q8BVY6
Myosin heavy chain	O08638
Na+/K+ ATPase 1	Q8VDN2
NADH-ubiquinone oxidoreductase	Q9DCT2
Nestin	Q6P5H2
Nexilin	Q7TPW1
NOL1/NOP2/sun family containing protein	Q8CDF9
Nucleoporin 50	Q9JIH2
Palladin	Q9CWW1
Peripherin 1	P15331
Phosphate carrier protein	Q8VEM8
Phosphoenolpyruvate carboxykinase	Q8BH04
Phosphofructokinase 1	P47857
Plectin-1	Q9QXS1
Polyadenylate binding protein 1	P29341
Polymerase I and transcript release factor	O54724
Proteasome 26S non-ATPase subunit 2	Q8VDM4
Proteasome 26S subunit ATPase 1	P62192
Protein kinase C	Q99JB8
Protein phosphatase 1	Q9DBR7
Serine/threonine-protein phosphatase 2B	P63328
Pyrroline-5-carboxylate synthetase	Q9Z110
Pyruvate kinase isozyme M2	P52480
Regulator of nonsense transcripts 1	Q9EPU0
Ribophorin I	Q91YQ5
40S ribosomal protein SA	P14206
RNA binding motif protein 17	Q8JZX4
Ryanodine receptor 1	Q80X16
Sarcalumenin	Q7TQ48
Coiled-coil domain containing 18	Q640L5
Sarcoplasmic/endoplasmic reticulum calcium ATPase 2	O55143
Sarcoplasmic/endoplasmic reticulum calcium ATPase 3	Q64518
Scribble homolog protein	Q80U72
Sec1 family domain containing protein 1	Q8BRF7
Sequestosome 1	Q64337
Sorbin and SH3 domain-containing protein 1	Q62417

(Table 1). Contd…..

Signal recognition particle receptor subunit beta	P47758
Beta-II spectrin	Q62261
SPFH domain protein 2	Q8BFZ9
Splicing factor, arginine/serine rich 1	Q6PDM2
Stress-70 protein	P38647
Striatin	O55106
Stromal interaction molecule 2	P83093
Synaptopodin 2-like protein	Q8BWB1
T-complex protein 1	P80315
Talin 1	P26039
Tangerin A	Q99MS7
182 kDa tankyrase 1-binding protein	P58871
Thrombospondin-1	P35441
Tight junction protein 4	Q9DCD5
Transferrin receptor protein 1	Q62351
Import inner membrane translocase subunit TIM50	Q9D880
Translocase of outer membrane TOM70	Q9CZW5
Trifunctional protein	Q5U5Y5
Tropomodulin 2	Q9JKK7
Tubulin beta	Q7TMM9
Ubiquinol cytochrome c reductase core protein 2	Q9DB77
Ubiquitin	P62991
Utrophin	Q9D1Z0
Vesicle docking protein	Q9Z1Z0
Vimentin	P20152
Serine/threonine-protein kinase WNK4	Q80UE6
Wolframin	P56695
Zinc finger CCCH domain-containing protein 11A	Q6NZF1

ProtA binds tightly to IgG-coated beads, which requires the use of TEV protease to elute materials under native conditions. For further purification, the eluate is incubated with calmodulin-coated beads in the presence of calcium. Finally, the complex of dysferlin and its associated proteins is released with EGTA. Fig. (**3**) shows a Coomassie-stained gel with proteins which were recovered from the purification of TAP-tagged dysferlin.

Bands shown in Fig. (**3**) were excised and processed for identification with MS. Table **1** shows the list of the identified proteins with their accession numbers of the UniProt database. Annexin A2, which was previously described as an interacting partner of dysferlin [12], can be found on this list.

A subset of the interacting partners was additionally assessed by co-IP and Western blots. Cell lysates were prepared from our mouse myoblast cell line to determine the interaction of endogenous dysferlin with the following three proteins from the list in Table **1**: striatin, adaptin alpha and utrophin. These three proteins were chosen because they are involved either in vesicular trafficking (striatin, adaptin alpha) or muscle regeneration (utrophin). Two different co-IPs were performed for confirming the TAP tag result. First, we used an anti-dysferlin antibody for co-immunoprecipitating the interacting partners of dysferlin. Second, the set-up for co-IP was reversed: we utilised an anti-striatin, -adaptin alpha or -utrophin antibody for co-immunoprecipitating dysferlin. We confirmed by Western blot that either the interacting partners of dysferlin (set-up one, Fig. **4**) or dysferlin (set-up two, Fig. **5**) were immunoprecipitated. As controls the co-IP procedure was performed in both set-ups without antibodies. The analysis of the co-IPs confirmed that proteins involved in vesicular trafficking or muscle regeneration do indeed interact with dysferlin.

Fig. (4). Immunoprecipitation of dysferlin-interacting partners with anti-dysferlin. The levels of the indicated proteins in the lysates before immunoprecipitation were detected by Western blot analysis (lane 1). To confirm interactions detected by the TAP tag technique, immunoprecipitations were conducted with a dysferlin-specific antibody (lane 2). As control, no dysferlin-specific antibody was added to the lysates (lane 3).

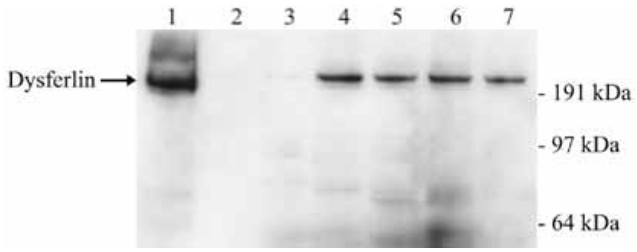

Fig. (5). Confirmation of the immunoprecipitation of dysferlin-interacting partners. Lane 1 shows the level of dysferlin in the lysate before immunoprecipitation. As controls no lysate were added to the beads (lane 2) or the lysate was not pre-incubated with any antibody (lane 3). For the immunoprecipitation antibodies against the following proteins were used: dysferlin (lane 4), adaptin alpha (lane 5), striatin (lane 6) and utrophin (lane 7).

DISCUSSION

Disruption of the plasma membrane is a common event in various normal cells. Plasma membrane repair is a basic cellular process required to reseal membrane disruptions [13-15]. The membrane repair machinery allows the cell to overcome physical injuries to the plasma membrane and is essential to prevent disruption-induced cell death. As the primary function of skeletal muscle is to generate physical force, muscle fibres are often exposed to varying degrees of mechanical stress. Therefore, muscle cells are often susceptible to damages of the plasma membrane and require efficient membrane repair machinery.

Dysferlin is a predicted transmembrane protein [8,16]. Mutations in the dysferlin gene cause proximal (LGMD2B) and distal (MM) forms of recessively inherited muscular dystrophies [3,4]. Recent work showed that dysferlin-null mice, like LGMD2B and MM patients, develop a slowly progressive muscular dystrophy [7]. The results of this work suggest a direct role of dysferlin in the repair process of plasma membrane in muscle fibres.

Nearly every major cellular process is carried out by assemblies of large complexes of proteins [17]. It can be assumed that this also pertains for the process of plasma membrane repair. Dysferlin is the first molecule to be discovered that is possibly involved in the membrane repair machinery in skeletal muscle. It is very probable that proteins which interact with dysferlin are associated with this machinery. Furthermore, these proteins are potential candidate genes for other muscular dystrophies. In this work we used the TAP tag purification approach for their identification.

The TAP tag technique allows a spectrometric analysis of dysferlin associated proteins. As usual with such procedures, the interactions must be confirmed by other techniques. This was done by co-IP for the following three proteins: striatin, adaptin alpha and utrophin.

Striatin is an intracellular protein and is involved in vesicular trafficking [18]. In the light of recent finding, a dysferlin-mediated membrane repair model was already postulated [19]. The model states that vesicles are targeted to disruption sites, where they fuse with each other and the plasma membrane. This causes an addition of membrane to the plasma membrane, thereby patching and resealing the disrupted membrane. Dysferlin is thought to play a role at the fusion step of this repair process by facilitating the docking and fusion of the vesicles with the plasma membrane through interactions with other proteins.

Adaptins are subunits of adaptor protein (AP) complexes. Four basic AP complexes have been described: AP-1, AP-2, AP-3 and AP-4. Each of these complexes is composed of two large adaptins, one medium adaptin and one small adaptin [20-22]. Adaptin alpha belongs to the group of large adaptins and is part of AP-2. Like striatin, AP complexes are also involved in vesicular trafficking [23].

Early time point of muscle regeneration is characterized by the activation of satellite cells: upon muscle damage quiescent satellite cells start proliferating, migrate to the injury site, and fuse together to repair the damaged fibres [24]. A second type of muscle membrane fusion relates to reseal membrane disruptions which occur under physiological conditions [19]. In both types of membrane fusion, it is thought that vesicles fuse with the plasma membrane to facilitate fusion of apposed membranes [25]. The induction of utrophin during muscle regeneration suggests an important role of it during this process [26-28]. Our TAP data show that dysferlin interacts with utrophin. This result was also confirmed by IP. Therefore, we propose that there is a link between muscle regeneration and the repair of muscle membrane and that dysferlin plays an important role during both processes. Inefficient regeneration of muscle fibres contribute to reduced muscle mass seen with age. Thus, dysferlin and its associated proteins could not only be important for disease states, but also for muscle degeneration associated with aging.

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