1 General introduction

Muscular dystrophy

Muscular dystrophies are a clinically and genetically heterogeneous group of degenerative muscle disorders, characterized by progressive muscle wasting and weakness with variable distribution and severity. Several characteristic clinical features, including distribution of predominant muscle weakness, disease course and age at onset as well as variable serum concentration of creatine kinase, muscle histology, and genetic inheritance are used to classify muscular dystrophy [1]. Since the discovery of dystrophin [2], a large number of genes either associated with, or linked to, various forms of muscular dystrophy (MD) have been identified [3]. A schematic diagram illustrates the location of MD-related proteins in the extracellular matrix, the sarcolemma, the sarcomere, the cytosol and the nucleus in Fig. 1 [4].

Fig. 1 Schematic diagram illustrating the location of proteins from the extracellular matrix, the sarcolemma, the sarcomere, the cytosol and the nucleus, involved in muscle limb girdle muscular and congenital muscular dystrophies. Proteins in red color are related to both disorders. Adapted from “Molecular etiopathogenesis of limb girdle muscular and congenital muscular dystrophies: boundaries and contiguities” [4].
Limb girdle muscular dystrophies

Limb girdle muscular dystrophies (LGMDs) are a genetically heterogeneous group of primary myopathies showing progressive weakness and wasting of the muscles of the pelvic and shoulder girdle, and ranging from severe forms with onset in the first decade with rapid progression to milder forms with later onset and a slower course [5,6]. LGMD often presents with a milder progression relative to the more common Duchenne muscular dystrophy (DMD) and the wide range of clinical heterogeneities in this group of muscular dystrophies can be partly attributed to the involvement of a large number of different genes as detailed in Table 1 [7]. LGMD is divided into six autosomal dominant forms (LGMD1A–F) and 11 autosomal recessive forms (LGMD2A–J).

The dysferlinopathies

Autosomal recessive LGMD2B is a predominantly proximal muscular dystrophy. Clinically, LGMD2B is a relatively mild disease with a late onset in the second or third decade and marked elevations of serum creatine kinase (CK) levels. The patients show normal mobility in childhood with a slowly progressive muscle weakness and wasting [8]. The anterior muscles of distal legs and distal arms are relatively normal in these patients, even at later stages of the disease.

Positional cloning revealed that LGMD 2B is caused by mutations in the gene encoding dysferlin. Currently, many mutations causing partial or complete loss of dysferlin have been identified in LGMD2B patients (refer to the Leiden Muscular Dystrophy database, http://www.dmd.nl/dysf_home.html). Mutations in dysferlin cause a number of clinically distinct muscle diseases including the proximal limb-girdle form of muscular dystrophy LGMD 2B [9] and two forms of distal myopathy; Miyoshi myopathy [10] and distal myopathy with anterior tibial involvement (DMAT) [11]. In these muscular dystrophies, the phenotypic differences are prominent at the early stages, but are difficult to recognize later in disease progression. It would be reasonable to use the term ‘dysferlinopathies’ to describe all the diseases due to dysferlin mutations. One interesting and unresolved feature concerns the heterogeneity in clinical presentation of the dysferlinopathy: identical dysferlin mutations have been shown to cause a variable phenotype within the same family and the same clinical presentation can be caused by
<table>
<thead>
<tr>
<th>Gene location</th>
<th>Gene product</th>
<th>Subcellular localization</th>
<th>Proposed function</th>
</tr>
</thead>
<tbody>
<tr>
<td>LGMD1A 5q22–q34</td>
<td>Myotilin</td>
<td>Sarcomere (Z-line)</td>
<td>Binds actinin, filamin and bundles F-actin</td>
</tr>
<tr>
<td>LGMD1B 1q11–q21</td>
<td>Lamin A/C</td>
<td>Nuclear membrane</td>
<td>Structural component of nuclear lamina</td>
</tr>
<tr>
<td>LGMD1C 3p25</td>
<td>Caveolin-3</td>
<td>Sarcolemma</td>
<td>Concentration of signalling molecules/biogenesis of T-tubules</td>
</tr>
<tr>
<td>LGMD1D 6q23</td>
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<td>LGMD1E 7q</td>
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<td>LGMD1F 7q</td>
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</tr>
<tr>
<td>LGMD2A 15q15.1–q21.1</td>
<td>Calpain-3</td>
<td>Cytosolic</td>
<td>Regulation of NF-κB/IκB in protection from apoptosis. Also binds titin. Cleaves filamin</td>
</tr>
<tr>
<td>LGMD2B 2p13</td>
<td>Dysferlin</td>
<td>Sarcolemma/cytosolic</td>
<td>Membrane repair</td>
</tr>
<tr>
<td>LGMD2C 13q12</td>
<td>γ-sarcoglycan</td>
<td>Sarcolemma</td>
<td>Stabilizes DGC at the sarcolemma, binds filamin</td>
</tr>
<tr>
<td>LGMD2D 17q12–q21.33</td>
<td>α-sarcoglycan</td>
<td>Sarcolemma</td>
<td>Stabilizes DGC at the sarcolemma</td>
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<td>LGMD2E 4q12</td>
<td>β-sarcoglycan</td>
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<td>Stabilizes DGC at the sarcolemma</td>
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<td>Sarcolemma</td>
<td>Stabilizes DGC at the sarcolemma, binds filamin</td>
</tr>
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<td>LGMD2G 17q11–q12</td>
<td>Telethonin</td>
<td>Sarcomere (Z-line)</td>
<td>Substrate for titin kinase</td>
</tr>
<tr>
<td>LGMD2H 9q31–q34.1</td>
<td>TRIM32</td>
<td>Cytosolic</td>
<td>E3-ubiquitin ligase involved in targeting proteins to the proteasome</td>
</tr>
<tr>
<td>LGMD2I 19q13.3</td>
<td>Fukutin-related protein</td>
<td>Golgi apparatus</td>
<td>Glycosylation of α-dystroglycan, the extracellular component of the DGC</td>
</tr>
<tr>
<td>LGMD2J 2q</td>
<td>Titin</td>
<td>Sarcomere</td>
<td>Molecular ruler protein specifying sarcomeric structure</td>
</tr>
<tr>
<td>LGMD2K 9q34.1</td>
<td>POMT1</td>
<td>Transmembrane protein, extracellular matrix and cytoplasm</td>
<td>Affects glycosylation of alpha-dystroglycan</td>
</tr>
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Table 1 Current state of knowledge on the genes and proteins involved in LGMD Adapted from "Limb-girdle muscular dystrophies--from genetics to molecular pathology" [7].
different mutations [12;13]. However, no correlation between the nature or location of the mutation and the clinical phenotype has been established. Therefore, additional factors distinct from dysferlin must be involved but, no genetic factors which modify these phenotypes are currently known. This thesis focused on the proteins in the skeletal muscle, dysferlin, AHNAK and calpain 3 (CAPN3), for which we have shown that they act in a single complex.

Dysferlin

The human dysferlin gene (Gene Symbol DYSF) was cloned simultaneously by Liu et al. as the disease gene for Miyoshi myopathy (MM) and by Bashir et al. as the disease gene for LGMD 2B in 1998 [9;10]. The dysferlin gene maps to 2p13, contains over 55 exons and spans over 150 kb of genomic DNA. The mRNA encodes a type-II membrane-associated protein belonging to the ferlin family that shows homology to spermatogenesis factor fer-1 gene of Caenorhabditis elegans [9]. The ferlin family also includes otoferlin, which is mutated in autosomal recessive non-syndromic prelingual deafness, DFNB9 [14], myoferlin [15] and Fer1L4 [16]. Myoferlin and Fer1L4 are currently not associated with disease. The ferlin family proteins (Fig. 2) [3] have a conserved structure characterized by the presence of a single C-terminal transmembrane domain and multiple C2 domains. C2 domains are Ca$^{2+}$-binding motifs of approximately 130 amino acid residues in length that were originally defined as the second-constant sequence (hence called C2) in protein kinase C [17]. The 237 kDa dysferlin contains a single carboxyl-terminal transmembrane domain and six C2 domains that are predicted to reside in a large cytoplasmic domain. Dysferlin has a ubiquitous distribution, but is predominantly expressed in skeletal muscle, in which dysferlin localizes at the plasma membrane of muscle fibers [18;19], as well as in cytoplasmic vesicles [20]. Recently, immunohistochemical and biochemical analyses of skeletal muscle fibers demonstrated the precise localization of dysferlin not only at the sarcolemma but also in T-tubules [21]. Myoferlin is highly homologous to dysferlin and abundantly expressed in both cardiac and skeletal muscle. Myoferlin is associated with the plasma membrane but, in contrast to dysferlin, it is also present in the nucleus of the muscle fibers [15]. The amino acid sequence of otoferlin shows 31% identity to dysferlin. Mouse otoferlin is expressed mainly in the cochlea, vestibule and brain, although a basal level of expression could also be
detected in several other tissues including lung, kidney, skeletal muscle and heart [14]. Fer1L4 is the most recently identified member of ferlin family and is most homologous to otoferlin [16].

![Conserved protein structure of the ferlin family.](image)

Fig. 2 Conserved protein structure of the ferlin family. All ferlin proteins have variable number of C2 domains throughout their length and a transmembrane domain at their C terminus. Sequence analysis of dysferlin and myoferlin and the longer isoform of otoferlin suggest the presence of six C2 domains, whereas data on the small isoform of otoferlin suggest only three C2 domains and those on Fer1L4 suggest five C2 domains. Although the *C. elegans* fer-1 protein is very similar to dysferlin in size, it has only four C2 domains. Adapted from “Dysferlin and the plasma membrane repair in muscular dystrophy” [18].

**The dysferlin protein complex**

Several observations suggest that dysferlin is associated with other proteins involved in various forms of LGMD. A possible transient physical interaction was observed between dysferlin and caveolin-3, which is involved in LGMD-1C. Secondary reduction of caveolin-3 is reported in LGMD2B/MM providing further support for a functional relationship between these proteins [22]. Recently, Hernández-Deviez *et al.* showed that caveolin-1 and 3 are required for trafficking of dysferlin from the Golgi complex to the plasma membrane and T-tubules and suggested that dysferlin may play a role in both membrane systems [23].

Secondary reduction has also been described for CAPN3 in LGMD 2B [24]. CAPN3 is a skeletal muscle-specific member of the calpain superfamily of non-lysosomal, Ca\(^{2+}\)-dependent cysteine proteases and mutations in CAPN3 cause autosomal recessive LGMD2A [25]. The reverse, a secondary dysferlin deficiency was also demonstrated in muscle membranes in LGMD2A patients by immunofluorescence microscopy [26], suggesting that there may be an association between dysferlin and CAPN3. Although dysferlin is not an integral component of the dystrophin-glycoprotein
complex (DGC), reduced sarcolemmal expression along with accumulation of intracellular staining of dysferlin was shown in DGC-linked muscular dystrophies [20]. Nevertheless, in muscle fibers of dysferlinopathy patients a structurally stable sarcolemma and a normal expression of DGC on muscle fibers were demonstrated. Expression profile analyses and in vitro investigations revealed that dysferlin normally associates with both annexins A1 and A2 in a Ca$^{2+}$ and membrane injury-dependent manner [27]. Recently, Cagliani et al. [28] performed genotype/phenotype correlations in a large number of dysferlinopathic patients. They observed higher expression levels of annexins A1 and A2 compared to controls and a significant correlation between annexin expression levels and clinical severity scores. They proposed that dysferlin is integrally involved in the patch fusion repair of membranes through its interaction with the annexins. It was reported by Matsuda et al. [29] that affixin is also a dysferlin-binding protein that shows altered localization in dysferlinopathy muscles. They precisely characterized the interacting domains of dysferlin and affixin by immunoprecipitation studies. It was suggested that affixin may participate in membrane repair with dysferlin. Recently, Ampong et al. found that dysferlin and caveolin-3 coprecipitated with the dihydropyridine receptor (DHPR), a protein complex localized in T-tubules, and dysferlin and DHPR were observed to partially colocalize by double immunofluorescent staining in skeletal muscle fibers. Based on these results, they suggested that dysferlin might be involved in the fusion of caveolin-3-containing vesicles with T-tubules [21].

Both mutations in the dysferlin gene and disruption of the dystrophin-glycoprotein complex can lead to reduced sarcolemmal expression and intracellular vesicles accumulation of dysferlin [20]. Electron microscopy examination of early abnormalities in non-necrotic muscle fibers of patients with a primary dysferlinopathy revealed replacement of the plasma membrane by one to multiple layers of small vesicles, subsarcolemmal vacuoles and membranous projections in the majority of fibers [30]. Based on the binding of the first C2 domain of dysferlin to phospholipids in a calcium-dependent manner, dysferlin has been proposed to play a role in vesicle trafficking and membrane fusion in muscle cells [31]. Therefore, the function of dysferlin is proposed to be related to maintain and repair the structural integrity of the muscle fibre plasma membrane. Recent work of Bansal et al. [32] supports a specific cellular role of dysferlin in the repair of muscle plasma membrane. They demonstrated that isolated dysferlin-null muscle fibers are defective in Ca$^{2+}$-dependent sarcolemma resealing. These
studies show that disruption of the muscle membrane repair machinery is responsible for dysferlin-deficient muscle degeneration and increases our understanding of normal muscle plasma membrane maintenance. A role of dysferlin protein was subsequently proposed in a new model of membrane repair [3]. According to this model, dysferlin facilitates vesicle docking and fusion with the plasma membrane by interacting with other dysferlin molecules and unknown proteins at the plasma membrane. The fusion of vesicles causes addition of membrane to the plasma membrane, thereby patching and resealing the disrupted membrane. Moreover, as discussed, dysferlin co-localises and interacts with the Ca\(^{2+}\) and lipid-binding proteins, annexins A1 and A2 [27]. They proposed that dysferlin is integrally involved in the patch fusion repair of membranes through its interaction with the annexins.

A more comprehensive knowledge of the dysferlin protein complex and their interactions is essential for understanding the normal function of this protein in muscle. Based on studies described in this thesis, we propose that AHNAK and CAPN3 are partners of dysferlin.

AHNAK

AHNAK (meaning 'giant' in Hebrew, also termed desmoyokin; MW 700 kDa) is a ubiquitously expressed giant protein of 5643 amino acids. Shtivelman et al. [33] first described it as a differentiation-related protein that localized in interphase nuclei and as a gene whose transcription is repressed in cell lines derived from neuroblastomas and other tumors. Hieda et al. [34] had also identified independently a large protein in keratinocytes, named desmoyokin, as a plasma membrane-associated protein localized to the desmosomal attachment plaques in stratified epithelium. Later studies showed that AHNAK and desmoyokin are identical and that AHNAK has a variable subcellular localization depending on the cell type [35].

AHNAK1 is encoded by an intronless gene located on human chromosome 11q12–13 [36] and has three main structural regions: an amino-terminal domain of 251 amino acids, a large central domain of ~4300 amino acids with multiple repeated units, most of which are 128 amino acids in length, and a carboxyl-terminal 1002 amino acids large domain. The central region is predicted to have antiparallel \(\beta\)-strands connected by intervening loops [33]. Several putative regulatory elements are clustered within the C-terminal region, including nuclear export localization signals [37], a leucine
zipper [33] and potential phosphorylation sites for protein kinase B and C [38].

A second AHNAK-like protein, AHNAK2, coded for by at least seven exons and located on chromosome 14q32, was identified in a search for genes homologous to AHNAK in the human genome reference sequence [39]. AHNAK2 is a 600 kDa protein composed of a large number of highly conserved repeat segments, which may have a series of linked, antiparallel β-strands, similar to AHNAK1. Some of the repeat segments contain short peptide segments that were identical with those found in the original AHNAK1 repeats [39]. AHNAK1-deficient mice show no obvious phenotype, and it is speculated that AHNAK2 can compensate for the loss of function of AHNAK1.

AHNAK is a ubiquitous protein expressed in a variety of cell types. In an epithelial cell model, AHNAK relocates from the cytosol to the cytosolic surface of the plasma membrane during the formation of cell-cell contacts and the development of epithelial polarity [38]. This targeting is reversible and regulated by Ca\(^{2+}\)-dependent cell-cell adhesion [40]. On the other hand, AHNAK is expressed in the nucleus and cytoplasm of non-epithelial cells. It also shuttles between nucleus and cytoplasm depending on extracellular calcium concentration, and the C-terminus is responsible for this translocation [37]. AHNAK is expressed in all muscle cells including cardiomyocytes, skeletal muscle cells, smooth muscle cells, fibromyocytes, and myoepithelial cells [41;42]. In the brain, AHNAK is expressed at the plasma membrane of brain endothelial cells forming specific blood-brain barriers [43].

Although the exact biological function of AHNAK is unknown, AHNAK has been implicated in essential biological functions based on its interaction with other proteins. \textit{In vitro}, AHNAK fragments bind and activate Phospholipase C\(\gamma\) (PLC\(\gamma\)) in the presence of arachidonic acid, suggesting that an intact AHNAK molecule contains multiple sites for PLC\(\gamma\) activation [44]. AHNAK is also a major target protein for the calcium- and zinc-binding protein S100B and the S100B-AHNAK interaction is proposed to participate in the S100B-mediated regulation of cellular Ca\(^{2+}\) homeostasis [45]. In cardiomyocytes, AHNAK interacts specifically with the β2 subunit of cardiac L-type calcium channels at the plasma membrane, and is suggested to play a role in protein kinase A (PKA)-mediated signal transduction pathway [41]. \textit{In vitro}, the carboxyl-terminal AHNAK region (aa 5262–5643) binds to G-actin and cosediments with F-actin. Data suggest a role for AHNAK in the maintenance of the structural and functional organization of the subsarcolemmal cytoarchitecture [46]. In resting
neuronal PC12 cells, AHNAK is localized within the lumen of specific vesicles called ‘enlargosomes’, and is redistributed to the external surface of the plasma membrane in response to large increases in Ca\(^{2+}\). These enlargosomes have been proposed to participate in cell membrane enlargement, differentiation and repair [47]. Being devoid of a transmembrane domain, the localization of AHNAK at the plasma membrane was explained by the interaction with the annexin 2/S100A10 complex in human epithelial MCF-7 cells, which regulates AHNAK plasma membrane localization [40]. The carboxyl-terminal AHNAK domain (aa 5262–5643) has actin-bundling capacity and exerts a stabilizing effect on muscle contractility [48].

All these proposed functions of AHNAK are based on in vitro studies using cell culture models. Therefore, the biological in vivo function of AHNAK/desmoyokin remains to be elucidated. One approach to further delineate the biological functions of AHNAK is to analyze its partners.

**CAPN3**

LGMD2A (MIM# 253600) or calpainopathy is considered to be the most frequent form of recessive LGMD [49]. The gene responsible for LGMD2A coding for CAPN3 (OMIM# 114240) was localized by linkage studies to the chromosomal region 15q15.1–15.3 and subsequently cloned by positional cloning [25]. To date, >150 distinct pathogenic mutations in the CAPN3 gene have been described according to the Leiden Muscular Dystrophy database (http://www.dmd.nl/capn3_home.html).

LGMD2A was the first example of a limb-girdle dystrophy that was caused by a defect in a non-structural protein, the enzymatic protein CAPN3. CAPN3 is a skeletal muscle-specific member of a calpain superfamily of non-lysosomal, Ca\(^{2+}\)-dependent, cysteine proteases [50]. CAPN3 contains four domains (I-IV) and differs from the ubiquitous calpains (m- and μ-calpain) mainly by three exclusive sequence inserts (NS, IS1, and IS2) and a monomeric functional structure. IS1 and IS2 are each thought to contribute to the rapid autolytic degradation of CAPN3 and removal of these sequences results in a stable active protein [51]. IS1 acts as an internal autoinhibitory propeptide, blocking the active site of p94 from substrates and inhibitors [52]. Autolysis in the active site IS1 domain is required for CAPN3 - mediated substrate cleavage and cytoskeleton remodelling [53]. Domain I has a regulatory role; domain II contains a catalytic site and is responsible for proteolytic functions; domain III has a C2-like domain usually found in
various proteins, which are known to interact with Ca$^{2+}$ and phospholipids; and domain IV represents the Ca$^{2+}$ binding module [54]. Recently, it was shown that CAPN3 activation is Ca$^{2+}$ dependent [55]. CAPN3 is not inhibited by calpain inhibitors e.g. calpastatin, a specific endogenous proteinaceous inhibitor for conventional calpains, E-64, or leupeptin [56;57]. Several cytoskeleton components were identified as substrates for CAPN3 and have linked CAPN3 to the regulation of cytoskeleton architecture. CAPN3 was shown to interact with the N2 and M-line region of titin [58] in the myofibrils [51;59]. This association with titin has been shown to protect CAPN3 from autolytic degradation, and the dissociation may lead to local activation of this enzyme in myofibrils. In addition, CAPN3 shows the ability to cleave a number of cytoskeletal proteins such as ezrin, talin, filamin, vinexin, fodrin, and titin [53;60]. A misalignment of A-bands is observed in the sarcomere of CAPN3-deficient mice [61]. So far, it is difficult to place all these observations in a precise model to explain the biological function of CAPN3 and why its mutations cause disease.

Two antibodies against dysferlin as immunological analysis tools, NCL-Hamlet and NCL-hamlet 2, are commercially available. Unfortunately, immunoprecipitation of dysferlin employing these antibodies yields inconsistent results largely preventing efficient analysis of the dysferlin protein complex. In order to analyze partners in the dysferlin complex by immunoprecipitation, we set out to select single domain antibody fragments against different domains of dysferlin from a non-immune llama-derived phage display library.

**Llama single-domain antibody fragments**

Hamers-Casterman and colleagues discovered a novel class of IgG antibodies in cameldids [62]. These unique antibody isotypes are devoid of light chains and interact with the antigen by virtue of only a single variable domain. The variable domain of heavy chain of heavy-chain antibody is referred to VHH (single-domain antibody fragments or HCAb fragments) [63] (Fig. 3). VHH exhibit a high level of specificity and affinity towards their corresponding antigens [64], with values of the dissociation constant ($K_D$) in the nanomolar range, which is very similar to the affinity of single-chain variable fragments (scFvs) [65] and most conventional antibodies [66]. The single-domain VHH antibody fragments have unique properties
[63], including their small size (<20 kDa), good solubility, high physical chemical stability [66] and can be produced at high levels in microorganisms [67], resulting in estimated production costs of €3/g. VHH antibody fragments have been shown to have great potential in many biotechnological applications [67;68] due to their unique characteristics.

Fig. 3 Schematic illustration of the conventional (top) and heavy-chain IgG antibodies (bottom) present in camelid serum. The entire light chain (curved lines) and CH1 domain (black) are absent in HCAb. The antigen-binding domains of conventional antibodies obtained after proteolysis (Fab) or after cloning, and expression of the gene VH and VL fragments are shown. A synthetic linker introduced between the VH and VL stabilises the VH–VL dimer and forms the scFv. The recombinant VHH, the variable domain of a heavy chain of HCAb is obtained after cloning and expression. The VHH is the minimal intact antigen-binding fragment that can be generated. Adapted from “Single domain camel antibodies: current status” [65].

**Antibody phage display**

Smith first introduced phage display technology in 1985 [69] and in the past years, phage-display has proven to be a very powerful technique to select peptides or proteins with specific binding properties [70;71]. One of the most successful applications of phage display has been the isolation of monoclonal antibody reagents using antibody phage display. Antibody phage display is an established technology for the rapid isolation of antibodies against virtually any antigen for therapy, research and diagnostics [72;73]. The principle of the phage display system is illustrated in Fig. 4.
Fig. 4 Schematic illustration of the principle of the phage display system. A basic selection for binders includes: Pan phage-display library against immobilized antigen to the immunoplate or PVDF membrane and after binding wash unbound phage, elute the bound phage and neutralize, and subsequently inoculate TG1 bacteria to amplify enriched phage for subsequent rounds of selection. After 2-3 rounds of selections the enriched phage pool is plated out as single colonies and amplified as individual clones for further analysis, such as ELISA and fingerprint analysis. Positive clones are sequenced and recloned into in production vector which is VSV and His-tagged for further purification and immunological application. Adapted from http://www.bio.anl.gov

Phage-display derived antibody reagents have some advantages related to time and labour over monoclonal antibodies generated by the hybridoma technology and polyclonal antiserum produced by animal immunization [74]. In addition, a large natural display library from variable gene repertoires allows isolation of antibodies with high affinity against any antigen, such as non-immunogenic or toxic antigens that are not feasible by classic techniques. Moreover, genes that encode phage antibodies can be easily genetically modified to improve their physical properties and affinities for their antigens. Finally, recombinant antibodies selected by phage display have been proved to be valuable tools for widespread application in proteomics, like immunocytotoxic chemical probes [75], in vivo immunomodulation [76] and antibody arrays [77].

Outline of this thesis

The limited knowledge of the role of muscle proteins in muscle cell biology has largely precluded the understanding of the pathophysiology of muscular dystrophies. Current data demonstrate a role for dysferlin in the repair of the muscle-fiber plasma membrane. A more comprehensive knowledge of the
dysferlin protein complex and its interactions is essential for understanding the normal function of this protein in muscle. The main objective of this research was to increase our knowledge of the pathological mechanisms underlying LGMD 2B, to better characterize each member of the dysferlin complex and, more importantly, to try to identify potential modifier genes that might modulate the clinical course in patients.

In chapter 2, by diverse selection methodologies on different truncated forms of recombinant dysferlin, VHH antibody fragments with specificity for two different dysferlin domains were selected from a non-immune phage display library. The selected llama antibody fragments are functional in Western blotting, immunofluorescence microscopy and immunoprecipitation. Using these antibody fragments, we found that CAPN3, which shows a secondary reduction in the dysferlinopathies, interacts with dysferlin.

In order to gain functional insight into the molecular mechanisms of dysferlin, we have searched for yet unknown proteins that interact with dysferlin in skeletal muscle. By co-immunoprecipitation coupled to mass spectrometry, we demonstrated that dysferlin interacts with AHNAK. The binding domains in dysferlin and AHNAK were mapped and the AHNAK expression in normal skeletal muscle dysferlinopathy was assessed by immunofluorescence analysis. Furthermore, the physiological relevance of AHNAK and dysferlin was demonstrated in regenerating muscle (Chapter 3).

Chapter 4 describes the proteolysis of AHNAK by CAPN3. Direct interaction of AHNAK and CAPN3 was studied by GST-pull down assays and specific AHNAK domains that were cleaved by CAPN3 were identified in a cell model. The biological relevance of this cleavage was also studied at cellular level.

Finally, in chapter 5 the selection strategies of antibody phage display and the biomedical application of VHH will be discussed. This chapter will mainly focus on the role of dysferlin complex in dysferlinopathy and future perspectives will be discussed in this chapter.

References


