CHARACTERIZATION OF DYSTROPHIN PROTEIN COMPLEXES IN THE RETINA

by

Paula Constance Demacio

A thesis submitted in conformity with the requirements for the Degree of Doctor of Philosophy
Graduate Department of Molecular and Medical Genetics
University of Toronto

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Doctor of Philosophy, 2001
Paula Constance Demacio
Molecular and Medical Genetics
University of Toronto

Thesis Abstract

The Duchenne Muscular Dystrophy gene, dystrophin, gives rise to a diverse family of protein isoforms that differ in structure, localization and function. Alternative splicing and internal promoter usage form the basis for this diversity and provide a framework for the creation of distinct dystrophin complexes. The retina is an excellent model and example of dystrophin's complexity as it allows for an in depth examination of the various functions of distinctive dystrophin protein complexes.

Three dystrophin isoforms, Dp427, Dp260 and Dp71, are expressed in the retina and have non-overlapping patterns of localization at synaptic and non-synaptic layers. We hypothesize that these isoforms each interact with specialized sets of proteins that serve to tailor their individual functions. This thesis is focused on characterizing both novel and known dystrophin-associated proteins in order to understand the diverse functions of these extended dystrophin complexes. I have identified α-catulin as a novel dystrophin-associated protein that interacts with a unique C-terminus of dystrophin and is localized to the Müller cell endfeet of the retina. I have also shown that isoforms of the dystrophin-associated protein dystrobrevin are differentially localized in the retina.
providing evidence for the existence of additional dystrophin complexes. My data indicate that the localization of each of these dystrophin-associated proteins is dependent upon the presence of its specific dystrophin partner. These findings provide new insight into the function of dystrophin in the retina and outline how the absence of specific isoforms and their extended protein complexes contributes to the disturbances in retinal neurotransmission observed in most Duchenne Muscular Dystrophy patients.
Acknowledgements

I would like to thank my supervisor, Dr. Peter Ray, for guiding me through the challenges and achievements of scientific research. Peter, thank you for being my mentor and for supporting my project interests. Drs. Johanna Rommens and Andras Nagy have been very valuable committee members. Thank you for your encouragement, support and helpful suggestions. Past and present lab members, especially Dr. Enrico Arpaia, Felipe Cisternas, Ben Isserlin and Dan Stevens have made the lab at The Hospital for Sick Children a great place to work. Your friendship, advice and useful discussions are greatly appreciated.

I am extremely lucky to have the most wonderful family in the world. Jeff, your love and time are the most valuable! Thank you for brightening my every day with your smile. I couldn't have done this without you. To my Mom, Dad, Wendy, Dave, Jodie, Stephen and Leanne, thank you for your love, support, encouragement, and for always being there for me. Finally, to Mr. & Mrs. D, Andrea and Peter, I couldn't ask for a better extended family!
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Chapter I

General Introduction
Dystrophin Protein Complexes: Unique Structure and Function
I.1 Duchenne Muscular Dystrophy

Duchenne Muscular Dystrophy (DMD) is a severe X-linked recessive disorder affecting 1/3300 male births worldwide (Emery, 1993). Before patients reach their fifth year of life, proximal muscle weakness and pseudohypertrophy of the calves become apparent. An infiltration of fat cells into the muscle tissue and reactive fibrosis accompany this progressive muscle degeneration which ultimately results in the complete loss of muscle function. The use of a wheelchair becomes a necessity early in the second decade of life and patients usually succumb to respiratory or cardiac failure in their early twenties. Becker Muscular Dystrophy (BMD), an allelic variant of DMD, has a later onset of disease symptoms, a milder phenotype and an increased survival rate.

Although the primary DMD phenotype is progressive muscle weakness, non-progressive cognitive defects and abnormal neurotransmission across the retina are also characteristic features of the disease (Emery, 1993). Almost 1/3 of patients have cognitive deficiencies characterized by defects in verbal IQ, language and reading ability (Karagan et al., 1980, Emery, 1987, Bresolin et al., 1994, for review see Mehler, 2000). The range of IQ scores is normally distributed but shifted approximately one standard deviation to the left of the population mean. A second common non-progressive neurophysiological abnormality observed in DMD patients is abnormal neurotransmission across the retina. In a dark-adapted state, many patients have abnormal retinal responses to a flash of light (Pillers et al., 1993, Cibis et al., 1993, Sigesmund et al., 1994, Fitzgerald et al., 1994). This is recorded as an altered response on an electroretinogram (ERG) and reflects an abnormal synaptic response.
I.2 The Dystrophin Gene

DMD is caused by mutations in the very large dystrophin gene (Burghes et al., 1987, Hoffman et al., 1987, Koenig et al., 1987). Occupying 2.5Mb of the X chromosome, dystrophin was the first major human disease gene to be identified by positional cloning (Monaco et al., 1986, Burghes et al., 1987, Koenig et al., 1987). The dystrophin gene contains 79 exons that give rise to a 14kb mRNA transcript (Koenig et al., 1987, Roberts et al., 1993). The presence of at least seven unique promoters of this gene, in addition to regions subject to alternative splicing, drastically increases the complexity and functional potential of its protein products.

The enormous dystrophin locus has an unusually high rate of new mutation, accounting for the defects observed in approximately 1/3 of patients. Overall, large intragenic deletions or duplications are the most common dystrophin mutations, present in 65% of patients (Forrest et al., 1988, Gillard et al., 1989, Hu et al., 1990). Small insertions, deletions or nucleotide substitutions are found scattered throughout the gene, while large deletions occur in two distinct hotspots (Roberts et al., 1994). The first region occurs at the 5' end of the gene between exons 2 and 19, and the second region occurs between exons 44 and 54 (Forrest et al., 1988, Beggs et al., 1990). Any mutation disrupting the translational reading frame leading to a premature translational stop causes the severe Duchenne form of muscular dystrophy (Monaco et al., 1988). Deletions that preserve the translational reading frame and result in the production of internally deleted protein products are usually responsible for the milder BMD phenotype (Koenig et al., 1989).
1.3 The Dystrophin Protein

The positional cloning of the dystrophin gene led to the identification and characterization of the dystrophin protein (Hoffman et al., 1987, Koenig et al., 1988, Ahn and Kunkel, 1993). Full-length dystrophin is a rod-shaped, 3685 amino acid protein with overall sequence homology to the α-actinins and spectrins (Hammonds, 1987, Davidson and Critchley, 1988, Koenig et al., 1988). Based on amino acid sequence analysis and partial proteolytic degradation experiments, dystrophin was predicted to contain several discreet domains (Figure 1.1) (Hoffman et al., 1987, Koenig et al., 1988). Dystrophin’s N-terminal 240 amino acids are homologous to the actin-binding regions of α-actinin and β-spectrin, predicting an actin-binding function for this domain (Hammonds et al., 1987, Koenig et al., 1988). Subsequent in vitro and in vivo studies confirmed the actin binding capability of dystrophin and revealed three distinct actin binding regions within the dystrophin N-terminus (ABS1, ABS2 and ABS3) (Levine et al., 1992, Way et al., 1992, Fabbrizio et al., 1993, Corrado et al., 1994). Each region shows high conservation with other members of the actin binding family, and is thought to contribute equally to the actin-binding function of dystrophin (Corrado et al., 1994). The globular actin binding amino-terminus is flanked by an extended, flexible rod domain consisting of 24 spectrin-like repeats that are interrupted by four hinge regions (Davidson and Critchley, 1988, Koenig and Kunkel, 1990). The proline-rich hinge regions are thought to provide increased flexibility to the central rod region by disrupting its repetitive organization (Cross et al., 1990, Koenig and Kunkel, 1990). However, the precise molecular function of the rod domain remains unclear, especially in light of the BMD patient and transgenic mouse evidence demonstrating that the middle portion of the rod
Figure I.1: Dystrophin Domain Structure. The full-length dystrophin protein, Dp427, is composed of several discreet domains schematically depicted in this figure. The N-terminal actin binding domain links dystrophin to the cytoskeleton. The extended rod domain contains four hinge regions (H1-H4) thought to confer flexibility to the protein. The WW and cysteine-rich (CYS) domains are responsible for binding the transmembrane dystroglycan protein complex, while the dystrophin C-terminal domain (C-term) mediates interactions with the syntrophins and dystrobrevins.
domain can be deleted without devastating consequences (Beggs et al., 1991, Phelps et al., 1995). Recently, an additional actin-binding site in exon 56 of the rod domain was identified, suggesting that this region may also contribute to the cytoskeletal anchoring of the dystrophin protein (Rybakova et al., 1996, Amann et al., 1998). A WW motif is immediately adjacent to the rod domain and, in part, mediates the binding between dystrophin and its membrane anchor, β-dystroglycan (Bork and Sudol, 1994, Huang et al., 2000). A short cysteine rich domain, also required for dystrophin's interaction with β-dystroglycan, follows the WW domain (Suzuki et al., 1992). The binding of β-dystroglycan to matrix-bound α-dystroglycan completes an important molecular link between the actin cytoskeleton, dystrophin, dystroglycan and the extracellular matrix. Finally, the C-terminal domain of dystrophin is a region unique to the dystrophin superfamily of proteins (Ahn and Kunkel, 1993, Koenig et al., 1988). These 320 amino acids contain the binding sites for the dystrophin-associated proteins syntrophin and dystrobrevin (Ahn and Kunkel, 1995, Suzuki et al., 1995, Ahn et al., 1996, Sadoulet-Puccio et al., 1997).

1.4 Dystrophin Function

An understanding of the Duchenne phenotype, coupled with information on dystrophin structure and protein partners, enabled the development of a functional hypothesis for dystrophin. The initial working model was hypothesized through analogy to the function of spectrin, since dystrophin is closely related by sequence and plasma membrane localization. Spectrin helps to maintain the flexibility and stability of membrane structures in erythrocytes by linking the intracellular cytoskeleton to the
membrane costameres (Craig et al., 1983, Pardo et al., 1983). Analogously, the "structural hypothesis" of dystrophin suggests that dystrophin and its associated dystroglycans form a structural link between the actin cytoskeleton and the basal lamina of the extracellular matrix (Ibraghimov-Beskrovnaya et al., 1992, Ahn and Kunkel, 1993). This scaffolding system is thought to maintain the structural integrity of the muscle membrane through the repeated rounds of muscle contraction and relaxation. In dystrophic muscle, contraction-based membrane stresses are thought to lead to membrane ruptures, effectively causing muscle necrosis (Petrof et al., 1993, Petrof, 1998). Such "delta lesions" are, in fact, observed in the early stages of the disease and are thought to lead to a leakage of muscle enzymes, imbalances in intracellular ionic concentrations, and eventually cell death (Morki and Engel, 1975, Hutter, 1992).

The existence of non-progressive, non-muscle phenotypes, in addition to the progressive degeneration of skeletal muscle, complicates the molecular interpretation of this disease. Although well-suited to explain the major DMD muscle phenotype, the structural hypothesis is a simplistic, generalized view of dystrophin function. There is now substantial evidence to suggest that dystrophin is more than a scaffolding protein. Much of this information is based on specific indirect protein interactions between dystrophin and several known signaling molecules. In fact, each subcomplex of dystrophin-associated proteins interacts with signaling proteins, coupling dystrophin to several signal transduction pathways.
I.5 Dystrophin-Associated Proteins

Dystrophin is a member of an expanding network of associated proteins. Its extended complex is composed of extracellular, cytoplasmic and integral membrane proteins, and forms unique associations in a cell-type and tissue-specific manner. It is through these interactions that dystrophin acquires the ability to perform its varied functions. In fact, the intimate association of these proteins suggests that the observed DMD phenotype may not simply reflect the function of dystrophin, but rather the function of the entire dystrophin associated complex. This is supported by the observation that the loss of dystrophin from the sarcolemma results in a reduction of the other components of the dystrophin protein complex (Ervasti et al., 1990, Ohlendieck and Campbell, 1991, Ohlendieck et al., 1993, Metzinger et al., 1997). This makes it imperative that the various members of the complex are identified and characterized.

Several members of the dystrophin protein complex have already been identified and have provided crucial information regarding the function of the dystrophin complex. However, the diversity of the complex continues to grow as new members are constantly being identified. Our current hypothesis is that the various functions of dystrophin are controlled by the composition of each individual extended dystrophin complex. Therefore, it appears as though a full understanding of dystrophin function will rely on the characterization of its associated proteins and an understanding of their contribution to the function of the complex. Currently, there are three main subcomplexes of dystrophin-associated proteins: the dystroglycans, the sarcoglycans and the cytoplasmic complex consisting of dystrobrevin and syntrophin (Figure I.2) (for review see Watkins et al., 2000).
Figure 1.2: The Dystrophin Protein Complex. The multiple domain structure of dystrophin facilitates the interaction with several associated proteins and protein complexes. Dystrophin’s N-terminus binds to f-actin, providing a link to the cytoskeleton. Transmembrane β-dystroglycan (β-DAG) serves to position/anchor the dystrophin complex to the plasma membrane, while α-dystroglycan (α-DAG) establishes a connection with the extracellular matrix. It is through these interactions with actin and the dystroglycan subcomplex that dystrophin forms a “structural bridge” thought to stabilize the muscle membrane. Although not directly bound to dystrophin, the sarcoglycan (SG) subcomplex of proteins and sarcospan (SP) are also associated with the dystrophin complex. Their specific roles remain to be determined. The C-terminal (C-term) domain of dystrophin binds cytoplasmic syntrophin (Syn) and dystrobrevin (DB). Syntrophin forms further connections with several signaling proteins including sodium channels (NaCh).
1.5.1 Dystroglycans

The dystroglycan subcomplex consists of two proteins that form a transmembrane link between dystrophin and the extracellular matrix (Ibragimov-Beskrovnaya et al., 1992, Ervasti and Campbell, 1993a). α- and β-dystroglycan are post-translationally cleaved products of a single gene (Ibragimov-Beskrovnaya et al., 1992, 1993). A precursor polypeptide is processed into an N-terminal 156kDa α-moiety and a 43kDa C-terminal β-moiety. α-dystroglycan is a highly glycosylated extracellular peripheral membrane protein that binds to laminin, an extracellular matrix protein, and to agrin, a protein known to be essential for the clustering of acetylcholine receptors at the neuromuscular junction (Campanelli et al., 1994, Gee et al., 1994, Talts et al., 1999). α-dystroglycan is also non-covalently bound to β-dystroglycan, linking this transmembrane protein to the extracellular matrix (Ervasti and Campbell, 1993).

β-dystroglycan is a transmembrane protein with a proline-rich motif in its cytoplasmic tail that interacts with dystrophin’s WW and cysteine-rich domains (Jung et al., 1995, Huang et al., 2000). Further contributions from the first half of dystrophin’s C-terminal domain appear to enhance the affinity of this interaction (Suzuki et al., 1992). In essence, β-dystroglycan is the membrane anchor that links α-dystroglycan and the extracellular matrix to intracellular dystrophin. The N-terminal actin-binding property of dystrophin is then able to further extend this link to include the cytoskeleton, forming a structural scaffold that it thought to stabilize the plasma membrane (Levine et al., 1992, Way et al., 1992). It is this molecular bridge that forms the basis for the structural hypothesis of dystrophin function.
In addition to anchoring dystrophin to the plasma membrane, there is accumulating evidence of several other protein interactions that suggest β-dystroglycan may have a role in cell signaling. β-dystroglycan has been shown to bind to Grb2, an SH2/SH3 adapter molecule involved in several intracellular signaling pathways (Yang et al., 1995b). The significance of this interaction remains to be elucidated, but Grb2 may mediate an interaction between β-dystroglycan and Focal Adhesion Kinase (p125FAK) (Cavaldesi et al., 1999). FAK is a non-receptor tyrosine kinase that interacts indirectly with dystroglycan and would serve to link the dystrophin complex to intracellular transduction pathways involved in processes such as membrane depolarization and neurotransmitter receptor activation (Ilic et al., 1997). The potential for dystroglycan to function in signaling pathways is strengthened by its interaction with the peripheral membrane protein, rapsyn, an essential component of the acetylcholine receptor clustering pathway at the neuromuscular junction (see section I.6) (Apel et al., 1995, Gautam et al., 1995, Cartaud et al., 1998). The dystroglycan complex therefore appears to play a major role in both the structural and signaling functions of the dystrophin protein complex.

Since the function of dystrophin appears to rely on the dystroglycan complex, a complete understanding of dystroglycan function is essential. α and β dystroglycan are ubiquitously expressed proteins that are essential for the development and assembly of basal laminas (Henry and Campbell, 1998). The lethal phenotype of dystroglycan null mice stresses the importance of these dystrophin-associated proteins (Williamson et al., 1997). In the absence of dystroglycan, knockout embryos have mislocalized laminin and collagen, and most importantly, lack Reichert’s membrane, which results in early
embryonic death. In DMD, an absence of dystrophin corresponds to a mislocalization of
dystroglycan expression at the membrane (Ervasti et al., 1990). It is interesting to note,
however, that this disruption in DMD patients does not result in embryonic death.
Although not fully elucidated, it is possible that this situation is due to an interaction
between dystroglycan and dystrophin paralogs (section I.10) and/or other dystrophin
isoforms (section I.7). For example, in human fetal muscle, the dystrophin paralog,
utrophin, is expressed along the sarcolemma and only becomes concentrated at the
neuromuscular junction in the adult stage (Nguyen et al., 1991, Ohlendieck et al., 1991,
Clerk et al., 1993). Since dystroglycan is known to bind to utrophin, this would allow for
the proper localization of at least some dystroglycan during the early embryonic period.
However, the fact that dystrophin/utrophin null mice are not embryonic lethal indicates
that other factors are involved in dystroglycan function.

I.5.2 Sarcoglycans

The sarcoglycans are a family of single-pass integral membrane glycoproteins.
This subcomplex is thought to indirectly associate with dystrophin through a lateral
association with the dystroglycan proteins (Ozawa et al., 1995, Suzuki et al., 1994). The
expression of α and γ-sarcoglycan is confined to skeletal and cardiac muscle, while β, δ
and ε-sarcoglycan have additional expression in smooth muscle cells (Straub et al.,
1999). Further, β and ε-sarcoglycan are not restricted to muscle and are part of the
dystrophin complex in several non-muscle tissues (Lim et al., 1995, Ettinger et al., 1997).
The exact function of each sarcoglycan protein is unknown, however they are required
for normal muscle function as inherited mutations in the sarcoglycan genes cause
different forms of limb girdle muscular dystrophy (LGMD). Mutations in the α-sarcoglycan, β- sarcoglycan, γ- sarcoglycan and δ- sarcoglycan genes are responsible for LGMD 2d, 2e, 2c, and 2f respectively (Roberds et al., 1994, Bönnemann et al., 1995, Lim et al., 1995, Noguchi et al., 1995, Nigro et al., 1996). The LGMD phenotype can be variable, ranging from Duchenne-like severe myopathies to much milder forms where patients do not require the use of a wheelchair (Beckmann and Bushby, 1996).

Immunofluorescence studies using skeletal muscle sections from sarcoglycanopathy patients indicate that the disruption of one sarcoglycan protein through gene mutation results in the instability of the entire complex (Bönnemann et al., 1995, Lim and Campbell, 1998). Coimmunoprecipitation and chemical cross-linking studies have been used to analyze the interactions between the individual sarcoglycan proteins in order to provide some initial biochemical clues to the organization and function of the complex. These experiments show there is a close physical association between core proteins β, γ and δ, with α less tightly associated, supporting the observation that the removal of one sarcoglycan can disrupt the entire complex (Chan et al., 1998). The complex is also disrupted in the absence of dystrophin, indicating that the proper localization and function of the sarcoglycan proteins depend on a stable interaction with the dystrophin complex. In contrast, dystrophin does not appear to require an intact sarcoglycan subcomplex for normal localization. Immunofluorescence staining of dystrophin in sarcoglycanopathy patients is either normal or only slightly reduced suggesting that the sarcoglycans may depend on dystrophin solely for membrane localization but not necessarily for their signaling or regulatory functions (Hack et al., 1998).
The recent identification of a new sarcoglycan interacting protein has provided some information about the potential function of the subcomplex. Filamin 2 (FLN2) was recently described as a γ- and δ-sarcoglycan interacting protein (Thompson et al., 2000). FLN2 is a member of a family of proteins that has been implicated in signal transduction, actin reorganization, force transduction and mechanoprotection. This provides yet another link between the dystrophin complex and signal transduction, and strengthens the idea that the extended dystrophin protein complex has an active signaling role.

1.5.3 Sarcospan

Sarcospan was recently identified as the previously unknown 25kDa protein that colocalizes and copurifies with the dystrophin protein complex (Crosbie et al., 1997). It is a ubiquitously expressed, multi-subunit protein that is integrally associated with the sarcoglycans (Crosbie et al., 1997). Sarcospan actually requires the expression of sarcoglycans for proper localization, as LGMD mice with a complete or partial loss of the sarcoglycan subcomplex also lack sarcospan (Crosbie et al., 1999). Structural analysis predicts that sarcospan contains four transmembrane-spanning helices, raising the possibility that it may be a pore-forming protein (Crosbie et al., 1997). Despite this prediction, the function of sarcospan is still unclear, since mice null for sarcospan display normal muscle function (Lebakken et al., 2000). In fact, it is questionable whether sarcospan is even required for the normal function of the dystrophin complex. In the absence of sarcospan, the expression of known dystrophin protein complex components is unaffected, sarcolemmal integrity is maintained and there are no major histological abnormalities observed in muscle (Lebakken et al., 2000).
1.5.4 Dystrobrevins

Dystrobrevin is a unique member of the dystrophin protein complex as it is both related to and associated with dystrophin (Ambrose et al., 1997, Sadoulet-Puccio et al., 1997). α-dystrobrevin was originally identified in Torpedo californica as a phosphoprotein associated with the cytoplasmic face of the postsynaptic membrane (Carr et al., 1989, Wagner et al., 1993). Sequence analysis demonstrated moderate homology to dystrophin throughout the cysteine-rich and C-terminal domains. The dystrobrevin N-terminal region contains two EF hand motifs, while the C-terminus exposes an extended dystrobrevin unique region (Figure I.3) (Ambrose et al., 1997). Heterodimerization between dystrophin and dystrobrevin occurs between the coiled-coil regions located near the C-terminal domain of each protein (Sadoulet-Puccio et al., 1997). Directly adjacent to this binding site is the binding site for syntrophin, another member of the dystrophin protein complex (Dwyer and Froehner, 1995).

Much like the complex gene structure observed with dystrophin on the X-chromosome, several transcripts arise from the α-dystrobrevin gene producing many unique protein isoforms (Figure I.3) (Blake et al. 1996a). Expression of α-dystrobrevin can occur from three promoters, each active in a tissue-specific manner (Holzfeind et al., 1999). In addition, alternative splicing at the 3' end of the gene greatly alters the C-terminal region of the protein that flanks the dystrophin-binding site (Blake et al., 1996a). α-dystrobrevin-1 contains a unique 188 amino acid C-terminal extension that is absent in α-dystrobrevin-2. α-dystrobrevin-3 lacks the syntrophin and dystrophin-binding sites in addition to this unique tail region. Three additional variable regions (vr1-vr3) within the gene allow for further diversification of these isoforms (Blake et al., 1996a, Ambrose et
Figure 1.3: Dystrobrevin Structure; schematic representation of the domain structure of isoforms. All isoforms contain an N-terminal EF-Hand (EF) motif and a ZZ domain (ZZ). The vr3 variable region, present in α-dystrobrevin-1 and α-dystrobrevin-2, is only expressed in muscle. A homologous region, site A, exists in β-dystrobrevin. α-dystrobrevin-1 and β-dystrobrevin contain an extended dystrobrevin-unique (DU) region. α-dystrobrevin-3 lacks the coiled-coil (CC) domain.
al., 1997). The vr3 region, which is specifically expressed in skeletal and cardiac muscle, actually mediates binding to a second syntrophin molecule, serving to regulate the stoichiometry of syntrophin binding to the dystrophin/dystrobrevin complex (Newey et al., 2000). This provides a unique mechanism for the specialization of specific dystrophin complexes, which in turn, directly affects the function of the complex.

Sequence homology led to the identification of β-dystrobrevin, a novel dystrobrevin isoform encoded by a separate gene (Figure I.3) (Peters et al., 1997b, Blake et al., 1998, Puca et al., 1998). The 2.5 kb β-dystrobrevin transcript can be alternatively spliced at four regions (A-D) to generate several protein isoforms (Peters et al., 1997b, Blake et al., 1998). Alternative splicing in variable region A removes amino acids 361 to 390 and occurs only in brain. Region B corresponds to the insertion of seven charged amino acids at position 518, while splicing in regions C and D creates transcripts lacking one or both of the last two coding exons (17 and 18). The β-dystrobrevin protein is homologous to α-dystrobrevin-1 with a highly similar N-terminal region, a cysteine-rich/C-terminal domain capable of binding dystrophin and syntrophin, and a slightly shorter dystrobrevin unique region of ~90 amino acids (Peters et al., 1997b, Blake et al., 1998).

It is quite evident that the complexity of the dystrobrevin proteins and their interaction with dystrophin present a unique potential for the functional diversification of the dystrophin protein complex. This is emphasized by the differential localization patterns of the various dystrobrevin isoforms. α-dystrobrevin-2 is localized along the sarcolemma and at the neuromuscular junction; whereas α-dystrobrevin-1 is highly restricted to the synaptic region (Peters et al., 1998). In contrast, β-dystrobrevin, is not
expressed in muscle but is abundant in brain and kidney (Peters et al., 1997b, Blake et al., 1998). This differential expression and localization allows for the generation of distinct dystrophin complexes that could perform specialized functions.

Although the individual functions of the dystrobrevin isoforms remain unknown, evidence suggests that dystrobrevin may be involved in the signaling aspects of the dystrophin protein complex. This is based primarily on the observation that α-dystrobrevin null mice display a muscular dystrophy phenotype despite having an intact sarcolemma (Grady et al., 1999). It is thought that the observed dystrophic symptoms are the result of altered signal transduction. Neuronal nitric oxide synthase (nNOS) is mislocalized and the entire nNOS signaling pathway is disrupted, implicating this signaling pathway with normal muscle function (Grady et al., 1999). Dystrobrevin has also been associated with some of the non-muscle DMD phenotypes. Initial studies characterizing dystrobrevin in the brain have shown that it is expressed in hippocampal, cerebellar and cortical neurons and have postulated that dystrobrevin may be involved in the cognitive impairments observed in DMD patients (Blake et al., 1998, Blake et al., 1999). The retinal expression, localization, protein partners and potential functions of the dystrobrevin isoforms are examined in Chapters III and IV.

I.5.5 Syntrophins

The syntrophins are a family of intracellular dystrophin-associated proteins that were originally identified due to their postsynaptic enrichment in the electric organ of Torpedo californica (Froehner, 1984, Froehner et al., 1987). The three syntrophin isoforms, α1, β1 and β2, are encoded by separate genes, but exhibit very similar domain
organizations (Ahn et al., 1996). Each protein contains two separate pleckstrin homology (PH) domains, which are modules of approximately 100 amino acids found in a wide variety of signaling proteins. The first PH domain of α1-syntrophin can bind to phosphatidylinositol lipids establishing an alternative mode of membrane interaction (Chockalingam et al., 1999). This domain is interrupted by a PDZ (PSD-95, Discs large, ZO-1) domain which has proven to be essential for syntrophin interactions with associated signaling proteins (Brennan et al., 1996, Gee et al., 1998, Schultz et al., 1998, Lumeng et al., 1999). A syntrophin-unique C-terminal domain follows the second PH domain and partially mediates the interaction between syntrophin and dystrophin (Yang et al., 1995a). In fact, all three syntrophin proteins are able to bind directly to the coiled-coil regions in the C-terminal domains of dystrophin and its autosomal paralogs, utrophin and dystrobrevin (Ahn et al., 1996). These interactions are mediated by the second PH domain together with the syntrophin-unique domain of syntrophin. The differential association of specific syntrophin isoforms with different dystrophin family members may facilitate the diversification of dystrophin complexes for distinct membrane specializations. The original model suggested that dystrophin (or utrophin), syntrophin and dystrobrevin associate in a 1:2:1 ratio allowing for the creation of numerous unique complexes (Peters et al., 1997a). The identification of additional syntrophin binding sites in dystrophin and dystrobrevin now indicates that there is the potential for four syntrophin isoforms to bind to each dystrophin/dystrobrevin complex (Newey et al., 2000).

Dystrophin's association with syntrophin has recently gained considerable attention since the PDZ domain of syntrophin has been shown to recruit several signaling
molecules to the dystrophin protein complex, including adult skeletal (SkM1) and cardiac (SkM2) muscle sodium channels, microtubule-associated serine/threonine kinases MAST205 and SAST, and neuronal nitric oxide synthase (nNOS) (Brenman et al., 1996, Gee et al., 1998, Schultz et al., 1998, Lumeng et al., 1999). Of particular importance is the fact that not all syntrophin-associated signaling proteins interact with all syntrophin isoforms. The interaction between syntrophin and sodium channels can occur with each syntrophin isoform, while kinase binding is limited to β2-syntrophin and nNOS binding is specific for α1-syntrophin (Brenman et al., 1996, Gee et al., 1998, Lumeng et al., 1999). This provides yet another level of dystrophin complex specialization.

The functional differences between the individual syntrophin proteins are beginning to be elucidated. The differential association of specific syntrophin isoforms with certain signaling proteins combined with the slight differences in the syntrophin isoform localization patterns suggests that these proteins are performing specialized roles and may confer unique functions to the dystrophin complex at particular membrane specializations. This is particularly evident at the neuromuscular junction where the absence of α1-syntrophin leads to structurally aberrant neuromuscular synapses (Adams et al., 2000). α1-syntrophin knockout mice do not display a dystrophic phenotype but do have abnormal postsynaptic membranes with significantly reduced levels of acetylcholine receptors and disorganized postjunctional folds (Adams et al., 2000). In addition, nNOS is absent from the sarcolemma. These findings support the hypothesis that syntrophins are modular adapter proteins that recruit or anchor signaling proteins to the dystrophin complex.
I.6 Dystrophin and Signaling

The characterization of dystrophin-associated proteins has provided substantial evidence to suggest that the dystrophin complex has a role in signaling in addition to its function in the stabilization of the plasma membrane. Since dystrophin itself is not a signaling molecule, it is thought that the dystrophin complex serves to position or anchor these associated signaling proteins at non-random locations on the membrane. This has led to the suggestion that the disruption of specific signaling pathways contributes to the dystrophic phenotype of DMD patients. For example, nNOS is normally anchored to the sarcolemma through a direct association with syntrophin, but is mislocalized to the interior of the muscle fibre in DMD patients and mdx mice (Brennan et al., 1995, Brennan et al., 1996). These findings led to the hypothesis that mislocalized nNOS may result in free radical toxicity causing the observed dystrophic muscle pathology. The absence of nNOS would abnormally augment blood vessel constriction, effectively decreasing blood flow to contracting muscle, resulting in a dystrophic phenotype (Thomas et al., 1998). Further evidence to support the signaling role of nNOS in the DMD muscle pathology stems from the knockout mouse study involving α-dystrobrevin. α-dystrobrevin null mice display a muscular dystrophy phenotype that appears to be a result of disrupted muscle cell signaling (Grady et al., 1999). These mice retain the components of the dystrophin protein complex as well as the integrity of the sarcolemma, but display the dystrophic features characteristic of a mdx mouse (see section I.11). The observation that nitric-oxide-mediated signaling is significantly impaired, led to the proposition that the absence of α-dystrobrevin and nNOS from the membrane results in the dystrophic phenotype by blocking the generation or transmission of an activity-
dependent signal from the muscle fibres (Grady et al., 1999). This cannot however, be
the sole explanation for the muscular dystrophy phenotype since the pathological changes
observed in nNOS/dystrophin double mouse mutants do not differ significantly from
those of the dystrophin-deficient mdx mice (Huang et al., 1993, Chao et al., 1998, Crosbie
et al., 1998). Furthermore, α1-syntrophin deficient mice, who are also lacking
sarcolemmal nNOS, do not display a dystrophic phenotype (Kameya et al., 1999, Adams
et al., 2000). Together, these analyses outline the complexity of the molecular basis of
DMD, and suggest that the phenotype is the result of both structural and signaling
defects. In fact, the non-muscle phenotypes of DMD are more easily explained by this
broadened functional hypothesis.

Dystrophin's role in the positioning or anchoring of receptors/channels is also
prominent at the synapse. Much of the work focussing on synapse structure and function
has been based on the neuromuscular junction (for review see see Hall and Sanes, 1993,
Sanes and Lichtman, 1999). At this specialized nerve-to-muscle contact, dystrophin is
localized in the troughs of the junctional folds at the post-synaptic face (Sealock et al.,
1991, Bewick et al., 1992). Through an association with its extended complex of
proteins, dystrophin functions in both the structural and signaling aspects of the
neuromuscular junction (Figure 1.4). The clustering of synaptic proteins at the
neuromuscular junction is induced by agrin, a neuronally-derived extracellular matrix
protein that binds to α-dystroglycan and the MuSK complex (Gee et al., 1994, Glass et
al., 1996a, reviewed in Bowe and Fallon, 1995). MuSK is a transmembrane muscle-
Figure 1.4: The Neuromuscular Junction. Dystrophin (Dp427) is a member of an extended complex of proteins at the post-synaptic face of the nerve-to-muscle synapse. Agrin is a neuronally derived extracellular matrix protein that binds to α-dystroglycan (alpha) and the muscle-specific kinase (MuSK) complex. It induces the clustering of acetylcholine receptors (AChRs) through the activation of MuSK. Its interaction with MuSK is indirect as it binds to an unknown muscle specific specificity component (MASC) rather than directly to MuSK. The clustering and phosphorylation (P) of AChRs is facilitated by rapsyn, a 43kDa protein that binds to AChRs, β-dystroglycan (beta) and indirectly to the ectodomain of MuSK via a rapsyn-associated transmembrane linker (RATL).
specific receptor tyrosine kinase that is required for the formation and maintenance of the neuromuscular junction (Valenzuela et al., 1995, Glass et al., 1996b). It is involved in the augmentation of synapse-specific transcription and the mediation of acetylcholine receptor (AChR) phosphorylation and clustering (Valenzuela et al., 1995, DeChiara et al., 1996, Gillespie et al., 1996, Glass et al., 1997). The activation of MuSK by agrin is an indirect process, occurring through the as yet unidentified muscle specific specificity component (MASC) (Glass et al., 1996a). In a similar fashion, the effect of MuSK on AChR clustering is also indirect, being mediated by a 43kDa cytoplasmic protein, rapsyn (Frail et al., 1988, Gillespie, 1996). The interaction between rapsyn and β-dystroglycan functionally links the MuSK signaling complex to the dystrophin protein complex (Gautam et al., 1995, Apel et al., 1995). Dystrobrevin and syntrophin are also concentrated at the neuromuscular junction and have been discussed in detail in sections I.5.4 and I.5.5 respectively. Together, these proteins form a very specialized complex at the neuromuscular junction, essential for post-synaptic structural and functional organization.

The localization of dystrophin to interneuronal synapses and the prevalence of both cognitive and retinal defects in DMD patients suggests that dystrophin may also be important for the structure and function of nerve-to-nerve contacts. However, in comparison to the neuromuscular junction, relatively little is known about interneuronal synapses. The identification of proteins such as β-dystrobrevin and Dp260 (see section I.7.2), members of the dystrophin complex that are not expressed in muscle but are highly expressed in neuronal cells of the central nervous system, provides evidence for the formation of unique complexes at the synapses of the central nervous system (D'Souza et
al., 1995, Peters et al., 1997b, Blake et al., 1998). Therefore, the identification and characterization of dystrophin and its associated proteins in the CNS provides a unique opportunity not only to investigate the function of dystrophin, but also to study aspects of interneuronal synaptic structure and function in general.

Since each dystrophin-associated protein contributes to the overall function of the complex, the regulation of complex components provides a method to control function. For example, the tissue and cell-type specific expression patterns of the syntrophin and dystrobrevin isoforms allow for the creation of several unique dystrophin protein complexes. The subsequent recruitment of associated signaling proteins could also tailor the specific functions of the complexes. Furthermore, as discussed in section I.5.5, not all syntrophin-associated signaling proteins interact with all syntrophin isoforms. The potential to create countless unique dystrophin protein complexes, each with a specific function, provides the framework for the multiple functions of the dystrophin complexes. Importantly, as new proteins associated with the extended dystrophin complex are identified, our knowledge of the proposed mechanisms continues to grow.

I.7 Dystrophin Isoforms

The complex gene structure of dystrophin allows for the generation of several distinct protein products that form a family of dystrophin isoforms (Ahn and Kunkel, 1993, Sadoulet-Puccio and Kunkel, 1996). These tissue-specific dystrophin isoforms have the capacity to interact with specific dystrophin-associated proteins creating further diversity in the composition of the dystrophin protein complexes. Seven independent promoters drive the expression of tissue-specific dystrophin transcripts (Figure I.5). The position of each promoter determines the size and protein domain structure of the
Figure 1.5: Dystrophin Isoforms. There are at least seven promoters scattered throughout the dystrophin gene that give rise to tissue-specific dystrophin isoforms with unique structures and functions. The position of the promoter determines the size and protein domain structure of each isoform. Three tissue-specific promoters drive the expression of full-length dystrophin transcripts: brain (B), muscle (M), purkinje (P). Each contains a unique first exon that is spliced in-frame to a common exon 2. The resultant proteins are 427kDa (Dp427). The positions of the remaining intronic promoters in relation to the exons of the dystrophin gene are depicted. Each generates a dystrophin isoform with a specific domain structure. Dp260, Dp116 and Dp71 all contain unique N-terminal domains. Abbreviations: H1-H4 (hinge regions within the rod domain), WW (WW domain), CYS (cysteine-rich domain), C-term (C-terminal domain).
individual isoforms. Each protein product is therefore named according to its predicted molecular weight.

I.7.1 Dp427

There are at least three distinct tissue-specific promoters that produce full-length dystrophin transcripts, resulting in a 427kDa protein (Dp427) (Klamut et al., 1990, Boyce et al., 1991, Gorecki et al., 1992, Holder et al., 1996). Each utilizes a unique first exon that is spliced in-frame to the common second exon of dystrophin. The muscle promoter, active in all muscle types (skeletal, cardiac and smooth) is located between the two promoters that control expression of dystrophin in the brain (Klamut et al., 1990). One brain promoter is active in hippocampal and cortical pyramidal neurons, while the other drives the expression of dystrophin in cerebellar Purkinje cells (Boyce et al., 1991, Holder et al., 1996).

In skeletal muscle, dystrophin is localized to the cytoplasmic face of the sarcolemma and is enriched at specialized regions including myotendinous and neuromuscular junctions (Carpenter et al., 1990, Sealock et al., 1991, Zubrzycka-Gaarn et al., 1988, 1991). In cardiac muscle, immunocytochemical studies have shown peripheral staining around cardiocytes and an additional association with T-tubules, an important difference between cardiac and skeletal muscle (Klietsch et al., 1993). In the brain, Dp427 is found in the soma and dendrites of cortical and hippocampal neurons and in cerebellar Purkinje cells (Lidov et al., 1990, Huard and Tremblay, 1992, Lidov et al., 1993). Its association with the post-synaptic membrane of neurons and its presence in post-synaptic densities suggest it may have a role in synapse structure or function (Lidov
et al., 1990, Kim et al., 1992). Dp427 is also localized to the first synaptic layer of the retina, the outer plexiform layer, where it is found in the photoreceptor termini (Pillers et al., 1993, D’Souza et al., 1995, Howard et al., 1998a). In contrast to brain, this punctate expression is localized to the pre-synaptic face of the interneuronal retinal synapse (Ueda et al., 1995, Schmitz and Drenckhahn, 1997a, 1997b, Ueda et al., 1998).

1.7.2 Dp260

Dp260 was recently identified in our laboratory as a retinal specific isoform of dystrophin (D’Souza et al., 1995). An internal promoter and a unique 39 base pair first exon are located in intron 29, initiating a transcript that is spliced in-frame to exon 30 (D’Souza et al., 1995). An isoform-specific antibody has localized Dp260 to the synaptic outer plexiform layer of the retina where it plays a role in synaptic neurotransmission (Howard et al., 1998a). Lower levels of Dp260 have been observed in brain and heart, however its precise localization in these tissues remains unknown (D’Souza et al., 1995, Paula Demacio, personal observation).

Since Dp260 is lacking the N-terminal actin-binding domain, there is still a question as to whether Dp260 can form a full “structural bridge” between the actin cytoskeleton and the dystroglycan complex. The presence of the additional actin-binding site in the rod domain, a region included in Dp260, suggests that an interaction with actin is possible (Rybakova et al., 1996, Amann et al., 1998). Despite this potential, it is clear that Dp260 does not perform a function identical to Dp427. Firstly, Dp427 is expressed throughout the body, while Dp260 is predominantly expressed in the retina. Secondly, the study of patients and mice with mutations affecting one or both of these isoforms
indicates that, unlike Dp260, Dp427 is not required for normal neurotransmission across the retina (Pillers et al., 1995, Kameya et al., 1997, Pillers et al., 1999).

1.7.3 Dp140

Dp140 is most highly expressed in the brain and kidney, but is absent in skeletal muscle, heart, lung, liver and spleen (Lidov and Kunkel, 1997). In the brain, Dp140 is expressed in the glial cells surrounding the microvasculature, while in the kidney, it is found in the developing tubules. The unique promoter and first exon of Dp140 are located in intron 44 generating a 7.5kb transcript (Lidov et al., 1995). In contrast to the other dystrophin isoforms, the 109 base pair first exon does not contain an initiator methionine, and as a result, translational initiation occurs at a methionine in exon 51 (Lidov et al., 1995). This generates a protein containing the last five spectrin repeats of the rod domain in addition to the WW, cysteine-rich and C-terminal domains.

1.7.4 Dp116

Dp116 is a peripheral nerve specific isoform that is localized to the external layer of Schwann cells and is concentrated at an important nerve conduction site, the node of Ranvier (Byers et al., 1993). The Dp116 promoter lies between exons 55 and 56 giving rise to a 5.2 kb transcript with a unique first coding exon that is spliced in frame to exon 56 (Byers et al., 1993). The resultant protein contains a unique N-terminus, two distal repeats of the rod domain, the WW, cysteine-rich and C-terminal domains.
1.7.5 Dp71

Dp71 is the smallest and most ubiquitously expressed dystrophin isoform. Its promoter and unique first exon are found in intron 62. This gives rise to a dystrophin protein with a unique 7 amino acid N-terminus preceding the cysteine-rich and C-terminal domains (Hugnot et al., 1992, Lederfein et al., 1992, Lederfein et al., 1993). The fusion of this unique N-terminus with the remainder of the dystrophin protein creates a new actin binding site allowing Dp71 to bind to the actin cytoskeleton (Howard et al., 1998b). Although Dp71 can be found in all tissues, it acquired the label of the "dystrophin non-muscle isoform" due to its low level of expression in mature skeletal muscle (Bar et al., 1990, Leiderfein et al., 1992, Lambert et al., 1993). A Dp71 knockout mouse model was created in which the unique first exon was replaced with a β-galactosidase reporter gene (Sarig et al., 1999). X-gal staining of null mice enabled a detailed study of Dp71 expression. It was observed that Dp71 is expressed in a tight stage and cell-type specific manner. In the CNS, Dp71 can be found surrounding the microvasculature and lining the endfeet of Müller glial cells at the retinal inner limiting membrane (Howard et al., 1998a). The absence of Dp71 results in abnormal retinal neurotransmission, linking this isoform to retinal synaptic function (Pillers et al., 1999).

The small size of Dp71 gave hope to the idea that this isoform may be used to circumvent the difficulties encountered in gene therapy efforts due to the huge size of full-length dystrophin. To test its potential to functionally substitute for Dp427, DMD mice transgenic for Dp71 under the muscle creatine kinase promoter were generated. Dp71 was able to reconstitute the dystrophin protein complex at the sarcolemma, but was not sufficient to eliminate the dystrophic phenotype (Cox et al., 1994, Greenberg et al., 1993).
1994). In fact, the muscle phenotype worsened, indicating that the structural differences between Dp71 and Dp427 are substantial enough that they are not functionally exchangeable.

I.8 Alternative Splicing of Dystrophin

Alternative splicing is a powerful mechanism able to increase the functional capacity of a gene. In addition to the tissue-specific promoter usage that generates a family of N-terminal truncated dystrophin isoforms, regulated variation in mRNA processing produces several alternatively spliced variants of the dystrophin transcripts, adding to the complexity of the dystrophin family of proteins. There are two major alternatively spliced regions in the C-terminal domain of the dystrophin gene (Figure I.6a) (Feener et al., 1989, Bies et al., 1992, Austin et al., 1995). Exons 71-74 can be spliced individually or in combination generating a series of in-frame spliced variants. Exclusion of exons 73 and 74 in any of these transcripts produces a functional protein that is lacking the syntrophin binding sites (Ahn and Kunkel, 1995, Suzuki et al., 1995). The elimination of the binding site for this dystrophin-associated protein may represent a mechanism for the specialization of dystrophin protein complexes.

Exon 78, the penultimate exon of dystrophin, is the other region susceptible to alternative splicing. Inclusion of this exon within the mRNA transcript generates a 13 amino acid, mainly hydrophilic C-terminal region (Figure I.6b). Exclusion of exon 78 alters the translational reading frame generating a 31 amino acid, mainly hydrophobic C-terminus (Figure I.6c). The expression of these unique termini is cell-type specific and developmentally regulated (Feener et al., 1989, Bies et al., 1992, Austin et al., 1995,
Figure 1.6: Alternative Splicing of the Dystrophin C-terminus. a) The two main regions of alternative splicing at the 3' end of the dystrophin gene are schematically depicted. Exons 71-74 can be spliced individually or in combination generating multiple in-frame spliced variants. The absence of exons 73 or 74 in any transcript results in a protein lacking the syntrophin binding sites. The other region susceptible to alternative splicing is exon 78. The alternative splicing of penultimate exon 78 substantially alters the C-terminus of the protein. b) Inclusion of exon 78 generates a 13 amino acid, mainly hydrophilic C-terminus. c) Exclusion of exon 78 changes the translational reading frame, resulting in a unique exon 79-encoded, 31 amino acid hydrophobic C-terminus.
Howard et al., 1998a). For example, in the retina, Dp427 and Dp260 predominantly contain the hydrophilic C-terminus at the outer plexiform layer, while Dp71 contains the hydrophobic C-terminus and is differentially localized to the inner limiting membrane (Howard et al. 1998a). At present, the functional impact of the hydrophobic C-terminus on the dystrophin complex is unknown. However, an examination of its sequence and tissue-specific expression across species shows a high level of conservation, indicating a functional significance for this splicing event (Figure 1.7) (Roberts and Bobrow, 1998). The observation that the hydrophobic C-terminus displays a unique tissue localization pattern and the prospect that the change in hydrophobicity likely alters the folding of the C-terminal region of dystrophin, suggest that a new protein binding site in the hydrophobic C-terminus may be exposed. In this way, the alternative splicing of exon 78 may represent yet another regulated mechanism for the creation of unique dystrophin complexes.

I.9 Dystrophin Isoform Diversity

It has been generally accepted that the dystrophin protein complex provides both structural support to the membrane and a scaffold for the positioning or anchoring of signaling molecules (Hardiman, 1994). The specialized functions of the specific complexes are dictated by the various dystrophin-associated proteins. The existence of several dystrophin isoforms, each having the potential to be alternatively spliced, and each containing some or all of the known protein binding sites, provides a platform for the generation of countless distinct protein complexes. Furthermore, as discussed in section I.7.5, the fact that Dp71 cannot functionally compensate for Dp427 indicates that
**Dystrophin Hydrophilic C-terminus**

<table>
<thead>
<tr>
<th>Species</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>GRNTPGKPMREDTM*</td>
</tr>
<tr>
<td>Mouse</td>
<td><em><strong>A</strong></em>***********</td>
</tr>
<tr>
<td>Chicken</td>
<td>*<strong>A</strong><em><strong><strong>V</strong></strong></em></td>
</tr>
<tr>
<td>Xenopus</td>
<td><strong>IV</strong><em>Q</em>**E*</td>
</tr>
<tr>
<td>Dogfish</td>
<td>L<em>DSY**QVK</em>V*</td>
</tr>
</tbody>
</table>

**Dystrophin Hydrophobic C-terminus**

<table>
<thead>
<tr>
<th>Species</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
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</tr>
<tr>
<td>Mouse</td>
<td>***---------------------------------------------------------------------</td>
</tr>
<tr>
<td>Chicken</td>
<td>S***********<em>TTTTDKVL</em></td>
</tr>
<tr>
<td>Xenopus</td>
<td>GT********<em><em>TTTTDK</em>L</em></td>
</tr>
<tr>
<td>Dogfish</td>
<td>RN********<em><em>TTTI</em>A</em>A*</td>
</tr>
</tbody>
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**Figure 1.7:** Sequence Conservation of the Dystrophin C-termini. Sequence analysis shows a high level of sequence conservation across species within the hydrophobic C-terminus suggesting a functional significance for the exon 78 alternative splicing event. Only different amino acids are indicated.
individual isoform structure is closely related to its function. In addition to structural
differences, each dystrophin isoform has a tissue-specific expression pattern with cellular
and subcellular specializations. The retina provides a perfect model to examine the
diversity of dystrophin (see section I.12). Dp71, the smallest dystrophin isoform is
localized to the non-synaptic inner limiting membrane and surrounding retinal blood
vessels where it contains the hydrophobic C-terminus (Howard et al., 1998a). This is in
great contrast to the localization of Dp260 and Dp427 at the synaptic outer plexiform
layer of the retina. In comparison with Dp71, the protein structures of these two
dystrophin isoforms differ at both termini. Dp427 and Dp260 each have unique N-
terminal structures, and mainly contain the hydrophilic C-terminus. The functional
significance underlying the different structures and functions of these dystrophin
isoforms remains unknown, but it is our hypothesis that the composition of the extended
complexes is different, thus defining the function of the distinct complexes.

I.10 Dystrophin Paralogs

Dystrophin is a member of a superfamily of structurally and functionally related
proteins that include utrophin, dystrophin-related protein 2 (DRP2), α-dystrobrevin and
β-dystrobrevin (Figure I.8) (Hoffman et al., 1987, Tinsley et al., 1992, Blake et al.,
1996a, Roberts et al., 1996). There is substantial overlap in localization, function and
protein interactions throughout this family of proteins, which complicates the
characterization of each individual member.

Utrophin (DRP1) has the most extensive sequence homology to dystrophin and
contains similar protein binding properties (Love et al., 1989). This autosomal gene
displays a conserved domain structure to dystrophin, is found on the long arm of human
Figure 1.8: Dystrophin Paralogs. The domain structure of the five members of the dystrophin superfamily of proteins is illustrated. Dystrophin and utrophin are the most similar containing an N-terminal actin-binding domain (N-term), an extended rod domain, a cysteine-rich domain (CYS), a WW domain (WW) and a C-terminal domain (C-term). DRP2, α-dystrobrevin and β-dystrobrevin each have unique N-terminal domains (shown in white). The dystrobrevins have C-terminal dystrobrevin-unique (DU) extensions in addition to the cysteine-rich and C-terminal domains.
chromosome 6 and encodes a protein of 395kDa (Buckle et al., 1990, Khurana et al., 1990, Blake et al., 1996b). The slight reduction in size compared to dystrophin is due to the absence of spectrin repeats 15 and 19 in the rod segment of the protein (Winder et al., 1995a). Similar to dystrophin, utrophin is abundantly expressed at differing levels in a wide variety of tissues and is able to bind to actin and members of the dystrophin protein complex (Khurana et al., 1991, Nguyen et al., 1991, Matsumura et al., 1992, Winder et al., 1995b, Blake et al., 1996b). In adult skeletal muscle, utrophin becomes concentrated at the neuromuscular junction where it is thought to play a role in the maintenance of synaptic structure (Khurana et al., 1991, Nguyen et al., 1991, Ohlendieck et al., 1991). Since there is no disease associated with mutations in utrophin and no naturally occurring mouse mutations, utrophin knockout mice were generated to observe any pathological consequences due to the absence of utrophin (Deconinck et al., 1997a, Grady et al., 1997). The resultant phenotype was minor, showing only a slight reduction in membrane folding at the neuromuscular junction in addition to a reduction in the total number of acetylcholine receptors. However, when the absence of utrophin is combined with the absence of dystrophin in a double knockout mouse, the phenotype is more severe than the combination of the two individual phenotypes, suggesting that utrophin and dystrophin have complementing rather than identical roles (Deconinck et al., 1997b). Postsynaptic membrane folds at the neuromuscular junction are virtually absent in these mice, body weight is reduced, abnormal breathing patterns occur and progressive muscle weakness is evident. The most severe consequence is premature death at 20 weeks of age. Since these mice exhibit all of the clinical features of DMD, it has been suggested that this represents a more valid murine model for the study of DMD.
The severe phenotype associated with the double knockout mouse is supported by the observation that in DMD mice, a deficiency of dystrophin is often associated with an upregulation of utrophin synthesis in the dystrophic muscle fibres, potentially providing functional compensation (Ohlendieck et al., 1991). Although it is not sufficient to eliminate the dystrophic phenotype of patients, compensation by utrophin may lessen the potential muscle damage that would occur if utrophin were also absent. The partial overlapping functions, along with the compensation demonstrates yet another difficulty in the molecular interpretation of the DMD phenotype.

DRP2 is another member of the dystrophin superfamily of proteins that is encoded by a separate gene on the X-chromosome (Xq22) (Roberts et al., 1996). Transcription initiation can occur from two adjacent start sites generating protein products that appear as a quartet of bands (100-120kDa) on a Western blot. The overall protein structure of DRP2 is most similar to dystrophin isoform Dp116, containing two spectrin-like repeats, a cysteine-rich and a C-terminal domain. It is expressed predominantly in the brain and spinal cord where it is found on neuronal dendrites and enriched in the post-synaptic density.

α and β-dystrobrevin are also members of the dystrophin family of proteins and have been discussed in detail in section I.5.4.

The existence of several dystrophin paralogs allows for the formation of countless specialized extended protein complexes that in some cases, have overlapping patterns of expression. Since the exact role of each dystrophin family member is unknown, it is difficult to unravel the functional contributions of each.
L11 Duchenne Muscular Dystrophy Mouse Models

Much of our understanding of dystrophin function has stemmed from the creation and use of DMD mouse models. These mice have facilitated studies examining functionally relevant regions of dystrophin, specific protein-protein interactions as well as detailed investigations of individual isoform functions. The original DMD mdx mouse arose spontaneously in the C57BL/10 strain, and has a nonsense mutation in exon 23 causing premature termination of the full-length dystrophin protein (Sicinski et al., 1989, Bulfield et al., 1984). With the exception of Dp427, all dystrophin isoforms are expressed normally. Four other mdx mutants, mdx<sup>2Cv</sup>-mdx<sup>5Cv</sup>, were created through N-ethylN-nitrosourea mutagenesis (Chapman et al., 1989, Cox et al., 1993). Each mdx allelic variant contains a unique mutation that affects a specific subset of dystrophin isoforms (Figure 1.9) (Im et al., 1996). The mdx<sup>2Cv</sup> mouse has a single base change in the splice acceptor sequence of intron 42 that leads to a series of aberrant splicing events (Im et al., 1996). As none of the resultant transcripts preserves the open reading frame, both Dp427 and Dp260 are disrupted. The mdx<sup>3Cv</sup> allelic variant has a mutant splice acceptor in intron 65 that results in a translational frameshift and the elimination of all dystrophin isoforms (Cox et al., 1993). It has been reported that low levels of this aberrant transcript can accumulate due to alternative splicing, leading to the production of dystrophin protein lacking part of the cysteine-rich domain encoded by exons 65 and 66 (Cox et al., 1993). This questions the validity of the mdx<sup>3Cv</sup> mouse as a model for DMD. However, when expressed in a transgenic mdx mouse, this internally deleted protein product is non-functional, explaining the identical phenotypes observed in mdx and mdx<sup>3Cv</sup> mice (Rafael et al., 1996). mdx<sup>4Cv</sup> mice have a C to T transition in exon 53 which creates a stop codon
Figure I.9: Duchenne Muscular Dystrophy Mouse Models. Five $mdx$ allelic variants exist, each with a unique mutation that affects a specific subset of dystrophin isoforms. The position of each mutation is depicted in relation to the various dystrophin promoters. The chart outlines the presence (+) or absence (-) of the dystrophin isoforms in the DMD mouse models.
and disrupts all dystrophin isoforms except Dp116 and Dp71 (Im et al., 1996). Finally, mdx5Cv mice have a single A to T transversion that creates a new splice donor, resulting in the deletion of 53 base pairs from exon 10 and premature translation termination. Similar to the mdx mouse, Dp427 is the only isoform affected (Im et al., 1996).

In comparison to the human DMD phenotype, all mdx mice display a much milder muscle phenotype (Coulton et al., 1988). Around the time of weaning, the dystrophin-deficient mice go through a phase of muscle necrosis, which is accompanied by an elevation in serum creatine kinase levels. Shortly thereafter, the skeletal muscle fibres recover through a rapid regeneration process and the mice appear physically normal and have a life span comparable to wild-type mice. Apart from a progressive degeneration of the mdx diaphragm, this is in great contrast to the ongoing muscle degradation observed in humans. The murine retinal phenotype, on the other hand, is very similar to that observed in humans, making the mouse an excellent model for the study of dystrophin in the retina (refer to section I.12) (Pillers et al., 1995, 1999). In fact, the existence of the various mdx mice expressing unique subsets of dystrophin isoforms has allowed for a detailed examination of the functional contribution of individual isoforms expressed in the retina.

I.12 Dystrophin in the Retina—A Case Study

The retina is a well-characterized and highly organized accessible tissue that provides an excellent in vivo system in which to study the complexities of dystrophin function. Not only does this neural tissue express several dystrophin isoforms, there is differential localization based on isoform size and C-terminal splicing. Further, the
available *mdx* mouse models and a measurable electroretinogram (ERG) phenotype allow for a detailed analysis of dystrophin function in this tissue.

The observation that many boys with DMD have abnormal retinal neurotransmission led to the study of dystrophin in the retina (Pillers et al., 1993, Cibis et al., 1993, Sigesmund et al., 1994, Fitzgerald et al., 1994). The retina is composed of three nuclear layers, which are separated by two synaptic plexiform layers (Figure I.10). Incident light enters the retina and reaches the photoreceptors (outer nuclear layer) at the back of the retina. This light signal is then transformed into electrical and chemical signals that are passed from the photoreceptors across the outer plexiform synaptic layer to the bipolar and horizontal cells of the inner nuclear layer (for review see Wu, 1994, Yau, 1994). The bipolar cells then transmit this information across the inner plexiform layer to the ganglion cell layer, the third nuclear layer of the retina. Finally, the signal is carried through the axons of the ganglion cells and out the optic nerve to the visual cortex of the brain. Müller glial cells span almost the entire retina and provide support, nourishment and regulation to these retinal neurons (Newman and Reichenbach, 1996).

This understanding of retinal structure has provided a wonderful platform for the initial studies of dystrophin function.

Western blot analysis and immunofluorescence studies show that three dystrophin isoforms, Dp427, Dp260 and Dp71, are expressed in the retina and are localized to three distinct areas: the outer plexiform layer (OPL), the inner limiting membrane (ILM) and surrounding retinal blood vessels (Figure I.11) (Pillers et al., 1993, D'Souza et al., 1995, Howard et al., 1998a). The subsequent development of isoform specific antibodies and the existence of several *mdx* mutant mouse models allowed for a more detailed
Figure I.10: Schematic Representation of Retinal Structure. The retina is a beautifully organized tissue composed of three nuclear layers and two synaptic layers. The outer nuclear layer (ONL) contains the photoreceptor rods (R) and cones (C), and the inner nuclear layer (INL) contains the bipolar (B) and horizontal (H) cells. The third nuclear layer, the ganglion cell layer (GCL), contains the ganglion (G) cells. The light-induced electrical and chemical signals produced by the photoreceptors are transmitted to the INL across the outer plexiform layer (OPL), the first synaptic layer of the retina. The signal is then passed across the inner plexiform layer (IPL) to the GCL. The axons of the ganglion cells then carry the signal to the optic nerve. Müller cells (M) are the principal glial cells of the retina and serve to nourish, support and regulate the retinal neurons. The Müller cells span almost the entire retina from the outer limiting membrane (OLM) to the inner limiting membrane (ILM). (Modified from Farber and Adler, 1986).
Figure L.11: Dystrophin in the Retina. Immunofluorescence staining showing dystrophin expression in the retina. a) A phase contrast image of a retinal section. b) A wildtype mouse retinal section stained with an antibody specific for the hydrophobic C-terminus of dystrophin (ACT-1). This antibody is known to recognize all three dystrophin isoforms expressed in the retina: Dp427, Dp260 and Dp71. Staining is observed at the outer plexiform layer (OPL), the inner limiting membrane (ILM) and surrounding retinal blood vessels. Other immunofluorescence experiments have demonstrated that the punctate staining at the OPL is due to Dp427 and Dp260, while Dp71 is localized to the ILM and microvasculature (Pillers et al., 1993, D’Souza et al., 1995, Howard et al., 1998a). Scale bar: 35μm.
investigation examining the expression patterns of the individual dystrophin isoforms. It is now well documented that Dp427 and Dp260 are expressed at the OPL, the first synaptic layer of the retina where the photoreceptors contact the bipolar and horizontal cells of the inner nuclear layer (D'Souza et al., 1995, Howard et al., 1998a). Dp71, on the other hand, is responsible for the staining observed at the non-synaptic ILM and surrounding retinal blood vessels (Howard et al., 1998a). This differential localization immediately suggests that the individual dystrophin isoforms have unique functions at the synaptic versus non-synaptic locations. A closer analysis of these isoforms revealed that the dystrophin isoforms at the OPL mainly contain the hydrophilic C-terminus, while Dp71 almost exclusively contains the hydrophobic C-terminus (Figure 1.12) (Howard et al., 1998a). This provides a potential mechanism for further specialization of the distinct dystrophin complexes in the different regions of the retina.

Any correlation between dystrophin localization and its potential role in retinal neurotransmission requires an understanding of the underlying events leading to the electroretinogram (ERG) recording. The ERG is a measure of the voltage change across the retina in response to stimulation by a flash of light. It is a direct reflection of the summed electrical potential generated by the cells of the retina. In a dark-adapted (scotopic) state, the onset of light results in neuronal activation and an increase in K⁺ concentration at the outer plexiform layer of the retina (Fishman and Sokol, 1990). The rapid removal of these K⁺ ions is essential to limit fluctuations in neuronal excitability. This is accomplished through a spatial-buffering mechanism performed by retinal Müller cells (Newman et al., 1984). The K⁺ is taken up by the Müller cells and effectively redistributed to regions of low K⁺ concentration at the Müller cell endfeet (Newman and
Figure I.12: Differential Localization of Dystrophin C-termini in the Retina. a) A phase contrast image of a retinal section. b) Wildtype mouse retinal section stained with an antibody specific for the dystrophin hydrophilic C-terminus shows localization at the outer plexiform layer (OPL). This coincides with Dp427 and Dp260 expression. c) Dp71 with the hydrophobic C-terminus, visualized using antibody ACT-1, is predominantly expressed at the inner limiting membrane (ILM) and surrounding retinal blood vessels. Scale bar: 35 μm.
The return current through the extracellular space generates a positive transretinal potential that is recorded as the ERG b-wave (Stockton and Slaughter, 1989).

Although there are no clinically manifesting retinal phenotypes associated with DMD, many patients and mdx mice have an ERG b-wave with a reduced amplitude and a significantly delayed implicit time (Pillers et al., 1993, Cibis et al., 1993, Sigesmund et al., 1994, Fitzgerald et al., 1994, Pillers et al., 1995, Pillers et al., 1999). Upon close investigation, there is a clear correlation between the position of the dystrophin mutation and the severity of the ERG phenotype (Figure I.13) (Pillers et al., 1995, Pillers et al., 1999). Mice with mutations at the 5' end of the gene (mdx and mdx^SCv), lack dystrophin isoform Dp427, but express Dp260 and Dp71 normally. These mice have normal ERG recordings, indicating that Dp427 is not essential for the production of the ERG b-wave (Cibis et al., 1993, Pillers et al., 1995, Pillers et al., 1999). Mice with mutations disrupting both Dp427 and Dp260 (mdx^SCv and mdx^4Cv) lack dystrophin expression at the OPL, but express Dp71 at the ILM and surrounding retinal blood vessels. ERG analysis of these mice shows an increase in b-wave implicit time, indicating that Dp260 is required for normal b-wave generation (Kameya et al., 1997, Pillers et al., 1999). Only when the retina is completely devoid of all three retinal dystrophin isoforms (mdx^3Cv) do the mice display a significant reduction in b-wave amplitude in addition to the increase in implicit time (Pillers et al., 1995, Pillers et al., 1999). These results demonstrate that Dp260 and Dp71 each has a unique, yet essential role in normal retinal neurotransmission.
<table>
<thead>
<tr>
<th>C57</th>
<th>mdx</th>
<th>Mdx$^{4Cy}$</th>
<th>Mdx$^{3Cy}$</th>
</tr>
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<tbody>
<tr>
<td>•Dp427</td>
<td>•Dp260</td>
<td>•Dp260</td>
<td>•Dp71</td>
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<td>•Dp260</td>
<td>•Dp71</td>
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**Figure I.13:** Electroretinogram analysis of Duchenne Muscular Dystrophy Mutant Mice. The electroretinogram (ERG) recordings for one wildtype mouse (C57) and three mdx allelic variants are illustrated. The position of the mdx mutation and the resultant disruption of specific dystrophin isoforms is directly related to the severity of the ERG. Wildtype C57 mice express Dp427, Dp260 and Dp71 and have an ERG b-wave with a normal amplitude and implicit time. Mdx mice have a mutation that disrupts Dp427 without affecting the expression of Dp260 or Dp71. These mice also have a normal ERG indicating that Dp427 is not essential for the generation of a normal ERG. Mdx$^{4Cy}$ mice do not express any dystrophin at the outer plexiform layer, but do express Dp71 at the inner limiting membrane and surrounding the microvasculature. Although these mice have a normal b-wave amplitude, the implicit time is significantly lengthened. Mdx$^{3Cy}$ mice are completely devoid of dystrophin and have the most severe ERG phenotype. The b-wave amplitude is severely reduced in addition to the increase in implicit time.
The clear genotype/phenotype correlation observed in DMD patients and mice suggests that the two smaller dystrophin isoforms expressed in the retina perform unique roles in the generation of a normal ERG b-wave. To determine the individual roles of these isoforms, the precise subcellular localizations were analyzed using electron microscopic techniques. At the OPL, dystrophin and its membrane anchor β-dystroglycan are located presynaptically on the photoreceptor plasma membrane that borders the lateral sides of the invaginated synaptic triad (Schmitz and Drenckhahn, 1997a, 1997b, Ueda et al., 1995, Ueda et al., 1998). This region is distal to the active synaptic zone that contains the synaptic ribbon, indicating that dystrophin’s effect on synaptic transmission is likely indirect. The Dp71 immunolabel lines the endfezt of the Müller glial cells where they contact the ILM and microvasculature (Howard et al., 1998a). This corresponds precisely to the locations of K⁺ efflux suggesting that Dp71 may be anchoring or clustering these channels at the appropriate places on the Müller cell membrane.

Despite our knowledge outlining the differential localization and function of dystrophin isoforms expressed in the retina, the molecular basis for these differences is unknown. Apart from an interaction with β-dystroglycan, nothing is known about the composition of the dystrophin complexes at the different layers of the retina. Importantly, there are no morphological abnormalities associated with the absence of dystrophin in the retina, suggesting that the dystrophin complex is not likely responsible for the mechanical stability of retinal cells (Blank et al., 1999). This suggests that the ERG phenotype is due to a disruption in dystrophin-associated signaling rather than
scaffolding. Since the function of dystrophin is determined by the composition of the extended protein complex, the characterization of these complexes will provide crucial information regarding the different functions of dystrophin in the retina.

1.13 Summary

A complete understanding of dystrophin’s multiple functions has been hindered by the lack of information surrounding the composition of the extended dystrophin protein complexes. In addition, the functional significance underlying the existence of numerous tissue-specific dystrophin isoforms and how their unique structures relate to function is unknown. The work in this thesis demonstrates that cell-type specific dystrophin complexes exist in the retina, the functions of which are determined by their specialized extended complexes of associated proteins.
Chapter II

Alternative Splicing of the Dystrophin C-terminus Exposes a New Protein Binding Site for α-catulin

I performed the majority of the experiments in this chapter. Jeff Wong, a former summer student in the lab, generated the dystrophin hydrophobic C-terminus bait construct. Felipe Cisternas, a former graduate student, performed the brain yeast two-hybrid library screen and I performed the heart yeast two-hybrid screen. Melanie Wong, a fourth year thesis student, performed the immunofluorescence on the human retinal sections (Figure II.9).
II.1 Abstract

The Duchenne Muscular Dystrophy gene, dystrophin, is a very large and complex gene that gives rise to a family of diverse protein isoforms. Part of this diversity arises through alternative splicing at the 3' end of the gene. The most common alternative splicing event involves exon 78 and significantly alters the carboxy terminus of the dystrophin protein. Splicing of this penultimate exon changes the C-terminus from a 13 amino acid hydrophilic region to a 31 amino acid mainly hydrophobic terminus. The retina offers a unique opportunity to study the functional significance of this splicing event since the dystrophin spliced isoforms are differentially localized in this tissue. Dp427 and Dp260 mainly contain the hydrophilic C-terminus and are localized to the synaptic outer plexiform layer. Dp71, on the other hand, contains the hydrophobic C-terminus and is found at the non-synaptic inner limiting membrane, outer limiting membrane and surrounding retinal blood vessels.

In this chapter, a yeast two-hybrid approach was used to show that the exon 78 splicing event is functionally significant in the creation of unique protein complexes in the retina. We show that α-catulin, an α-catenin related protein, binds specifically to the hydrophobic C-terminus of dystrophin and colocalizes with Dp71 at the inner limiting membrane, outer limiting membrane and surrounding retinal blood vessels. The localization of α-catulin is disrupted in dystrophin-deficient retinas, suggesting that α-catulin plays a role in the altered neurotransmission observed in Duchenne Muscular Dystrophy patients and mice.
II.2 Introduction

The dystrophin gene gives rise to numerous isoforms through the selective use of multiple internal intronic promoters, creating various N-terminal truncated protein products (Ahn and Kunkel, 1993, Sadoulet-Puccio and Kunkel, 1996). The complexity of these isoforms is further increased through alternative splicing at the 3' end of the gene, producing unique C-termini that may serve to functionally diversify the protein products (Bies et al., 1992, Feener et al., 1989, Austin et al., 1995). One of the main splicing events involves the penultimate exon 78. Inclusion of this exon within the mRNA transcript generates a 13 amino acid hydrophilic C-terminal region. Exclusion of exon 78 results in a translational frameshift, leading to the production of an extended 31 amino acid, mainly hydrophobic C-terminus (HCT). In comparison to the hydrophilic C-terminus, the HCT displays differential tissue expression and localization patterns suggesting a unique functional role for this spliced region (Feener et al., 1989, Bies et al., 1992, Austin et al., 1995, Howard et al., 1998a). Moreover, the high sequence conservation of the dystrophin HCT across species further supports a functional significance for this splicing event (Roberts and Bobrow, 1998).

The retina is a unique tissue in which to study the functional significance of the differential promoter usage and splicing events that occur in the dystrophin gene. The three dystrophin isoforms expressed in the retina have non-overlapping patterns of localization based on isoform size and splicing. Dp427 and Dp260 are both found at the synaptic outer plexiform layer (OPL) predominantly containing the hydrophilic C-terminus (Pillers et al., 1993, D'Souza et al., 1995, Howard et al., 1998a). Dp71, on the other hand, almost exclusively contains the HCT at the non-synaptic inner limiting
membrane (ILM) and surrounding retinal microvasculature (Howard et al., 1998a). This regulated differential localization at synaptic versus non-synaptic retinal layers, with specific C-termini for the different isoforms strongly suggests that unique functions may result from distinct protein-protein interactions at each location.

Most Duchenne Muscular Dystrophy (DMD) patients and dystrophic mice have abnormal neurotransmission across the retina caused by the absence of the smaller dystrophin isoforms (Pillers et al., 1993, Cibis et al., 1993, Sigesmund et al., 1994, Fitzgerald et al., 1994, Pillers et al., 1999). In mouse models it has been shown that the absence of Dp260 or Dp71 generate different changes in retinal neurotransmission, however the molecular basis underlying the specific role of each isoform remains unclear (Kameya et al., 1997, Pillers et al., 1995, Pillers et al., 1999). In particular, there is no information on how the splicing of exon 78 and the subsequent altering of the dystrophin C-terminus may affect function. We hypothesize that the expression of the alternatively spliced HCT of dystrophin plays an integral part in the formation and function of unique dystrophin protein complexes in the retina. Specifically, we propose that this unique C-terminus interacts with a specialized protein, or group of proteins, that may serve to tailor the specific function of the extended dystrophin complex. We have therefore used a yeast two-hybrid approach to identify proteins that interact with the extended dystrophin HCT.

II.3 Results

II.3.1 Yeast Two-Hybrid Library Screen

The dystrophin hydrophobic C-terminus (HCT) displays a unique localization pattern in the retina (Howard et al., 1998a) and in the heart (P. Demacio, unpublished
observation). In order to identify proteins that interact with this specialized region of dystrophin, we used a dystrophin hydrophobic C-terminus bait construct (amino acids 3668 to 3698) fused to the Gal4 binding domain to screen a mouse brain yeast two-hybrid library and a human heart yeast two-hybrid library. The brain library was used in lieu of a retinal library, as a retinal yeast two-hybrid library with sufficient clonal complexity was not available.

For the heart library screen, yeast strain CG1945 was sequentially transformed with the dystrophin HCT bait construct and ~150μg of target human heart cDNA plasmids (Clontech). Transformed yeast were plated on SD selection media lacking histidine to select for colonies in which a protein-protein interaction was occurring. 60 His⁺ clones were obtained and were subsequently subjected to a filter β-galactosidase assay to confirm the His reporter results (data not shown). 21 His⁺lacZ⁺ colonies were obtained and the activation domain plasmids were isolated, sequenced and identified using BLAST analysis (http://blast.bioinfo.sickkids.on.ca). As a confirmation to verify the specific interaction with the dystrophin HCT and to eliminate false positives due to activation by the library plasmid alone, each His⁺lacZ⁺ activation domain plasmid was reintroduced into yeast alone, with the dystrophin HCT bait and with two non-specific activation domain plasmids, pVA3 and pLAM and subjected to β-galactosidase assays (data not shown). The clones identified as potential dystrophin HCT interacting proteins are outlined in Table II.1.

Several potential interacting proteins were identified in both yeast two-hybrid screens, however, one protein, α-catalin, was obtained once from each library. Since this interaction with the hydrophobic C-terminus of dystrophin was positive in two
Table II.1: Heart Yeast-Two Hybrid Clone Descriptions

<table>
<thead>
<tr>
<th>Clone Number</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>16.1, (4.5)</td>
<td>Human α-catenin</td>
</tr>
<tr>
<td></td>
<td>*Clone (4.5) was obtained from the brain library screen.</td>
</tr>
<tr>
<td>9.1, 13.1, 1.1</td>
<td>Human NAD(P)H menadione oxidoreductase 2</td>
</tr>
<tr>
<td>26.2</td>
<td>Human basic helix-loop-helix TF</td>
</tr>
<tr>
<td>20.1</td>
<td>Human succinate dehydrogenase iron-protein subunit</td>
</tr>
<tr>
<td>13.4</td>
<td>Human ADA3-like protein</td>
</tr>
<tr>
<td>15.1, 30.3</td>
<td>Human peroxisome biogenesis factor 10</td>
</tr>
<tr>
<td>13.5, 10.2, 18.2</td>
<td>Human mitochondrial DNA</td>
</tr>
<tr>
<td>16.2</td>
<td>Human cytosolic malate dehydrogenase</td>
</tr>
<tr>
<td>4.1, 7.1, 8.1, 13.3, 15.2, 23.3, 25.1, 29.2</td>
<td>novel</td>
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independent libraries representing two different tissues from two different species, α-catulin was selected as a candidate for further study.

α-catulin is a newly identified protein that has modest homology to both α-catenin and vinculin (Zhang et al., 1998, Janssens et al., 1999). Although sequence homology predicts potential talin, β-catenin and/or actin binding sites, essentially nothing is known about its function, localization or protein partners (Janssens et al., 1999).

II.3.2 *In Vitro* Binding Assays

An *in vitro* binding assay was used as an independent means to assess the interaction between the dystrophin hydrophobic C-terminus and α-catulin. Two separate dystrophin hydrophobic C-terminus glutathione S-transferase (GST) fusion constructs were created (Figure II.1), expressed in bacteria, batch purified and tested for expression using Western blot analysis (Figure II.2). The first was identical to the yeast two-hybrid bait construct and included only the 31 amino acids that comprise the dystrophin hydrophobic C-terminus (GST-HCT) (amino acids 3668-3698). To ensure that this short region of the dystrophin protein was able to fold properly, an extended dystrophin construct, including amino acids 3486 to 3698, was created (GST-LZHCT). α-catulin was prepared using an *in vitro* transcription/translation strategy generating full-length $^{35}$S-labelled protein. To test for interactions between the dystrophin hydrophobic C-terminus and α-catulin, the α-catulin protein was incubated with the bead-conjugated GST-fusion proteins in a pull-down experiment. In this assay, full-length α-catulin bound to both dystrophin hydrophobic C-terminus proteins but did not interact with the GST-alone or the GST-PHR1 fusion proteins used as negative controls (Figure II.3). This
**Figure II.1:** Structure of the Fusion Proteins Used in the *In Vitro* Binding Assays. Two dystrophin hydrophobic C-terminus fusion proteins were constructed. GST-HCT corresponds to the 31 amino acids (amino acids 3668-3698) that comprise the hydrophobic C-terminus, while GST-LZHCT is an extended construct that includes amino acids 3486 to 3698. GST-DCT is a dystrophin hydrophilic C-terminal fusion (amino acids 3077-3679). The control fusion proteins are GST-alone and GST-PHR1. PHR1 is a retinal specific protein.
Figure II.2: Western Blot Analysis of GST Fusion Proteins. The two dystrophin HCT fusions (GST-LZHCT and GST-HCT) and the dystrophin hydrophilic C-terminal fusion (GST-DCT) were purified and tested for expression using Western blot analysis and antibodies specific for GST, the dystrophin hydrophobic C-terminus (ACT-1) and the dystrophin hydrophilic C-terminus (1583). Proteins of predicted sizes were observed for each fusion.
Figure II.3: *In vitro* Binding Assays with α-catulin. 35S-labeled full-length α-catulin was incubated with dystrophin HCT fusion proteins (GST-LZHCT and GST-HCT) and negative control fusion proteins (GST-alone and GST-PHR1) to test for interactions. Bound proteins were eluted, separated by SDS-PAGE and visualized by autoradiography. α-catulin bound to both dystrophin proteins but did not bind to control fusion proteins.
confirms the specific binding reaction between dystrophin and α-catenin, supporting our initial yeast two-hybrid results. This result also demonstrated that the C-terminal 31 amino acids of the dystrophin hydrophobic C-terminus are both required and sufficient for α-catenin binding.

To further test the specificity of this protein interaction, the binding experiment was repeated using a GST fusion protein containing the hydrophilic C-terminus of dystrophin (see Figure II.1). α-catenin bound specifically to the HCT of dystrophin, and did not bind to the dystrophin hydrophilic C-terminal protein (GST-DCT) (amino acids 3077 to 3679) (Figure II.4). This is the first evidence demonstrating a specific protein interaction with the hydrophobic C-terminal region of dystrophin and strongly suggests a functional significance for this alternative splicing event.

To determine which region of α-catenin binds to the hydrophobic C-terminus of dystrophin, the binding assay was used to test various regions of α-catenin produced and labeled with 35S in vitro (Figure II.5). As can be seen from the results of this analysis, the N-terminal region (amino acids 1-373) of α-catenin mediates binding to dystrophin.

II.3.3 α-catenin Expression

To assess the tissue-specific expression pattern of α-catenin mRNA we performed full-length PCR on a multi-tissue panel of human cDNAs. Figure II.6a shows ubiquitous expression in all tissues tested, including retina, corresponding to the previously reported Northern and Dot blot expression analysis (Zhang et al., 1998, Janssens et al., 1999). A multi-tissue western blot was then performed to analyze the expression of α-catenin at
Figure II.4: *In Vitro* Binding Assay - Testing the Specificity of the α-catulin/Dystrophin Interaction. $^{35}$S-labeled full-length α-catulin protein was incubated with GST fusion proteins corresponding to the dystrophin hydrophobic (GST-LZHCT) and hydrophilic (GST-DCT) C-termini. The interaction between α-catulin and dystrophin was specific for the HCT.
**Figure II.5: In Vitro Binding Assay - Identifying the Dystrophin Binding Region on the α-catulin Protein.** (a) Binding occurred between the dystrophin HCT (GST-LZHCT) and α-catulin fragments A and D. This indicates that the N-terminal 373 amino acids of α-catulin mediated binding to the dystrophin HCT. (b) Schematic representation of $^{35}$S-labeled α-catulin fragments used in the binding experiment.
**Figure II.6**: α-catulin expression in adult mouse tissues. (a) Full-length RT-PCR of α-catulin indicates ubiquitous expression. (b) Immunoblot using an anti-α-catulin polyclonal antibody on total protein extracts. The antibody recognized a major band of ~84kDa that corresponds to α-catulin.
the protein level (Figure II.6b). A primary band of ~84kDa, corresponding to the predicted molecular weight of α-catulin, is present in brain, heart, kidney and muscle, but was not observed in lung. An identical control blot stained with an anti-actin antibody was performed to ensure the protein samples were not degraded. A strong band corresponding to actin was present in all protein samples (data not shown).

II.3.4 α-catulin Localization in the Retina

If the binding of α-catulin to dystrophin is biologically significant both molecules should show co-localization. Therefore, once it had been established that α-catulin was expressed in the retina, its localization in this neural tissue was investigated by immunostaining retinal sections with an α-catulin polyclonal antibody. Figure II.7b shows a strong signal at the inner limiting membrane (ILM), surrounding the retinal microvasculature and at a region corresponding to the outer limiting membrane (OLM). While dystrophin localization to the ILM and surrounding retinal blood vessels is well established, localization to the OLM of the retina had not previously been reported thus prompting a closer investigation (Howard et al., 1998a). An identical signal at the OLM was also present in the section stained with the antibody specific for the dystrophin hydrophobic C-terminus (Figure II.7c). To characterize the dystrophin signal at the OLM, immunofluorescence analysis of retinal sections using antibodies specific for both the dystrophin hydrophobic and hydrophilic C-termini was performed. Results confirmed that the signal at the OLM was specific for the dystrophin HCT (Figure II.8). An antibody specific for human Dp71 was then used on human retinal sections to determine if the signal at this location was a result of Dp71 expression or another
Figure II.7: Immunofluorescence Analysis of α-catulin Localization in the Retina. Serial sections from a frozen mouse retina were prepared and stained with α-catulin and dystrophin HCT antibodies to observe the expression pattern of α-catulin and to look for colocalization between α-catulin and the dystrophin HCT. Panel (a) is a phase contrast image of a section showing the cellular layers of the retina including the inner limiting membrane (ILM), the outer plexiform layer (OPL) and the outer limiting membrane (OLM). α-catulin localizes to the ILM, OLM and retinal microvasculature (b), where it colocalizes with the dystrophin HCT (c). A control section omitting the primary antibody is shown in panel (d). Scale bar: 35μm.
Figure II.8: Dystrophin Expression at the Outer Limiting Membrane of the Retina. Serial retinal sections were stained with dystrophin antibodies to analyze the novel dystrophin expression observed at the outer limiting membrane (OLM). The signal at the OLM was observed with the dystrophin antibody specific for the HCT (b), but not with the antibody specific for the dystrophin hydrophilic C-terminus (DCT) (c). The inner limiting membrane (ILM) and outer plexiform layer (OPL) are labeled for reference. Scale bar: 35μm.
dystrophin isoform. Figure II.9b shows strong staining at the OLM indicating that, similar to the expression observed at the ILM and microvasculature, the dystrophin signal at the OLM is due to Dp71. Since Dp71 contains the HCT (Dp71-HCT) at the ILM, surrounding retinal blood vessels and at the OLM, the localization pattern of α-catenin is in accordance with our previous observation that α-catenin binds specifically to the hydrophobic C-terminus of dystrophin. The specific interaction between α-catenin and Dp71, one of three dystrophin isoforms expressed in the retina, provides evidence for the existence of unique dystrophin complexes in the retina that are created as a result of an alternative splicing event.

The hydrophobic C-terminus of dystrophin is also expressed at low levels at the outer plexiform synaptic layer (OPL) of the retina (see Figure II.7c). The staining pattern observed is a punctate signal corresponding to dystrophin expression at the photoreceptor termini. Although there was some generalized α-catenin staining at the OPL (Figure II.7b), it did not have a punctate appearance and may represent non-specific background staining without distinguishable dystrophin colocalization. Similar generalized α-catenin staining was observed within the ganglion cell layer, a region devoid of dystrophin expression.

II.3.5 α-catenin Expression in mdr Mutant Mice

The absence of dystrophin results in a disruption in the localization of most dystrophin-associated proteins (Ervasi et al., 1990, Ohlendieck and Campbell, 1991, Ohlendieck et al., 1993, Metzinger et al., 1997). To determine if dystrophin is required for α-catenin expression, immunofluorescence analysis of two Duchenne Muscular
Figure II.9: Dp71 at the Outer Limiting Membrane. Immunofluorescence of human retinal sections was performed to determine if the staining at the outer limiting membrane (OLM) is due to Dp71 expression. Panel (a) is a phase contrast image of a human retinal section. Panel (b) shows strong staining at the OLM with an antibody specific for Dp71. Panel (c) is a control section omitting the primary antibody. Scale bar: 35μm.
Dystrophy mouse models deficient in specific dystrophin isoforms was performed (Figure II.10). The *mdx* mouse has a missense mutation in exon 23 that eliminates expression of Dp427 (Sicinski et al., 1989). All of the shorter isoforms are unaffected and are expressed normally. In the retina, this includes Dp260 at the OPL and Dp71 at the ILM, OLM and blood vessels (Figure II.10e). The *mdx* \textsuperscript{3cv} mouse has a mutation in the splice acceptor in intron 65 that disrupts expression of all dystrophin isoforms. Immunofluorescence staining of *mdx* retinal sections showed normal α-catenin localization at the ILM, OLM and blood vessels indicating that Dp427 is not required for α-catenin localization (Figure II.10b). In contrast, staining of retinal sections from the *mdx* \textsuperscript{3cv} mouse clearly showed that α-catenin localization is severely disrupted when Dp71-HCT is absent (Figure II.10c). This confirmed our *in vitro* findings that α-catenin binds to the HCT of dystrophin, and also raises the possibility that the absence of α-catenin in dystrophin-deficient retinas could be a key factor in the abnormal neurotransmission observed in DMD patients and mice.
Figure II.10: α-catenin Localization in Dystrophin-Deficient Retinal Sections (mdx and mdx<sup>3Cy</sup>). α-catenin localization is normal in both the wild-type (a) and mdx (b) sections, but is severely disrupted in the mdx<sup>3Cy</sup> retinal section (c). Corresponding sections stained with an antibody specific for the dystrophin HCT are shown in panels (d-f). Scale bar: 35μm.
**Discussion**

We have identified a new dystrophin protein complex that involves a previously unidentified binding site in dystrophin. The binding of dystrophin to α-catenin occurs through an interaction between the N-terminal region of α-catenin and the 31 amino acids that form the hydrophobic C-terminus (HCT) of dystrophin. We determined that α-catenin is concentrated at the ILM, OLM and surrounding retinal blood vessels where it binds to the HCT of dystrophin isoform Dp71. It did not appear to colocalize with dystrophin isoforms Dp427 or Dp260 at the outer plexiform layer. α-catenin localization is severely disrupted when Dp71 is absent indicating that this interaction is required for normal localization of α-catenin in the retina.

Alternative splicing can be a key component in the generation of functional diversity within a gene and is often tightly regulated in a cell-type- or developmental-specific manner. It has the power to govern a wide range of biological processes from male versus female fate-determination in *Drosophila* to specific protein binding capabilities in mammals (Smith and Valcárcel, 2000). It is well known that alternative splicing is an important mechanism for modulating the protein interactions within the extended dystrophin complex. The alternative splicing of dystrophin (exons 73-74) and dystrophin-associated protein dystrobrevin (vr3 region) removes syntrophin binding sites, regulating the stoichiometry of potential protein binding partners (Ahn and Kunkel, 1995, Suzuki et al., 1995, Newey et al., 2000). The complex alternative splicing of agrin, an extracellular matrix protein that interacts with α-dystroglycan, determines its ability to cluster acetylcholine receptors at the neuromuscular junction (Ferns et al., 1992, Ferns et al., 1993). We have identified another example of the biological significance of
alternative splicing in dystrophin. The 31 amino acid hydrophobic C-terminus that is generated through the regulated splicing of exon 78 constitutes a previously undescribed dystrophin protein binding site for α-catulin. It is well established that the three dystrophin isoforms expressed in the retina have specific localization patterns providing an initial scaffold for the creation of distinct complexes (Pillers et al., 1993, D’Souza et al., 1995, Howard et al., 1998a). The exon 78 alternative splicing event and the resultant novel protein interaction then provide a mechanism for further specialization of these unique dystrophin protein complexes. The colocalization of α-catulin with the Dp71-HCT at the Müller cell endfeet suggests that the expression of α-catulin shows cell-type specificity. This regulated expression would permit the creation of the unique Dp71/α-catulin complexes in retinal Müller cells.

Our immunofluorescence data showed that α-catulin colocalizes with the dystrophin HCT at the ILM, surrounding retinal blood vessels and at the OLM, a site not previously documented to possess dystrophin complexes. Previous research has shown that Dp71-HCT lines the plasma membrane at the endfeet of Müller glial cells where they contact the ILM and retinal blood vessels (Howard et al., 1998a). We have now shown that Dp71-HCT and α-catulin are also expressed at the OLM, a region corresponding to the Müller cell apical microvilli that project into the subretinal space and surround the photoreceptors. The OLM contains special plaque-bearing adhering junctions in which cadherins mediate connections between the photoreceptors and the Müller glial cells in a precise planar array (Matsunaga et al., 1988, Wöhrn et al., 1998, Honjo et al., 2000). It provides the structural framework required to establish and maintain the positional alignment of the photoreceptors and the Müller cells. Similar to the situation at the ILM
and surrounding retinal blood vessels, dystrophin expression at the OLM is HCT-specific, supporting the hypothesis that the HCT plays an integral role in the formation and function of the dystrophin complex in this specialized region of the retina.

While the data suggests that α-catulin is indeed an important member of the dystrophin protein complex, the specific role of α-catulin in the dystrophin complex remains unclear. The localization of Dp71 and α-catulin at the Müller cell endfeet terminating at the ILM, OLM and blood vessels suggests that the α-catulin/dystrophin complex may have a structural role in maintaining membrane architecture necessary for the optimal orientation of membrane molecules. Alternatively, the complex may mediate interactions with the extracellular matrix and neighbouring cells to maintain the precise radial alignment of the Müller glial cells. Although the specific function of α-catulin is unknown, its homology to vinculin and α-catenin facilitates the development of a functional hypothesis. α-catulin shares sequence similarities with vinculin and α-catenin, two proteins that are members of cell adhesion and signaling complexes (Rüdiger, 1998, Janssens et al., 1999). These functionally related proteins found at adherens junctions, participate as cytoplasmic members of transmembrane complexes that mediate interactions between the actin cytoskeleton and neighbouring cells or the extracellular matrix (Rimm et al., 1995, Gumbiner and McCrea, 1993, Rüdiger, 1998). Vinculin and α-catenin are both members of the cadherin signaling complex involved in Ca^{2+}-dependent cell-cell adhesion. Although similar in structure and function, each links to the cadherin complex in a unique fashion. α-catenin associates with the cadherins through an interaction with β-catenin, while vinculin binds to α-catenin (Huber et al., 1998, Obama and Ozawa, 1997, Weiss et al., 1998). Unlike α-catenin, vinculin serves a
dual role and is also a key component in cell-matrix contacts formed by the heterodimeric transmembrane integrin receptors. Within these complexes, vinculin binds to talin, a prominent structural protein that binds directly to the intracellular domain of the integrins (Jones et al., 1989, Gilmore et al., 1992).

α-catenin, the newly identified dystrophin-associated protein, shares sequence similarities with vinculin and α-catenin at 19 and 25% respectively (Janssens et al., 1999). This apparent limited homology is significant since the observed sequence conservation occurs in putative functional domains, implying similar functions (Figure II.11). The putative β-catenin and talin binding regions of α-catenin would provide a link between the dystrophin complex and the cadherin and/or integrin adhesion complexes. Of significance is the fact that members of the integrin and/or cadherin families are localized to the ILM, Müller cell processes surrounding blood vessels and at the OLM, colocalizing with α-catenin (Brem et al., 1994, Honjo et al., 2000). All three areas have important cell-matrix or cell-cell structural requirements necessary to maintain the morphology of Müller cells. α-catenin's similarity to vinculin suggests it may have the diversity to function in both cell-matrix and cell-cell adhesion complexes. Therefore, it is possible that α-catenin is performing a structural role anchoring the Müller cells, maintaining their radial morphology. We have previously presented data suggesting that Dp71 with the hydrophobic C-terminus is involved in the remodeling of the actin cytoskeleton in myoblasts during differentiation (Howard et al., 1999). It is possible that this function and the function of Dp71 in the retina are mediated through an interaction with α-catenin.
Figure II.11: α-catulin Protein Structure. Schematic representation of the α-catulin protein in comparison to α-catenin and vinculin. The β-catenin (β-cat), talin and actin binding sites in α-catenin and vinculin are shown. The predicted functional domains in α-catulin are also illustrated.
Müller cells are an integral component of retinal structure and function. In addition to supporting and nourishing the neuronal cells, Müller cells modulate the regulation of K⁺ flux in the retina (Newman and Reichenbach, 1996). In response to light, there is a release of K⁺ from active neurons at the two synaptic layers of the retina, the outer plexiform layer and the inner plexiform layer (Fishman and Sokol, 1990). The K⁺ is rapidly taken up by the Müller cells, causing depolarization and the redistribution of K⁺ to specific cellular regions through a spatial-buffering current (Newman et al., 1984). In vascularized mammalian retinas, K⁺ efflux occurs from three conductance hotspots that have high densities of potassium channels, Müller cell endfeet at the ILM, Müller cell apical microvilli (OLM) and Müller cell endfeet terminating on blood vessels (Newman and Reichenbach, 1996, Nagelhus et al., 1999). The net result of this K⁺ flow is a return current and a positive transretinal potential that translates into the electroretinogram (ERG) b-wave (Stockton and Slaughter, 1989). This is ultimately dependent upon the non-random distribution of potassium conductance within the Müller cells. Our observations that Dp71 and α-catenin are localized precisely to the three areas where K⁺ efflux occurs, coupled with the knowledge that most DMD patients and mice have abnormal ERG b-waves, suggest that this protein complex may be involved in anchoring or positioning these K⁺ channels at specific sites on the Müller cell membrane. The generation of a normal b-wave requires a non-random distribution of potassium conductance in Müller cells (Newman and Frishman, 1991). A uniform distribution would result in a shorter current loop and a smaller transretinal voltage (b-wave). In the absence of the Dp71/α-catenin complex, the K⁺ channels may not be properly clustered,
leading to the diminished ERG b-wave observed in DMD patients. Therefore, it is possible that α-catulin is performing a structural role stabilizing the Müller cell membrane, perhaps maintaining the membrane morphology required for the clustering of K⁺ channels and effective K⁺ flux.

In summary, we have identified a new member of the dystrophin protein complex that interacts directly with the alternatively spliced hydrophobic C-terminus of dystrophin. This novel protein binding site provides a new mechanism for the assembly of distinct protein complexes in the retina allowing for specialized functions at specific cellular layers. Since α-catulin and the dystrophin hydrophobic C-terminus are both broadly expressed, further research will likely show that this complex is functional in other tissues as well. The identification of a novel protein binding site within the dystrophin hydrophobic C-terminus provides new opportunities for the analysis of dystrophin function.

II.5 Materials and Methods

Yeast Two-Hybrid Library Screening

A yeast two-hybrid bait construct was created by fusing the cDNA of the dystrophin HCT (corresponding to amino acids 3668 to 3698) to the binding domain of Gal4 in the vector pGBT9 (Trp1, amp'). To obtain yeast cells containing the binding domain plasmid, a small scale transformation was performed. 100μl of competent CG1945 yeast cells were transformed with 0.5μg of pGBT9-HCT plasmid DNA. For the library screen 5.5ml of competent CG1945/pGBT9-HCT cells were transformed with ~110 μg of mouse brain cDNA or ~150μg of human heart cDNA fused to the Gal4
activation domain in the pACT2 vector (Leu2, amp') (Matchmaker System, Clontech). The amino acid sequence of the HCT is identical in mouse and human allowing for the use of the same bait construct in both library screens. More than $4 \times 10^5$ brain library cotransformants and $1 \times 10^6$ heart library cotransformants were plated on SD selection plates lacking Trp, Leu and His and incubated at 30°C. The brain and heart library screens were performed in the presence of 15mM and 20mM 3-aminotriazole respectively. The concentration of 3-aminotriazole required to suppress the leaky His expression endogenous to the CG1945 yeast strain was determined prior to the screens. Competent CG1945 cells were transformed with the pGBT9-HCT bait construct and pTD1, a non-specific activation domain plasmid. Transformed cells were plated on Trp, Leu, and His deficient plates supplemented with 10, 20 or 30mM 3-aminotriazole and incubated at 30°C for 5 days. Since the dystrophin HCT and pTD1 do not interact, the background levels of colony growth could be analyzed and the concentration of 3-aminotriazole required to suppress the leaky His expression could be selected.

60 His$^+$ colonies from the heart library screen were picked after 3, 4 or 5 days of growth and tested for β-galactosidase expression using a filter assay to confirm the presence of a protein-protein interaction (see section below). Plasmids from 21 His$^+$lacZ$^+$ colonies were purified using the following procedure. Yeast cultures (2ml) were grown to saturation in SD selection media, pelleted and resuspended in 200μl lysis buffer (2% Triton X-100, 1% SDS, 100mM NaCl, 10mM Tris-HCl pH 8.0, 1.0mM EDTA). 0.3g of acid-washed glass beads and 200μl phenol:chloroform:isoamyl alcohol (25:24:1) were added and the mixtures were vigourously vortexed for 5 minutes. The samples were centrifuged at 14 000 rpm for 10 minutes, the supernatant was collected and the DNA
was precipitated with 2.5 volumes of ethanol and 1/10 volume of 3M NaOAc. Following a wash in 70% ethanol, the DNA was resuspended in 10mM Tris pH 8.0. A QIAEX extraction kit (Qiagen) was used for plasmid purification.

It was then necessary to isolate the activation domains from each plasmid mixture. Competent *Escherichia coli* MH6 (Leu') cells were transformed with each plasmid mixture and plated on LB-ampicillin plates to obtain ampicillin resistant clones. To select for colonies containing the pACT2 Leu+ library activation domain plasmids, individual colonies were streaked onto leucine deficient M9 plates. Colonies containing the activation domain plasmids were then grown up in liquid LB and DNA was isolated using the QIAprep Spin Miniprep Kit (Qiagen). The 21 positive clones were then analyzed by sequencing (USB Cycle Sequencing Kit) using a pACT2-specific forward primer (5'-tggtcstatggccatgga-3').

To further verify the specific interactions between the dystrophin HCT and the novel interacting proteins identified in the screen, each activation domain plasmid was reintroduced into yeast either alone, with the dystrophin HCT bait or with control Gal4 binding domain plasmids pVA3 (murine p53) and pLAM (laminC) and subjected to a filter β-galactosidase assay.

**β-Galactosidase Filter Assay**

Each His+ colony isolated from the yeast two-hybrid selection was streaked on a Whatman filter (No. 3) and grown overnight at 30°C on an SD media plate. Filters were then flash frozen for approximately 5 seconds in liquid nitrogen and placed colony side up on a piece of Whatman paper (No. 40) presoaked with 2.5ml of Z-buffer (60mM
Na₂HPO₄•7H₂O, 40mM NaH₂PO₄•H₂O, 10mM KCl, 1mM MgSO₄, 30mM β-Mercaptoethanol) with a final concentration of 0.2mg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). All filters were then incubated at 30°C. β-galactosidase activity (blue colour) was assayed every 30 minutes over the course of 4 hours. Colonies that turned blue contained a positive protein-protein interaction and were scored from + (light blue) to ++++ (dark blue) by inspection. Colonies that remained white were classified as containing a negative protein-protein interaction.

**In Vitro Binding Assays**

Three GST fusion constructs were generated using the pGEX 2TK or 4T1 vectors (Pharmacia). Two dystrophin HCT constructs were made. GST-HCT encodes the last 31 amino acids of the hydrophobic C-terminus (amino acids 3668-3698). An extended version, GST-LZHCT, includes the coiled-coil region of dystrophin (amino acids 3486-3698). A dystrophin hydrophilic C-terminal fragment encoding amino acids 3077 to 3679 was obtained by RT-PCR (see RT-PCR section below). The GST-PHR1 construct was a gift from Rahim Ladak (McInnes lab, University of Toronto, Canada) and encodes the PHR1 gene.

Induced fusion proteins were batch purified on Glutathione Sepharose 4B (Pharmacia) and tested for expression using Western Blot analysis (see Western Blotting section below). α-catulin fragments were amplified from the full-length mouse yeast two-hybrid clone 4.5 via PCR using primers allowing for in vitro translation (Table II.2). [³⁵S]methionine-labeled proteins were translated using in vitro T7-coupled transcription/translation according to the manufacturer's instructions (TnT, Promega).
Table II.2: Primers used for the amplification of α-catenin fragments.

<table>
<thead>
<tr>
<th>α-catenin Fragment</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Amino Acids Encoded</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>5'gctaatacgactcacttagggacca gaccaccatggccgctttccagtc3'</td>
<td>5'gctcttttgcttctgtgg3'</td>
<td>1-373</td>
</tr>
<tr>
<td>B</td>
<td>5'gctaatacgactcacttagggacca accatggccgcggagctggaactc3'</td>
<td>5'ctctctctcttccttaaac3'</td>
<td>374-531</td>
</tr>
<tr>
<td>C</td>
<td>5'gctaatacgactcacttagggacca ccaccatgggagctggaaccttc3'</td>
<td>5'tcagttttagacatcccatgg3'</td>
<td>531-731</td>
</tr>
<tr>
<td>D</td>
<td>5'gctaatacgactcacttagggacca gaccaccatggccgctttccagtc3'</td>
<td>5'ctctctctcttccttaaac3'</td>
<td>1-531</td>
</tr>
<tr>
<td>E</td>
<td>5'gctaatacgactcacttagggacca accatggccgcggagctggaactc3'</td>
<td>5'tcagttttagacatcccatgg3'</td>
<td>374-731</td>
</tr>
<tr>
<td>Full Length</td>
<td>5'gctaatacgactcacttagggacca gaccaccatggccgctttccagtc3'</td>
<td>5'tcagttttagacatcccatgg3'</td>
<td>1-731</td>
</tr>
</tbody>
</table>
5μl of the α-catalin translation mixtures were incubated for 2 hours at 4°C with 50μl of the bead-conjugated GST-fusion slurries (~25μg) in TBST (10mM Tris pH 8.0, 0.1% v/v Tween-20, 150mM NaCl). After centrifugation, the unbound supernatant was kept for analysis and the pellet was washed 5 times with 200μl of TBST. Beads were resuspended in SDS-sample buffer and boiled for 5 minutes to elute the bound proteins. Equivalent amounts of input, unbound and bound fractions were separated by SDS-PAGE and examined by autoradiography.

**Antibodies**

Full-length mouse α-catalin was amplified from the yeast two-hybrid clone 4.5 and subcloned into pGEX-4T1 to generate a GST fusion protein for antibody production. The construct was fully sequenced on both strands. Induced fusion protein was batch purified from *Escherichia coli* using Glutathione Sepharose 4B (Pharmacia). The protein was then eluted in fractions using 20mM reduced glutathione, dialyzed against PBS and used to inject New Zealand white rabbits for the production of a polyclonal antibody (Research Genetics). The serum was affinity purified using column chromatography (Research Genetics). The α-catalin antibody was demonstrated to specifically recognize α-catalin fusion proteins (data not shown). Polyclonal antibodies ACT-1 and 1583 have been previously described and are specific for the dystrophin hydrophobic and hydrophilic C-termini respectively (Howard et al., 1998a, Bulman et al., 1991). NME1 is a polyclonal antibody specific for human Dp71 (Howard et al., 1998a). Monoclonal anti-GST was obtained from a commercial supplier (Santa Cruz Biotechnology).
**Western Blotting**

Aliquots from all bead-conjugated fusion proteins were centrifuged and resuspended in SDS-sample buffer. C57 protein extracts were prepared by homogenizing mouse tissues in sample buffer (0.05M Tris pH 6.8, 2% w/v SDS, 10% v/v glycerol, 0.36M B-Mercaptoethanol). All proteins were boiled, separated on an SDS-PAGE gel and electrophoretically transferred to a PVDF membrane (BIORAD) in transfer buffer (0.24% w/v Tris base, 1.16% w/v glycine, 0.01%w/v SDS). The membranes were then blocked overnight at 4°C in 5% powdered milk in TBST (10mM Tris pH 8.0, 150mM NaCl, 0.05% v/v Tween-20). Proteins were incubated in TBST and 1% milk for 2 hours at room temperature with the following primary antibody dilutions: GST(1/2000), ACT-1 (1/500), 1583 (1/2500), α-catenin (1/2500). After three 10 minute washes in TBST, the membranes were incubated for 1 hour with an HRP-conjugated anti-rabbit (or anti-mouse for GST Western) secondary antibody (Amersham) at a dilution of 1/5000. Following three 10 minute washes in TBST, proteins were detected by enhanced chemiluminescence (Amersham).

**RT-PCR**

The dystrophin hydrophilic C-terminus was amplified by RT-PCR to create a GST fusion construct (GST-DCT). Oligo dT primed mouse muscle cDNA was amplified in a nested PCR reaction using the following first set of primers (Dp71-F1: 5’-gagacccaaaccacctgttgg-3’ and Dp71-R1: 5’-tgcccaaatcatctgcaîgtgg-3’) followed by a second reaction (Dp71-F2: 5’-actggaattcgcaccacccaatgacagc-3’ and Dp71-R2: 5’-acgtctcagctacatgtgtcctctcattgg-3’). PCRs were performed with Advantaq Plus.
(Clontech) under the following amplification conditions: 95°C x 1 min, and 30 cycles of 95°C x 30 s, 55°C x 30 s, 68°C x 2 min. The product was gel purified and cloned into the pGEX-4T1 vector (GST-DCT).

Full-length nested RT-PCR of the α-catulin gene was performed using first-strand cDNA templates (Clontech) for all tissues except retina. Human retinal RNA was reverse transcribed using an oligo dT primer. All PCR reactions were performed using Elongase (Gibco BRL) and the following primers for the first round: (for: 5'agtcggtctgctagacc' and rev: 5'gatctgtaaattacagac'3') followed by a second round with: (for: 5'gatagaccgaggggctgg' and rev: 5'acaatcagttcatgac'3'). Reaction conditions were 95°C for 1 min., followed by 30 cycles of 95°C for 30 s, 55°C for 30 s and 68°C for 2 min.

**Immunofluorescence**

C57, mdx and mdx<sup>3Cv</sup> eyes and human retinas were isolated, embedded in OCT (Miles) and immediately frozen in liquid nitrogen. Human eyes were obtained from the Eye Bank Laboratory of the Canadian Institute for the Blind (Toronto, Ontario). 7μm sections were cut on a Leica microtome and mounted on silane-coated slides at -20°C. Mouse retinal sections were brought to room temperature and then fixed for 5 minutes at -20°C in methanol followed by 3 minutes in acetone at -20°C. The following steps were performed at room temperature unless otherwise stated. Fixed sections were rehydrated in 3 ten minute washes of PBS and then incubated in 0.3% Triton X-100, 1% bovine albumin in PBS for 45 minutes. Sections were then blocked in 10% goat serum in PBS for 45 minutes followed by 2% goat serum in PBS for 30 minutes. Polyclonal α-catulin
antibody was diluted (1/10 000) in 0.1% goat serum, 1% bovine albumin in PBS. Sections were incubated at 4°C overnight with the primary antibody followed by 12 five minute washes in PBS. Negative controls were treated identically except for the omission of the primary antibody. CY3-conjugated anti-rabbit secondary antibody (Jackson) was diluted 1/200 in 0.1% goat serum, 1% bovine albumin in PBS and applied to the sections for 1 hour. Sections were washed 3 times for 15 minutes in PBS, mounted in Immunoflo (ICN) and examined using a Leica epifluorescent microscope (Aristoplan). Antibodies ACT-1 (1/300), 1583 (1/1000) and NME-1 (1/1000) were used as previously described (Howard et al., 1998a).
Chapter III

Characterization of Dystrobrevin in the Retina

The work in this chapter is my own. Dr. Derek Blake (University of Oxford, UK) supplied the dystrobrevin isoform-specific antibodies.
III.1 Abstract

Dystrophin, the product of the Duchenne Muscular Dystrophy gene, is expressed as a family of protein isoforms which differ in localization, structure and function. A subset of these isoforms is found in specialized extended protein complexes in the retina and has been shown to be required for normal retinal electrophysiology. Full-length dystrophin (Dp427) and dystrophin isoform Dp260 are localized to the outer plexiform layer of the retina; whereas Dp71 is found at the inner limiting membrane and surrounding retinal blood vessels. Electretinogram analysis of Duchenne Muscular Dystrophy patients and mouse models indicates that the two shorter dystrophin isoforms, Dp260 and Dp71, have unique and essential roles in the retina. We and others have suggested that the differential localization patterns and specific functions are due to distinct protein-protein interactions at each location in the retina.

We describe the expression and localization of the dystrobrevins, a family of dystrophin-associated proteins. Using western blotting and immunofluorescence analysis dystrobrevin isoforms α-dystrobrevin-1 and β-dystrobrevin were shown to be expressed in the retina but were differentially localized. α-dystrobrevin-1 colocalized with Dp71 at the inner limiting membrane and blood vessels, whereas β-dystrobrevin colocalized with Dp427 and Dp260 at the synaptic outer plexiform layer. In mutant mice deficient for specific dystrophin isoforms we found that dystrobrevin localization was dependent upon the presence of its specific dystrophin partner. Our data provide evidence for the existence of distinct dystrophin protein complexes in the retina that perform unique functions, and suggest that disruption of specific dystrobrevin-dystrophin complexes
contributes to the abnormal retinal electrophysiology observed in Duchenne Muscular Dystrophy patients.

III.2 Introduction

The functions of dystrophin isoforms in the brain and retina and their involvement in the cognitive defects and retinal disturbances observed in Duchenne Muscular Dystrophy (DMD) patients are not well understood. The retina is an ideal tissue for the study of the dystrophin in the central nervous system (CNS) since it has defined synaptic layers, a known dystrophin expression profile and a measurable phenotype in DMD patients and mice. As discussed in section I.12, we and others, have previously shown that dystrophin isoforms Dp427 and Dp260 are expressed at the synaptic outer plexiform layer (OPL) of the retina, while Dp71 is localized to the non-synaptic inner limiting membrane (ILM), outer limiting membrane (OLM) (see Chapter II) and blood vessels (Pillers et al., 1993, Schmitz and Drenckhahn, 1997a, Howard et al., 1998a, D’Souza et al., 1995, Kameya et al., 1997). The availability of several DMD mutant mouse models has facilitated a detailed examination of dystrophin isoform involvement in retinal electrophysiology as revealed by electroretinogram (ERG) analysis, and has shown that Dp260 and Dp71 are required for specific components of the ERG (see section I.11 and I.12) (Pillers et al., 1999). Together with their distinct localization patterns in the retina, these results suggest that Dp260 and Dp71 have essential but different functions in retinal neurotransmission which are likely due to differential protein interactions resulting in isoform specific complexes.
Dystrobrevin is a member of the dystrophin related protein family and was originally identified as an 87kDa protein in the synaptic electric tissue of *Torpedo californica* (Carr et al., 1989, Wagner et al., 1993). Similar to dystrophin, the mammalian dystrobrevin genes encode a family of isoforms consisting of several splice variants of α-dystrobrevin and the recently identified β-dystrobrevin (refer to Figure 1.3) (Blake et al., 1996a, Blake et al., 1998, Holzfeind et al., 1999, Puca et al., 1998, Peters et al., 1997b, Blake at al., 1998). α-dystrobrevin is a component of the post-synaptic membrane complex at the neuromuscular junction and copurifies with acetylcholine receptors and other postsynaptic proteins (Peters et al., 1998, Nawrotzki et al., 1998). Recent studies of α-dystrobrevin null mice suggest that this protein functions to position signaling proteins at the neuromuscular junction (Grady et al., 1999). Coupled with the knowledge that dystrophin and dystrobrevin interact through their coiled-coil domains, these findings suggest that dystrophin is required to position a dystrobrevin signaling protein complex at the neuromuscular junction independent of its role in sarcolemmal stabilization (Sadoulet-Puccio et al., 1997). β-dystrobrevin, on the other hand, is highly expressed in neurons of the cortex, hippocampus and cerebellum, but is not detectable in muscle (Blake et al., 1998, Puca et al., 1998, Peters et al., 1997b). Its role in the CNS is still unknown.

In this chapter, we show that α-dystrobrevin-1 and β-dystrobrevin are differentially expressed in the retina and require dystrophin for proper localization. This provides evidence for the existence of distinct dystrophin complexes in the retina, and suggests that the disruption of specific dystrobrevin-dystrophin complexes is a key factor in the retinal electrophysiology observed in DMD patients.
III.3 Results

III.3.1 Dystrobrevin expression in the retina

To explore the role of dystrobrevin in retinal function we first determined which isoforms of this family of proteins were expressed in retinal tissue. Figure III.1 shows the results of a western blot of mouse retinal protein extracts probed with an antibody, \( \beta \)CT-FP, which recognizes \( \alpha \)-dystrobrevin-1, \( \alpha \)-dystrobrevin-2 and \( \beta \)-dystrobrevin isoforms (Blake et al., 1998). As can be seen in this figure, \( \alpha \)-dystrobrevin-1 and \( \beta \)-dystrobrevin are expressed in the retina whereas \( \alpha \)-dystrobrevin-2 is not. A similar pattern of dystrobrevin isoform expression is observed in brain tissue. Lung protein was included on the Western as a control to show the migration of the non-muscle \( \alpha \)-dystrobrevin-2 isoform.

III.3.2 Dystrobrevin localization in the retina

Having determined that dystrobrevin expression in the retina shows isoform specificity, we next wanted to determine if dystrobrevin colocalizes with dystrophin. Serial retinal sections were prepared from wild-type CD-1 mice and stained with \( \beta \)CT-FP and ACT-1, antibodies specific for dystrobrevin and dystrophin respectively. Figure III.2b shows a strong punctate signal corresponding to dystrobrevin expression at the outer plexiform layer (OPL), the first synaptic layer of the retina. In addition, dystrobrevin is localized to the inner limiting membrane (ILM) and blood vessels. Dystrophin is also expressed at these sites indicating that, at the light microscope level, dystrobrevin colocalizes with dystrophin at the OPL, ILM and blood vessels of the retina (Figure III.2c). Dystrophin is also found localized to the outer limiting membrane of the...
Figure III.1: Western Blot Analysis of Dystrobrevin Expression in the Retina and Brain. The βCT-FP antibody shows the expression of α-dystrobrevin-1 and β-dystrobrevin, but not α-dystrobrevin-2 in the retina. This corresponds the dystrobrevin expression profile observed in brain. Lung protein was included to show the migration of non-muscle α-dystrobrevin-2.
Figure III.2: Immunofluorescence Analysis of Dystrobrevin Localization in the Retina. Serial sections of frozen retina were prepared from CD-1 mice and stained with dystrobrevin and dystrophin antibodies to define the expression pattern of dystrobrevin in the retina, and to determine whether dystrobrevin colocalizes with dystrophin. a) A phase contrast image of a mouse retinal section. b) Antibody βCT-FP recognizes both dystrobrevin isoforms expressed in the retina (α-dystrobrevin-1 and β-dystrobrevin) and shows a strong punctate signal at the synaptic OPL. Staining was also observed at the ILM and blood vessels. c) Antibody ACT-1 is specific for the hydrophobic C-terminus of dystrophin and shows a colocalization with dystrobrevin at the OPL, ILM and blood vessels. The * indicates dystrophin staining at the outer limiting membrane. d) A control section omitting the primary antibody. Scale bar: 35μm.
retina (Figure III.2c), however, there is no dystrobrevin staining observed at this retinal layer.

III.3.3 Differential localization of dystrobrevin isoforms

It has been established that the dystrophin isoforms expressed in the retina (Dp427, Dp260 and Dp71) are differentially localized (Howard et al., 1998a, Kameya et al., 1997, Schmitz and Drenckhahn, 1997a, Chapter II). Dp427 and Dp260 are expressed at the synaptic OPL; whereas Dp71 is found at the non-synaptic ILM, outer limiting membrane and surrounding the retinal blood vessels. ERG analysis of mouse mutants deficient in specific dystrophin isoforms has demonstrated that the dystrophin isoforms have specific functions likely resulting from differential protein-protein interactions. To investigate this we examined the localization of specific dystrobrevin isoforms to determine if these protein isoforms display unique localization patterns providing functional specificity to the individual dystrophin complexes in the retina. Antibodies specific for the individual isoforms of dystrobrevin were used to stain frozen wild-type mouse retinal sections. Antibody α1CT-FP was raised against the unique C-terminus of α-dystrobrevin-1 and detects only this isoform of dystrobrevin (Blake et al., 1998). This antibody produced a strong signal at the ILM and at the blood vessels (Figure III.3a) coinciding with the expression of the smallest dystrophin isoform, Dp71. Dp71 is known to be expressed at the expanded endfeet of Müller cells at the inner border of the retina (ILM), the outer limiting membrane and in the perivascular endfeet of Müller cells that surround blood vessels (Schmitz and Drenckhahn, 1997a, Chapter II). The absence of α-
Figure III.3: Unique Localization Patterns of Dystrobrevin Isoforms. Serial frozen retinal sections were stained with a panel of isoform specific dystrobrevin antibodies to observe any potential differential localization. \( \alpha \text{CT-TP} \) recognizes \( \alpha \)-dystrobrevin-1 and produces strong staining at the ILM and blood vessels (a). \( \alpha \text{2N} \) is specific for \( \alpha \)-dystrobrevin-2 and shows no expression of this isoform in the retina (b). \( \beta \)-dystrobrevin is seen as a punctate signal at the OPL using isoform specific antibody \( \beta \text{417} \) (c). Antibody \( \beta \text{CT-TP} \) was included for reference to show the expression of all dystrobrevin isoforms in the retina (d). Control sections omitting the primary antibodies are shown in the right-hand column (e-h). Scale bar: 35\( \mu \)m.
dystrobrevin staining at the outer limiting membrane indicates that the dystrophin complexes differ between the ILM/blood vessels and the outer limiting membrane.

The western blot analysis indicated that α-dystrobrevin-1 and β-dystrobrevin were the only dystrobrevin isoforms expressed in the retina. To confirm our western blot finding that α-dystrobrevin-2 is not expressed in the retina, retinal sections were stained with α2N, an antibody specific for α-dystrobrevin-2 (gift from Dr. Derek Blake of the University of Oxford, UK). As expected, no staining was observed in the retina (Figure III.3b).

Antibody β417 was raised against a synthetic peptide corresponding to amino acids 414-429 of β-dystrobrevin and is specific for this isoform (Loh et al., 2000). The localization of β-dystrobrevin is confined to the outer plexiform layer (Figure III.3c). This is identical to the expression pattern of dystrophin isoforms Dp427 and Dp260.

III.3.4 Dystrobrevin expression in mdx mutant mice

The co-localization experiments described above suggest that dystrobrevin localization is determined by interactions with specific dystrophin isoforms. To test this we examined dystrobrevin localization in a series of DMD mutant mice lacking specific dystrophin isoforms. Figure III.4a outlines the dystrophin isoforms expressed in the retinas of these mdx allelic variants. The mdx mouse has a mutation in exon 23 of the dystrophin gene that disrupts the expression of Dp427 at the OPL in the retina but does not affect expression or localization of isoforms Dp260 or Dp71 (Sicinski et al., 1989). Figure III.4(c,g,k) shows normal expression of α-dystrobrevin-1 and β-dystrobrevin isoforms indicating that the presence of Dp260 at the synaptic OPL, and Dp71 at the
Figure III.4): An Examination of Dystrobrevin Localization in *mdx* Retinas.

a) A summary of dystrophin isoform expression in the retinas of *mdx* allelic variant mice.

<table>
<thead>
<tr>
<th>Wildtype</th>
<th><em>mdx</em></th>
<th><em>mdx</em>^{4Cv}</th>
<th><em>mdx</em>^{3Cv}</th>
</tr>
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<tbody>
<tr>
<td>Dp427</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Dp260</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Dp71</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

b-u) (next page): Our panel of dystrobrevin antibodies was used on frozen retinal sections from DMD mutant mice (*mdx*, *mdx*^{4Cv} and *mdx*^{3Cv}) to examine the expression/localization of dystrobrevin in dystrophin-deficient tissue. The left-hand column shows wildtype dystrobrevin expression (b, f, j), and panel (n) shows wildtype dystrophin expression. α-dystrobrevin-1 expression, detected by antibody α1CT-FP, is normal in *mdx* and *mdx*^{4Cv} retinas, but is severely disrupted in *mdx*^{3Cv} sections (c, d, e). β-dystrobrevin expression, detected by antibody β417, is normal in the *mdx* retina, but not in *mdx*^{4Cv} or *mdx*^{3Cv} sections (g, h, i). In addition, the βCT-FP antibody, which recognizes α-dystrobrevin-1 and β-dystrobrevin, was used to show disruption of β-dystrobrevin at the OPL in *mdx*^{4Cv} and *mdx*^{3Cv} and α-dystrobrevin-1 at the ILM and blood vessels in *mdx*^{3Cv} retinal sections (k, l, m). The dystrophin ACT-1 antibody was included as a reference to show that the disruption of dystrobrevin at a particular layer in the retina corresponds to the absence of dystrophin at that location (o, p, q). Control sections omitting the primary antibody are shown in the bottom row (r-u). Scale bar: 35μm.
<table>
<thead>
<tr>
<th></th>
<th>CD-1</th>
<th>( \text{mdx} )</th>
<th>( \text{mdx4Cv} )</th>
<th>( \text{mdx3Cv} )</th>
</tr>
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<tbody>
<tr>
<td>( \alpha \text{CT-FP} )</td>
<td></td>
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<tr>
<td>( \beta \text{417} )</td>
<td></td>
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<td></td>
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<tr>
<td>( \beta \text{CT-FP} )</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>( \text{ACT-1} )</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-primary</td>
<td></td>
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</tbody>
</table>
ILM/blood vessels are sufficient to maintain proper localization of specific dystrobrevin isoforms.

The \textit{mdx}^{4Cv} mouse does not express either Dp427 or Dp260 at the OPL, but does express Dp71 at the ILM and blood vessels (Figure III.4p). We observed a severe disruption of $\beta$-dystrobrevin expression at the OPL coinciding with the absence of Dp427 and Dp260 at this synaptic layer (Figure III.4h, l). In contrast, a strong signal was maintained at the ILM and retinal blood vessels indicating that the Dp71/\textit{\alpha}-dystrobrevin-1 interaction was unaffected in these mutant mice (Figure III.4d and l).

The \textit{mdx}^{3Cv} mouse has a splice acceptor mutation in intron 65 that disrupts all dystrophin isoforms (Figure III.4q) (Cox et al., 1993). Figure III.4(e, i, m) shows a severe disruption of dystrobrevin at the OPL, ILM and blood vessels, indicating that dystrophin is required for proper expression of both dystrobrevin isoforms in the retina.

### III.3.5 Utrophin expression in the retina

In the absence of dystrophin, we observed a severe disruption of dystrobrevin localization in the retina. The expression, however, was not completely abolished. This could be due to an interaction between dystrobrevin and the dystrophin homologue, utrophin. To examine the expression of utrophin in the \textit{mdx} mice, indirect immunofluorescence experiments were performed. The utrophin monoclonal antibody, NCL-DRP2 was used to stain retinal sections from the \textit{mdx} allelic variants described above. Figure III.5 shows expression of utrophin at the OPL, ILM and blood vessels in all strains tested, confirming that the residual dystrobrevin expression seen in dystrophin-deficient retinal sections could be due to a dystrobrevin/utrophin interaction.
Figure III.5: Urophin Localization in the Retina. a) A phase contrast image of a mouse retina. A utrophin antibody was used on retinal sections from wildtype (b) and DMD mutant mice (c, d, e). Staining was observed at the OPL, ILM and blood vessels on all sections indicating that utrophin colocalizes with dystrobrevin in the retina. Scale bar: 35µm.
III.4 Discussion

We and others have shown that dystrophin isoforms Dp260 and Dp71 are required for normal neurotransmission across the retina (Pillers et al., 1993, Cibis et al., 1993, Sigesmund et al., 1994, Pillers et al., 1999). The retinal electrophysiological abnormalities observed in most DMD patients can be readily measured through the use of an electroretinogram (ERG), a recording of the voltage change across the retina in response to a flash of light (Stockton and Slaughter, 1989). The b-wave component of the ERG is generated by a flux of potassium ions between the outer plexiform layer (OPL) and the Müller cell endfeet that terminate at the inner limiting membrane (ILM) and blood vessels, the exact locations of dystrophin expression. When dystrophin is absent from these locations, the ERG b-wave is abnormal (Pillers et al., 1999). We have now shown that specific dystrobrevin isoforms colocalize with dystrophin in the retina and are dependent upon the presence of their specific dystrophin partners for proper localization. Using western blotting and indirect immunofluorescence techniques to characterize dystrobrevin expression and localization in the retina, we have shown that both α-dystrobrevin-1 and β-dystrobrevin are expressed in the retina but are differentially localized. α-dystrobrevin-1 is found at the ILM and surrounding retinal blood vessels, colocalizing with dystrophin isoform Dp71. β-dystrobrevin is co-localized at the synaptic OPL with Dp427 and Dp260. In the absence of Dp71, there is a severe disruption of α-dystrobrevin-1 at the ILM and blood vessels. Similarly, β-dystrobrevin requires the expression of dystrophin (Dp427/Dp260) at the OPL for proper localization. The existence of these specialized dystrophin/dystrobrevin complexes at unique locations in the retina suggests that the individual complexes perform specific functions.
It is well established that the dystrophin isoforms expressed in the retina perform unique roles in the generation of a normal electroretinogram (ERG) b-wave (Pillers et al., 1999). In the absence of Dp427, mdx mice have a normal ERG, indicating that Dp427 is not required for normal retinal neurotransmission (Cibis et al., 1993, Pillers et al., 1995, Pillers et al., 1999). The additional absence of Dp260, however, results in an ERG recording in which the b-wave implicit time is significantly lengthened, reflecting a delay in signal transmission at the OPL (Pillers et al., 1999). Dystrophin mutations resulting in the disruption of Dp427, Dp260 and Dp71 result in an ERG recording with a significant reduction in b-wave amplitude in addition to the increase in implicit time (Pillers et al., 1995, Pillers et al., 1999). These results demonstrate that Dp260 and Dp71 each has a unique, yet essential role in normal retinal neurotransmission which is likely due to distinct protein-protein interactions at each location in the retina. Our results showing that the dystrobrevin isoforms are differentially expressed in the retina support this hypothesis and suggest that dystrobrevin may be a key factor in the specialized functions of the dystrophin complexes in the retina.

The function of α-dystrobrevin within the dystrophin complex is unclear. At the neuromuscular junction α-dystrobrevin-1 is thought to be involved in the synaptic function of the nerve-to-muscle contact (Peters et al., 1998). α-dystrobrevin null mice display a muscular dystrophy phenotype, impaired nitric-oxide-mediated signaling and defects in postsynaptic differentiation, despite the fact that the distribution of all other components of the dystrophin protein complex remains intact (Grady et al., 1999). The mislocalization of nNOS at the membrane, combined with the maintenance of sarcolemmal integrity, led to the proposition that α-dystrobrevin does not have a
structural role at this location, but rather acts as a scaffold for the positioning of signaling molecules at the synapse (Grady et al., 1999). This suggests that the dystrophic phenotype is related to the absence of a dystrobrevin signaling function.

Although α-dystrobrevin-1 has a non-synaptic localization in the retina, it is possible that it is performing a similar role and could be serving to anchor or cluster specific receptors or channels at the Müller cell endfeet. Müller cells, the principal glial cells of the retina, serve to support, nourish and regulate the synaptic function of the neurons (Newman and Reichenbach, 1996). It is these specialized glial cells that facilitate the flux of potassium ions across the retina in response to the onset of light (Newman et al., 1984). In response to a light stimulus there is a release of potassium ions from the OPL of the retina (Fishman and Sokol, 1990). These ions are then redistributed to regions of low potassium concentration via retinal Müller cells. Two major efflux hotspots occur at the peravitreous and perivascular endfeet, precisely corresponding to the regions of Dp71/α-dystrobrevin-1 expression (Newman and Reichenbach, 1996). A positive transretinal potential, recorded as the electroretinogram (ERG) b-wave, is the end result of this potassium flow (Stockton and Slaughter, 1989). In the absence of the dystrophin/dystrobrevin complex in mdx3Cv mice, the b-wave amplitude is severely reduced (Pillers et al., 1995, Pillers et al., 1999). This suggests that the complex may play a critical part in the clustering or positioning of potassium channels at the Müller cell endfeet. Therefore, although the localization of this dystrophin complex is non-synaptic, it has a profound effect on retinal neurotransmission. In parallel with the function of α-dystrobrevin at the neuromuscular junction, it is quite possible that the retinal ERG phenotype observed in DMD patients and mice is directly related to the
absence of dystrobrevin and indirectly to a deficiency of dystrophin. An analysis of dystrophin expression and ERG recordings of dystrobrevin null retinas would conclusively answer this question.

β-dystrobrevin is a newly characterized member of the dystrophin-related protein family with unknown function (Blake et al., 1998, Peters et al., 1997b, Puca et al., 1998). Since β-dystrobrevin is not expressed in muscle, we cannot draw functional parallels between muscle and the central nervous system. The fact that β-dystrobrevin is absent from muscle would indicate that it does not have a role in maintaining the structural integrity of the sarcolemmal membrane, rather it may have a specific neuronal function. It is possible that β-dystrobrevin provides the adaptation or specificity required for the dystrophin complex to function at interneuronal synapses. Dystrophin has been localized presynaptically on the photoreceptor plasma membrane that borders the lateral sides of the invaginated synaptic triad photoreceptors (Schmitz and Drenckhahn, 1997a, 1997b, Ueda et al., 1995, Ueda et al., 1998). Although the precise localization of β-dystrobrevin in the retina has not been determined by electron microscopy, since it is known to bind to dystrophin, it is presumably expressed in the same subcellular compartment of the presynaptic photoreceptor termini. It has not been established whether Dp427 and Dp260 are precisely colocalized within the OPL or whether they have slightly different subcellular localization patterns at this synaptic layer. These isoforms could have overlapping functions or they may perform unique roles at the OPL. Since β-dystrobrevin localization is maintained in mdx mice where Dp260 expression is normal, we can conclude that β-dystrobrevin forms a functional complex with Dp260 at this synaptic layer. It is unknown whether β-dystrobrevin also binds to Dp427 at the OPL.
Since β-dystrobrevin and the dystrophin complex are not found at the second synaptic layer of the retina, the inner plexiform layer, the complex is not likely involved in the general structure or function of interneuronal synapses. However, the increased b-wave implicit time observed when the dystrophin/dystrobrevin complex is absent reflects its requirement for normal OPL function. This suggests that the dystrophin/dystrobrevin complex may be involved in the immobilization of specific signaling molecules at the OPL or the stabilization of the elaborate invaginated morphology of the presynaptic face of the photoreceptors.

The differential colocalization of dystrobrevin isoforms is evidence that dystrobrevin may provide a mechanism for the assembly of distinct dystrophin protein complexes at unique locations in the retina. The function of the individual complexes could then be further specialized by the incorporation of other dystrophin-associated proteins such as syntrophin. A model has been proposed in which dystrophin, syntrophin and dystrobrevin form complexes with a stoichiometry of 1:2:1 (Peters et al., 1997a). More recently it has been shown that certain dystrophin/dystrobrevin complexes in muscle have the capacity to bind four syntrophin molecules providing further flexibility in the creation of specific extended protein complexes (Newey et al., 2000). Syntrophins are known to interact with the dystrophin complex and with members of signaling pathways such as voltage-gated sodium channels (SkM1 and SkM2), microtubule-associated serine/threonine kinases (MAST and SAST), and neuronal nitric oxide synthase (nNOS), and are thought to function as adapter proteins anchoring or recruiting signaling proteins or ion channels to the membrane (Gee et al., 1998, Schultz et al., 1998, Brenman et al., 1996, Lumeng et al., 1999). Syntrophin isoforms are also known to have
specific expression patterns and differential associations with interacting proteins. For example, the β2-syntrophin isoform is specifically expressed at the neuromuscular junction and binds MAST, whereas α1-syntrophin is localized on the sarcolemma and does not bind MAST (Lumeng et al., 1999, Peters et al., 1994). A primary complex of specific dystrophin and dystrobrevin isoforms in the retina could recruit specific subsets of syntrophin molecules to the complex, providing the means for specific channel clustering and positioning. Preliminary studies show that α1-syntrophin coimmunoprecipitates with Dp71 in cultured rat retinal Müller cells (Claudepierre et al., 2000a, Claudepierre et al., 2000b). An examination of syntrophin isoform expression in the retina will be valuable to obtain additional evidence for the functional differences between the unique dystrophin complexes at the OPL and ILM.

In addition to the dystrophin/dystrobrevin complexes formed in the retina, dystrobrevin has also been shown to interact with the dystrophin autosomal homologue, utrophin (Peters et al., 1998). Here we have shown that utrophin colocalizes with dystrobrevin at the ILM, OPL and surrounding the microvasculature in wildtype and mdx allelic variants. In skeletal muscle, utrophin is usually found concentrated at the crest of the junctional folds of the neuromuscular junction, but is upregulated in DMD patients and mice and is found along the sarcolemma (Helliwell et al., 1992, Nguyen et al., 1991). This partial compensation, however, is not sufficient to eliminate the symptoms of DMD. In the retina, an interaction between utrophin and dystrobrevin may explain the low level of dystrobrevin expression observed in mdx^{4Cv} and mdx^{3Cv} retinal sections. However, any potential compensation by utrophin/dystrobrevin complexes that may be occurring in the
absence of dystrophin, is not sufficient to prevent the abnormal neurotransmission observed in DMD patients.

In summary, we have shown that α-dystrobrevin-1 and β-dystrobrevin are expressed in the retina and are differentially localized to the ILM/blood vessels and OPL respectively. This provides evidence that the dystrophin protein complexes perform unique functions in the different layers of the retina. Evidence for the existence of unique dystrobrevin-containing complexes has been previously shown in brain neurons and glia as well as in the kidney, emphasizing the functional significance of distinct dystrobrevin/dystrophin complexes (Blake et al., 1999, Loh et al., 2000). We have also shown that dystrobrevin requires dystrophin for proper expression, suggesting that dystrobrevin is playing a role, along with dystrophin, in the mechanism that allows for the generation of a normal ERG b-wave. A detailed examination of the ERG from the α-dystrobrevin knockout mouse would provide further insight into the proposed involvement of the dystrobrevin family of proteins in neurotransmission across the retina.

III.5 Materials and Methods

Antibodies

Table 1 outlines the antibodies used and their specificities.

Antibodies ACT1, βCT-FP, α1CT-FP and β417 have previously been described (Howard et al., 1998a, Blake et al., 1998, Loh et al., 2000). α2N was a gift from Dr. Derek Blake of the University of Oxford, UK.
Table III.1: Antibodies and their specificities

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Protein Specificity</th>
</tr>
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<tbody>
<tr>
<td>βCT-FP</td>
<td>β-dystrobrevin, α-dystrobrevin-1 and α-dystrobrevin-2</td>
</tr>
<tr>
<td>ACT-1</td>
<td>All dystrophin isoforms with an alternatively spliced C-terminus (lacking exon 78)</td>
</tr>
<tr>
<td>α1CT-FP</td>
<td>α-dystrobrevin-1</td>
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<tr>
<td>α2N</td>
<td>α-dystrobrevin-2</td>
</tr>
<tr>
<td>β417</td>
<td>β-dystrobrevin</td>
</tr>
<tr>
<td>NCL-DRP2</td>
<td>Utrophin</td>
</tr>
</tbody>
</table>
Western Blotting

Protein extracts were prepared by homogenizing mouse tissues in sample buffer (0.05M Tris pH 6.8, 2% SDS, 10% glycerol, 0.36M B-Mercaptoethanol). Approximately 100µg of protein of each tissue were loaded on a 4-12% polyacrylamide gradient gel (Novex) and separated at 120 volts for 2 hours. Proteins were electrophoretically transferred to a PVDF membrane (BIORAD) in transfer buffer (0.24% w/v Tris base, 1.16% w/v glycine, 0.01%w/v SDS). The membrane was then blocked overnight at 4°C in 5% powdered milk in TBST (10mM Tris pH 8.0, 150mM NaCl, 0.05% Tween-20). Primary antibody βCT-FP was applied at a 1:1000 dilution in 1% milk in TBST for 2 hours at room temperature. The membrane was washed 3 times in TBST for 10 minutes. Horseradish peroxidase-labelled anti-rabbit secondary antibody (Amersham) was applied at a 1:2500 dilution in 1% milk in TBST for 1 hour at room temperature. The membrane was again washed 3 times for 10 minutes in TBST. Dystrobrevin was detected using an Enhanced Chemiluminescence kit as recommended (Amersham).

Indirect Immunofluorescence

Mouse eyes were embedded in OCT (Miles) and immediately frozen in liquid nitrogen. 7µm sections were cut on a Leica microtome and mounted on silane-coated slides at -20°C. All slides except those used with antibodies β417 and NCL-DRP2 were fixed for 15 minutes in 2% paraformaldehyde (ACP) at room temperature. Slides were then incubated in 0.2% sodium borohydride (Sigma) for 15 minutes followed by 0.1% Triton X-100 (Sigma) for 15 minutes at room temperature. NCL-DRP2 (utrophin) sections were fixed in acetone for 4 minutes at -20°C and allowed to dry at room
temperature for 20 minutes. β417 (β-dystrobrevin) sections were unfixed. Fixed sections were washed in filtered PBS for 10 minutes prior to antibody incubation.

All sections were blocked in 10% horse serum in PBS for 45 minutes, followed by a 10 minute wash in PBS. Primary antibodies were diluted in 1% horse serum, 1% fetal calf serum, 0.03% BSA in PBS at the following concentrations: ACT1 (1:300), βCT-FP (1:500), α1CTFP (1:500), β417 (1:25), α2N (1:20), and NCL-DRP2 (1:6). Sections were incubated for 2 hours at room temperature with the primary antibody and then washed three times for 10 minutes in PBS. Negative controls were treated identically except for the omission of primary antibodies. All sections were incubated with biotinylated anti-rabbit IgG antibodies (Amersham) at a 1:200 dilution in 1% horse serum, 1% fetal calf serum, 0.03% BSA in PBS except those with the NCL-DRP2 primary antibody for which biotinylated anti-mouse IgG antibodies (1:200) (Vector) were used. The secondary antibody incubations were performed for 1 hour at room temperature, followed by three 10 minute washes in PBS. All sections were incubated with CY-3 conjugated to streptavidin (Jackson) at a dilution of 1:200 in 1% horse serum, 1% fetal calf serum, 0.03% BSA in PBS for 1 hour at room temperature. Sections were washed three times for 10 minutes in PBS, mounted in Immunoflo (ICN) and examined using a Leica epifluorescent microscope (Aristoplan).
Chapter IV

Identification of β-dystrobrevin Interacting Proteins

The work in this chapter is my own. Ivan Blasutig, a fourth-year thesis student, generated the β-dystrobrevin yeast two-hybrid construct and helped to perform the yeast two-hybrid screen under my direct supervision.
IV.1 Abstract

Dystrophin, the gene defective in Duchenne Muscular Dystrophy, gives rise to several protein isoforms that are part of a specialized extended protein complex. The various functions of these dystrophin complexes are becoming more apparent with the continual identification and characterization of associated proteins. β-dystrobrevin, a newly characterized member of the dystrophin-related protein family, is highly expressed in the central nervous system but is not found in muscle. It is found in brain neurons and at the first synaptic layer of the retina, suggesting it may provide the adaptation or specificity required for the dystrophin complex to function at interneuronal synapses. We used a yeast two-hybrid approach to identify β-dystrobrevin interacting proteins to provide further insight into β-dystrobrevin’s functional contribution to the dystrophin complex. We screened a mouse brain library and identified the type 1α regulatory subunit of cAMP-dependent protein kinase, α1-liprin and its brain specific homologue α2-liprin. These interactions in yeast were confirmed using an independent in vitro binding assay.

The regulatory subunits of cAMP-dependent protein kinase are known to bind to anchoring proteins that serve to compartmentalize the kinase within the cell. Similarly, liprins function to localize leukocyte common antigen-related (LAR) tyrosine phosphatases at specific sites on the plasma membrane, possibly regulating their interaction with the extracellular environment. The potential interactions between cAMP-dependent protein kinase/β-dystrobrevin and liprin/β-dystrobrevin would serve to link β-dystrobrevin and the dystrophin complex to kinase and phosphatase signaling
pathways respectively. Further research will verify these findings and provide important information regarding the function of β-dystrobrevin at interneuronal synapses.

IV.2 Introduction

Dystrophin is a member of a multi-molecular complex that is thought to have a structural role in membrane stabilization in addition to serving as a scaffold for the positioning or clustering of specific signaling molecules at the membrane. In the Duchenne Muscular Dystrophy (DMD) disease state, the absence of dystrophin is often accompanied by a disruption of dystrophin-associated proteins, making the molecular analysis of DMD more difficult (Ervasti et al., 1990, Ohlendieck and Campbell, 1991, Ohlendieck et al., 1993, Metzinger et al., 1997). Since the function of dystrophin is accomplished through its intimate association with an extended complex of proteins, a complete understanding of dystrophin function requires the identification and characterization of each associated protein.

α-dystrobrevin is a dystrophin-related and dystrophin-associated protein that was originally identified as post-synaptic phosphoprotein in the electric organ of Torpedo californica (Carr et al., 1989, Wagner et al., 1993). The α-dystrobrevin protein is actually expressed as a family of spliced isoforms, generated through the regulated use of several unique promoters and alternative splicing at three internal variable regions (Blake et al. 1996a, Ambrose et al., 1997, Holzfeind et al., 1999). α-dystrobrevin-1 is expressed primarily at the neuromuscular junction, whereas α-dystrobrevin-2 can be found along the entire sarcolemma, suggesting that the different isoforms have unique functions (Peters et al., 1998). α-dystrobrevin-1 is subject to tyrosine phosphorylation and
copurifies with acetylcholine receptors, suggesting a role in synapse structure and function (Carr et al., 1989, Balasubramanian et al., 1998). The phenotype of the α-dystrobrevin knockout mouse supports the predicted synaptic role for this dystrophin-associated protein, as null mice develop muscular dystrophy as well as impaired acetylcholine receptor aggregation (Grady et al., 1999). The absence of structurally impaired muscle fibres, the maintenance of the dystrophin complex and the altered nNOS signaling in the α-dystrobrevin knockout mice suggest that the observed dystrophic phenotype is the result of a disruption of muscle cell signaling rather than the loss of sarcolemmal stabilization (Grady et al., 1999). This provides strong support for a signaling function for the entire dystrophin complex.

In 1997, a second dystrobrevin gene was identified with 77% nucleotide identity to the 3’ end of α-dystrobrevin (Peters et al., 1997b, Blake et al., 1998, Puca et al., 1998). This novel member of the dystrophin superfamily, β-dystrobrevin, displays a unique pattern of expression. It is highly expressed in the central nervous system (CNS) and kidney, but unlike α-dystrobrevin, is absent in muscle (Peters et al., 1997b, Blake et al., 1998). In the CNS, β-dystrobrevin is enriched at the post-synaptic membranes of neurons in the cortex and hippocampus where it forms distinct complexes with dystrophin and the dystrophin-associated protein syntrophin (Blake et al., 1999). Non-progressive neurophysiological abnormalities, including cognitive impairments and abnormal retinal neurotransmission are common phenotypic elements of DMD (Fillers et al., 1993, Cibis et al., 1993, Sigesmund et al., 1994, Fitzgerald et al., 1994, Mehler, 2000). This suggests that β-dystrobrevin plays an important role in the functioning of the dystrophin complex in the CNS where it may provide the specialization required for
dystrophin to function at interneuronal synapses. The localization of β-dystrobrevin at the first synaptic layer of the retina, the outer plexiform layer, and its disrupted localization in dystrophin-deficient retinas, further support this hypothesis (Chapter III).

To further characterize the function of β-dystrobrevin in the CNS, we used a yeast two-hybrid approach to identify β-dystrobrevin interacting proteins. The type 1α regulatory subunit of cAMP-dependent protein kinase and two liprin homologues were identified in this screen as novel β-dystrobrevin interacting proteins. The regulatory subunits of the cAMP-dependent protein kinase are thought to perform two distinct roles. They function as inhibitors of the catalytic subunits of the kinase in addition to anchoring the entire protein complex at specific intracellular sites (Taylor et al., 1990, Lester and Scott, 1997). Members of the liprin family of proteins are known to localize transmembrane leukocyte common antigen-related (LAR) tyrosine phosphatases to specific sites on the plasma membrane (Serra-Pagès et al., 1998). The interactions of these proteins with β-dystrobrevin may serve to link the extended dystrophin protein complex to kinase and phosphatase signaling pathways.

IV.3 Results
IV.3.1 Yeast Two-Hybrid Construct

To identify β-dystrobrevin interacting proteins we generated a full-length β-dystrobrevin bait construct for use in a yeast two-hybrid library screen. RT-PCR of mouse brain RNA was performed to obtain a full-length clone, which was fused in-frame to the Gal4 binding domain (Gal4-BD) in the pGBT9 vector (TRP1, amp'). To ensure that no mutations were introduced during the multiple rounds of PCR, and to identify the
splicing status of our clone, the entire construct was sequenced. Results indicated that
our construct was alternatively spliced at variable regions B and C (Figure IV.1). This
corresponds to a seven amino acid insertion at position 518 (B) and the absence of exon
17 (C). Transcripts with both of these splicing events predominate in brain indicating
that our clone represents a functional CNS isoform (Blake et al., 1998).

To ensure that our bait construct would be suitable for use in a yeast two-hybrid
screen, a Western blot was performed to analyze the expression of the β-dystrobrevin
hybrid protein. Yeast protein extracts were separated by SDS-PAGE, and a Western blot
was performed using antibodies against dystrobrevin and the Gal4-BD. Figure IV.2
shows that the Gal4-BD/β-dystrobrevin hybrid protein is stably expressed in Y190 as it is
recognized as the expected ~88kDa hybrid protein by both primary antibodies.

To determine if our bait construct had intrinsic DNA-binding and/or
transcriptional properties which would cause it to auto-activate the system, we
transformed our β-dystrobrevin bait construct into yeast either by itself or with the non-
specific activation domain hybrid pTD1 (SV40 large T-antigen) and screened for β-
galactosidase expression using a filter assay (Table IV.1). The β-dystrobrevin construct
did not auto-activate the system (data not shown).

As a final test to ensure that our β-dystrobrevin hybrid protein was able to fold
properly, we used the β-galactosidase filter assay to test for an interaction between
dystrobrevin and dystrophin, a known dystrobrevin-interacting partner (Table IV.1).
Yeast co-transformed with the Gal4-BD/β-dystrobrevin bait and a Gal4 activation
domain plasmid containing dystrophin isoform Dp71 turned blue very quickly in the filter
assay indicating a strong interaction between the two proteins (data not shown).
A: Deletion of amino acids 361-390
B: Insertion of EEEQKQA at position 516
C: Deletion of amino acids 573-602 (exon 17)
D: Deletion of amino acids 603-608 (exon 18)

Figure IV.1: Schematic Representation of the β-dystrobrevin Protein Structure. The functional domains including the EF hands, ZZ motif and coiled-coil (CC) region are boxed. The four alternative splicing regions (A-D) are indicated. The β-dystrobrevin construct used in the yeast two-hybrid experiment is spliced at regions B and C.
Figure IV.2: Western Blot Analysis Confirming the Stable Expression of the Gal4-β-dystrobrevin Protein in Yeast. Panel a) Western blot using the βCT-FP antibody which recognizes both α-dystrobrevin (α-db) and β-dystrobrevin (β-db). The two bands observed in the brain positive control lane correspond to α-dystrobrevin and β-dystrobrevin. The Gal4-BD/β-dystrobrevin (Gal4-β-db) fusion protein migrates at the expected 88kDa. The lower band in the Y190 Gal4-β-dystrobrevin lane (marked with an *) most likely represents an N-terminal degradation product, as a corresponding band is not present on the Western using the anti-Gal4-BD antibody. The Y190 negative control lane corresponds to protein from yeast cells that do not contain the Gal4-BD/β-dystrobrevin fusion protein. Panel b) A comparable blot stained with a Gal4-Binding Domain specific antibody. The hybrid Gal4-β-dystrobrevin (Gal4-β-db) protein of approximately 88kDa is visualized.
Table IV.1: Yeast Two-Hybrid Transformations and β-Galactosidase Filter Assay Results

<table>
<thead>
<tr>
<th>Gal4 Binding Domain Plasmid</th>
<th>Gal4 Activation Domain Plasmid</th>
<th>β-Gal Result (colony colour)</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-dystrobrevin</td>
<td>none</td>
<td>white</td>
<td>To ensure the β-dystrobrevin fusion does not autoactivate</td>
</tr>
<tr>
<td>β-dystrobrevin</td>
<td>pTD1 (SV40 Large T-antigen)</td>
<td>white</td>
<td>To confirm that the β-dystrobrevin bait does not interact with non-specific proteins</td>
</tr>
<tr>
<td>β-dystrobrevin</td>
<td>Dystrophin (Dp71)</td>
<td>blue</td>
<td>A positive control to ensure the β-dystrobrevin bait folds properly</td>
</tr>
<tr>
<td>β-dystrobrevin</td>
<td>pACT2: library cDNA</td>
<td>blue</td>
<td>To identify β-dystrobrevin interacting proteins</td>
</tr>
<tr>
<td>none</td>
<td>pACT2: library cDNA</td>
<td>white</td>
<td>To confirm that the library activation domain fusion does not autoactivate</td>
</tr>
<tr>
<td>pVA3 (murine p53 hybrid vector)</td>
<td>pACT2: library cDNA</td>
<td>white</td>
<td>To eliminate library clones that bind to non-specific binding domain fusion proteins</td>
</tr>
<tr>
<td>pLAM (laminC hybrid vector)</td>
<td>pACT2: library cDNA</td>
<td>white</td>
<td>To eliminate library clones that bind to non-specific binding domain fusion proteins</td>
</tr>
<tr>
<td>pVA3 (murine p53 hybrid vector)</td>
<td>pTD1 (SV40 Large T-antigen)</td>
<td>blue</td>
<td>A positive transformation control</td>
</tr>
<tr>
<td>pLAM (laminC hybrid vector)</td>
<td>pTD1 (SV40 Large T-antigen)</td>
<td>white</td>
<td>A negative transformation control</td>
</tr>
</tbody>
</table>
IV.3.2 Yeast Two-Hybrid Library Screen

In the absence of an available retinal yeast two-hybrid library, a commercially available mouse brain cDNA library with over $3 \times 10^6$ unique clones fused to the Gal4 activation domain in the pACT2 vector (Leu2, amp') was selected for the screen (Clontech). Saccharomyces cerevisiae strain Y190 was sequentially transformed with the β-dystrobrevin bait construct and 110μg of mouse brain cDNA. The physical separation of the two functionally independent Gal4 domains allowed for a powerful selection that would only permit the growth of colonies in which a protein-protein interaction was occurring. This would drive the expression of both the His3 nutritional reporter and the lacZ enzymatic reporter, permitting the identification of proteins that interact with our β-dystrobrevin bait. Transformed yeast were plated on SD media lacking histidine as an initial selection for interacting proteins. The screen was performed in the presence of 30mM 3-aminotriazole which acted as a competitive inhibitor of the His3 protein, suppressing any leaky His expression endogenous to the Y190 yeast strain. 56 His$^+$ clones were obtained after 3, 4 or 5 days of growth and were subsequently screened for β-galactosidase expression to confirm the His reporter results (Table IV.1). 52 His$^+$lacZ$^+$ colonies were obtained and the activation domain library plasmids were isolated, sequenced and identified using BLAST analysis (http://blast.bioinfo.sickkids.on.ca). To confirm the selection and to eliminate false positives due to activation by the library plasmid alone, each activation domain clone was reintroduced into yeast alone, with the β-dystrobrevin bait and with two non-specific bait hybrids, pVA3 and pLAM (Table IV.1). 45 His$^+$lacZ$^+$ clones were confirmed as potential β-dystrobrevin interacting proteins. Table IV.2 outlines the β-dystrobrevin interacting proteins.
Table IV.2: Yeast Two-Hybrid Clone Descriptions

<table>
<thead>
<tr>
<th>Clone Identification</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.4, 8.1, 8.2, 10.1, 10.2, 14.4, 17.2, 22.1, 25.3, 28.1, 28.4, 28.5</td>
<td>Type Iα regulatory subunit of cAMP-dependent protein kinase</td>
</tr>
<tr>
<td>7.1</td>
<td>α1-liprin (LAR interacting protein)</td>
</tr>
<tr>
<td>17.1</td>
<td>α2-liprin (LAR interacting protein)</td>
</tr>
<tr>
<td>2.5, 14.1, 17.5, 21.2, 24.1, 25.1</td>
<td>Kinesin Heavy Chain</td>
</tr>
<tr>
<td>13.1, 23.1</td>
<td>Brain β-3 Spectrin</td>
</tr>
<tr>
<td>2.1</td>
<td>Necdin</td>
</tr>
<tr>
<td>2.2</td>
<td>JM1 protein</td>
</tr>
<tr>
<td>9.1</td>
<td>Leucine Rich Repeat</td>
</tr>
<tr>
<td>11.1</td>
<td>Sorting Nexin</td>
</tr>
<tr>
<td>15.1</td>
<td>Valosin Containing Protein</td>
</tr>
<tr>
<td>15.2</td>
<td>Pancortin-2</td>
</tr>
<tr>
<td>19.5</td>
<td>α cardiac myosin heavy chain</td>
</tr>
<tr>
<td>22.4</td>
<td>Putative Transmembrane GTPase</td>
</tr>
<tr>
<td>26.1</td>
<td>Talin</td>
</tr>
<tr>
<td>28.2</td>
<td>KIAA0373</td>
</tr>
<tr>
<td>30.1</td>
<td>Nuclear Pore Glycoprotein p62</td>
</tr>
</tbody>
</table>
obtained from the yeast two-hybrid screen. Several of these clones represent excellent candidates for further study. Multiple clones of the type $1^\alpha$ regulatory subunit of cAMP-dependent protein kinase were identified in the screen. Of the 12 isolates, 8 independent clones were represented, providing strong evidence for an interaction with $\beta$-dystrobrevin. cAMP-dependent protein kinase is the major receptor for cAMP in eukaryotic cells and is involved in countless signal transduction pathways (Taylor et al., 1990). $\alpha$1-liprin and its brain-specific homologue, $\alpha$2-liprin, were selected for further analysis because the two liprin homologues, that are 72% identical each bind to $\beta$-dystrobrevin. Liprins are a family of transmembrane leukocyte common antigen-related (LAR) phosphatase binding proteins that are thought to localize LARs to specific sites on the plasma membrane (Serra-Pagès et al., 1995, Serra-Pagès et al., 1998). To further characterize these potential novel interactions, in vitro binding assays were performed.

IV.3.3 In vitro Binding Assays

In vitro binding assays were used as an independent method to confirm the $\beta$-dystrobrevin/cAMP-dependent protein kinase and $\beta$-dystrobrevin/liprin interactions observed in yeast. A glutathione S-transferase (GST)/$\beta$-dystrobrevin fusion construct was made and transformed into E. coli for the purification of a GST-$\beta$-dystrobrevin fusion protein. Negative control proteins included a GST-alone protein and a dystrophin hydrophobic C-terminal GST-fusion (amino acids 3668-3698). All constructs were expressed in bacteria, batch purified and tested for expression using Western blot analysis (Figure IV.3). The purified fusion proteins were conjugated to glutathione sepharose 4B and incubated with $^{35}$S-labelled proteins (cAMP-dependent protein kinase R1$\alpha$, $\alpha$1-liprin
Figure IV.3: Western Blot Analysis of GST Fusion Proteins. Three fusion proteins, GST-alone, GST-dystrophin and GST-β-dystrobrevin were purified and tested for expression using antibodies specific for GST, dystrophin and dystrobrevin. The GST antibody recognized all fusion proteins (top row), while antibodies βCT-FP and ACT-1 recognized the β-dystrobrevin and dystrophin fusions respectively.
or α2-liprin) which had been transcribed and translated in vitro. After a two-hour incubation at 4°C and five brief washes, bound protein was eluted from the beads by boiling in water, run on an SDS-polyacrylamide gel and visualized by autoradiography. Figure IV.4 shows that the type Iα regulatory subunit of cAMP-dependent protein kinase and both liprin homologues bind specifically to β-dystrobrevin and not to the GST-alone or GST-dystrophin controls, confirming the yeast two-hybrid results.
Figure IV.4: *In vitro* Binding Assays. $^{35}$S-labeled type I$^\alpha$ regulatory subunit of cAMP-dependent protein kinase, $\alpha$1-liprin and $\alpha$2-liprin prepared by *in vitro* transcription/translation were incubated with a GST-\(\beta\)-dystrobrevin fusion protein and negative control fusion proteins (GST-alone and GST-dystrophin) to test for specific interactions. Bound proteins were eluted, separated by SDS-PAGE and visualized by autoradiography. In this assay, the type I$^\alpha$ regulatory subunit of cAMP-dependent protein kinase and both $\alpha$1-liprin and $\alpha$2-liprin bound to $\beta$-dystrobrevin and did not bind to control fusion proteins.
IV.4 Discussion

β-dystrobrevin is a unique member of the dystrophin protein complex that is not expressed in muscle. Its expression is mainly restricted to the central nervous system and the kidney where it is thought to play a key role in the specialized function of the dystrophin complexes in these tissues (Peters et al., 1997b, Blake et al., 1998). To further understand β-dystrobrevin’s contribution to the function of the dystrophin complex, we performed a yeast two-hybrid experiment to identify novel interacting proteins. Several potential interacting proteins were identified, and the interactions with the type 1α regulatory subunit of cAMP-dependent protein kinase, α1-liprin and its brain-specific homologue, α2-liprin, were selected for further characterization.

cAMP-dependent protein kinase (PKA) is a ubiquitously expressed holoenzyme that is the major receptor for cAMP in eukaryotic cells (reviewed in Taylor et al., 1990). In the absence of the diffusible second messenger cAMP, PKA exists as an inactive tetramer containing two regulatory subunits and two catalytic subunits (Taylor et al., 1990). To date, genes encoding four regulatory subunits (RIα, RIβ, RIIα and RIIβ) and three catalytic subunits (Cα, Cβ, and Cy) have been identified (reviewed in Scott, 1991). The binding of two cAMP molecules to each regulatory subunit activates the kinase by dissociating the catalytic subunits (Beebe and Corbin, 1986). PKA is then able to act as a modulator of several biological processes including ion transport, cell differentiation, gene transcription, development and synaptic transmission. The precise subcellular localization of PKA is an important prerequisite to its function. PKA is tethered to subcellular sites near specific substrates through an association with anchoring proteins called AKAPs (cAMP-dependent protein kinase anchoring proteins) (Lester and Scott,
1997). These AKAPs bind to the regulatory subunits of PKA and anchor the tetrameric complex to structural proteins, membranes or cellular organelles (Figure IV.5). For example, the binding of PKA to AKAP15 targets the kinase to voltage-gated brain sodium channels and voltage-gated skeletal muscle calcium channels (Tibbs et al., 1998).

Although most AKAPs bind the type II regulatory subunits of PKA, recent studies have identified proteins that can also bind the type I regulatory subunits (Huang et al., 1997). In our yeast two-hybrid screen, the type Iα regulatory subunit of PKA was identified as a potential β-dystrobrevin binding protein. It is therefore possible that β-dystrobrevin functions as an AKAP and serves to position PKA complexes containing the type I regulatory subunit. In addition to binding to the regulatory subunit of PKA, to function as an AKAP, β-dystrobrevin would also require a unique targeting domain to anchor the kinase to specific intracellular sites. This could be readily accomplished through β-dystrobrevin’s interaction with dystrophin. This would tether the β-dystrobrevin/kinase complex to the actin cytoskeleton and to specific sites on the plasma membrane. This interaction would serve to link the entire dystrophin complex to a protein kinase signaling pathway.

The other major focus of this chapter is the identification of two liprin homologues as β-dystrobrevin interacting proteins. The liprin genes give rise to a family of proteins that bind to leukocyte common antigen-related (LAR) transmembrane protein-tyrosine phosphatases (Serra-Pagès et al., 1995, Serra-Pagès et al., 1998). They are subdivided into α-type and β-type liprins based on sequence homology and binding characteristics (Figure IV.6). Within the α-subtype, there are four human family members and one C. elegans ortholog. Fewer β-liprins exist with only two known
Figure IV.5: The cAMP-dependent Protein Kinase Complex. This complex is composed of two catalytic subunits (C) and two regulatory subunits (R). An anchoring protein (AKAP) serves to tether the kinase to specific subcellular structures to compartmentalize the holoenzyme within the cell.
Figure IV.6: Overall structure of α and β liprins. N-terminal coiled-coil regions and C-terminal liprin homology (LH) domains are schematically depicted for α1- and β1-liprin. The yeast two-hybrid liprin clones obtained from the library screen with β-dystrobrevin are included. The α1-liprin yeast two-hybrid clone includes the last 763 out of 1202 amino acids, while the α2-liprin yeast two-hybrid clone was almost full-length including 1240 of the 1257 amino acids.
human members and one C. elegans ortholog. Both α and β liprins have N-terminal coiled-coil motifs that mediate homodimerization and heterodimerization within each liprin subtype (Serra-Pagès et al., 1998). The C-terminal non-coiled-coil 250 amino acid domain, or liprin homology (LH) region, displays a high degree of sequence conservation throughout all members of the liprin protein family (Serra-Pagès et al., 1998). Within this region, there are 34 amino acids that are completely conserved in all six human and two C. elegans liprin proteins. 95 other amino acids in the LH domain are conserved in at least six of the eight family members. This extensive sequence conservation suggests that this region is functionally significant and may mediate important protein-protein interactions. It has been established that the LH domain of α-liprin interacts with the intracellular phosphatase domain of LAR family members and also permits heterodimerization with β-liprins (Serra-Pagès et al., 1995, Serra-Pagès et al., 1998).

Transmembrane LARs are broadly expressed protein tyrosine phosphatases that are thought to play a role in the communication between the external and internal environments of a cell (Pulido et al., 1995). Co-expression studies indicate that α-liprins serve to localize these LARs at non-random locations on the plasma membrane (Serra-Pagès et al., 1998). LAR/liprin complexes are concentrated at sites of cell-cell and cell-matrix adhesion where they are thought to be involved in cellular communication, in addition to mediating specific linkages between the extracellular environment and the cytoskeleton (Serra-Pagès et al., 1998). Our finding that liprin binds to β-dystrobrevin, coupled with the colocalization of dystrophin, dystrobrevin and liprin/LAR complexes at focal adhesions, suggest that the dystrophin complex may play a critical role in the non-random localization of liprin/LAR complexes (Serra-Pagès et al., 1995, Serra-Pagès et
al., 1998). This novel interaction between \(\beta\)-dystrobrevin and \(\alpha\)-liprin would link the dystrophin/dystroglycan complex to the transmembrane phosphatase signaling pathway, broadening the signaling potential associated with the dystrophin complex.

In the past, the yeast two-hybrid strategy has been successfully utilized to identify dystrophin, syntrophin and sarcoglycan interacting proteins (Castelló et al., 1996, Lumeng et al., 1999, Thompson et al., 2000). It is a powerful technique that can recognize even transient protein-protein interactions. A potential drawback, however, is the loss of cell-type specificity in the creation of a yeast two-hybrid library. Although \(\beta\)-dystrobrevin bound both \(\alpha_1\) and \(\alpha_2\)-liprin in our screen, it is unknown whether both of these events represent biologically relevant interactions. Northern blot analysis has shown that \(\alpha_1\)-liprin is ubiquitously expressed, whereas \(\alpha_2\)-liprin expression is restricted to the central nervous system (Serra-Pagès et al., 1998). \(\beta\)-dystrobrevin is only expressed in the kidney, hippocampal and cortical neurons, and in retinal photoreceptor cells (Chapter III, Peters et al., 1997b, Blake et al., 1998). It will be crucial to determine if the cellular localization of both liprin proteins overlaps that of \(\beta\)-dystrobrevin. It is quite possible that in vivo, \(\beta\)-dystrobrevin interacts with only one of the \(\alpha\)-lirpins and that the other interaction was observed in yeast simply because of the high degree of homology in the C-terminal region of liprin proteins.

The identification of \(\alpha\)-liprin as a \(\beta\)-dystrobrevin interacting protein provides new insight into the function of \(\beta\)-dystrobrevin in the central nervous system. Much of the liprin functional data has been derived from experiments on the \textit{Caenorhabditis elegans} liprin ortholog, SYD-2 (Zhen and Jin, 1999). SYD-2 shares approximately 50% sequence identity with human \(\alpha_1\)-liprin and can interact with both mammalian and
Drosophila LAR in a yeast two-hybrid system, indicating that it is a functional ortholog of liprin (Serra-Pagès et al., 1998, Zhen and Jin, 1999). SYD-2 is expressed at presynaptic terminals of neurons, but is not directly associated with the synaptic vesicles (Zhen and Jin, 1999). A mutation in the *C. elegans* syd-2 (synapse-defective 2) gene results in several mild behavioural defects, including sluggish locomotion and defective egg laying, that indicate a slight impairment in synaptic transmission (Zhen and Jin, 1999). In addition, several ultrastructural synaptic abnormalities were observed that affect the size of the active zone and the distribution of presynaptic proteins (Zhen and Jin, 1999). Although the total number of synaptic vesicles per synapse is unaffected, electron microscopy studies show that the electron-dense active zone of *syd-2* mutants is significantly lengthened. In addition, there is a diffused localization of several presynaptic proteins (synaptotagmin, syntaxin and RAB-3). It was therefore proposed that through its interaction with transmembrane phosphatases, SYD-2 may have a structural role at the pre-synaptic face and/or recruit other molecules to the active zone (Zhen and Jin, 1999).

This scenario is very similar to the situation involving the dystrophin complex in the retina, and several parallels can be drawn between the synapses of *C. elegans* and mammalian retinal synapses. At the outer plexiform synaptic layer, the dystrophin complex is located presynaptically on the photoreceptor plasma membrane that borders the lateral sides of the invaginated synaptic triad (Schmitz and Drenckhahn, 1997a, 1997b, Ueda et al., 1995, Ueda et al., 1998). Similar to SYD-2, dystrophin and its associated proteins are not components of the synaptic vesicles, but instead are localized just distal to the active synaptic zones that contain the synaptic ribbon (Schmitz and
Drenckhahn, 1997a, 1997b). At this synaptic layer, β-dystrobrevin is postulated to act as a scaffold for the positioning or clustering of signaling proteins at the membrane (Chapter III), similar to the proposed function of SYD-2. In the absence of dystrophin and β-dystrobrevin, retinal neurotransmission is partially impaired, but no morphological changes have been observed in the retina (Pillers et al., 1993, Cibis et al., 1993, Sigesmund et al., 1994, Fitzgerald et al., 1994, Pillers et al., 1999). Similar to the mild synaptic defects observed in the syd-2 mutant, this defect is subtle, and does not result in an embryonic lethal DMD phenotype. The similarities in protein localization, proposed function and mutation phenotype between SYD-2 and the dystrophin complex provide strong evidence for an interaction between the dystrophin-associated protein, β-dystrobrevin, and α-liprin.

It was originally proposed that the association between β-dystrobrevin and dystrophin at interneuronal synapses provides a mechanism for the assembly of specialized membrane complexes (Blake et al., 1999). However, the molecular basis underlying this hypothesis was unknown. We have demonstrated an interaction between β-dystrobrevin and two members of the liprin family of proteins. Not only does this association link β-dystrobrevin and the dystrophin complex to a phosphatase signaling pathway, it also provides a mechanism by which liprins localize LARs at non-random locations along the membrane. Further research will provide important information regarding the function of β-dystrobrevin at interneuronal synapses.

It is noteworthy, however, that dystrophin was not identified in the screen. The β-galactosidase filter assay assessing the interaction between β-dystrobrevin and dystrophin, performed prior to the library screen, confirmed that the β-dystrobrevin bait
was able to bind to the Gal4 activation domain/dystrophin fusion. This verified that the β-dystrobrevin bait was able to fold properly in yeast. The absence of dystrophin clones identified in the screen may be due to a low library representation of rare clones, such as dystrophin.

IV.5 Materials and Methods

Yeast Two-Hybrid Constructs

RT-PCR was used to obtain a full-length mouse β-dystrobrevin clone. Brain RNA was reverse transcribed using an oligo dT primer. β-dystrobrevin specific primers (for: 5' - gagagaattcatgattgaggaagc-3' and rev: 5'-caacgtcgcacagcagagtaacc-3') and Elongase (Gibco BRL) were then used for PCR amplification under the following conditions: 94°C x 1 min. followed by 30 cycles of 94°C x 30 s, 55°C x 30 s, 68°C x 2.5 min. Gel purified product was subcloned in-frame, downstream of the Gal4 Binding Domain in the pGBT9 vector (Clontech). The pACT2-Dystrophin (Dp71) activation domain plasmid was isolated from the Clontech yeast two-hybrid mouse brain library by a fellow graduate student in the lab.

Control plasmids for the yeast two-hybrid system included pVA3, pTD1 and pLAM. pVA3 (TRP1, amp') encodes a Gal4-BD-murine-p53 fusion protein in a pGBT9 backbone. pVA3 is known to bind to pTD1 and is used as a positive control. It is also used in co-transformations with the library activation domain plasmids to eliminate false positive activation domain fusions that bind to non-specific proteins. pTD1 (LEU2, amp') encodes a Gal4-SV40 large T-antigen fusion protein in pGAD3F. pLAM (TRP1,
amp') is another false positive detection plasmid encoding a Gal4-human-laminC fusion protein in pGBT9.

**Sequencing**

The entire β-dystrobrevin yeast two-hybrid bait construct and approximately 200 nucleotides from the 5' end of each yeast two-hybrid interacting clone were sequenced using a USB cycle sequencing kit. The following pACT2 specific forward primer (pGAD2) was used to sequence all yeast two-hybrid activation domain clones: 5'-tggtcatagcagcagga-3'.

**β-Galactosidase Filter Assay**

Individual His' colonies from the yeast two-hybrid selection were streaked on Whatman filters (No. 3) and grown overnight at 30°C on an SD media plate. Filters were then flash frozen in liquid nitrogen and placed colony side up on a piece of Whatman paper (No. 40) presoaked with 2.5ml of Z-buffer (60mM Na₂HPO₄•7H₂O, 40mM NaH₂PO₄•H₂O, 10mM KCl, 1mM MgSO₄, 30mM β-Mercaptoethanol) with a final concentration of 0.2mg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Filters were incubated at 30°C and analyzed every 30 minutes over the course of 4 hours for β-galactosidase activity. A blue colour indicated a positive protein-protein interaction and was scored from + (light blue) to ++++ (dark blue). Colonies that remained white were classified as containing a negative protein-protein interaction.
Western Blot Analysis

Untransformed yeast and yeast transformed with a β-dystrobrevin bait construct were grown in 10ml of the appropriate SD selection media to an OD$_{600}$ of 0.5-0.8. Cultures were cooled on ice for 15 minutes, pelleted at 4°C for 10 minutes at 3000rpm, resuspended in sterile water, repelleted at 14 000rpm for 5 minutes and frozen at −20°C for 15 minutes. Frozen cell pellets were cracked in 100μl prewarmed (60°C) cracking buffer (1% β-mercaptoethanol, 1% SDS, 6M urea, 0.1mM EDTA, 40mM Tris-HCl pH 6.8, 10% glycerol), protease inhibitor cocktail, and glass beads. The mixtures were vortexed for 5 minutes, heated to 70°C for 10 minutes, vortexed again for 5 minutes and centrifuged at 14 000rpm for 5 minutes at 4°C. The supernatant was removed and the pellet was re-extracted in 50μl cracking buffer. Supernatants were pooled and stored at −80°C.

Brain tissue extracts were prepared by the homogenization of frozen mouse brain tissue in sample buffer (0.05M Tris pH 6.8, 2% w/v SDS, 10% v/v glycerol, 0.36M β-Mercaptoethanol).

Induced GST-fusion proteins, GST-β-dystrobrevin, GST-alone, and GST-dystrophin (see In vitro Binding Assay section below), were batch purified on Glutathione Sepharose 4B (Pharmacia).

All protein was boiled in water, loaded on SDS-polyacrylamide gels and separated at 120V for 2 hours. Protein was then electrophoretically transferred to PVDF (BIORAD) membranes in transfer buffer (0.24% w/v Tris base, 1.16% w/v glycine, 0.01%w/v SDS). Membranes were blocked at 4°C overnight in TBST (10mM Tris pH 8.0, 150mM NaCl, 0.05% v/v Tween-20) with 5% powdered milk. Primary antibodies
dystrobrevin (1/500), Gal4 Binding Domain (1/500), GST (1/2000) and dystrophin ACT-1 (1/500) were applied to the membrane for 2 hours at room temperature in TBST with 1% powdered milk. The polyclonal dystrobrevin antibody, βCT-FP, recognizes both α and β-dystrobrevin and has been previously described (Blake et al., 1998). ACT-1 recognizes the hydrophobic C-terminus of dystrophin and has been previously described (Howard et al., 1998a). The monoclonal Gal4 Binding Domain and GST antibodies were obtained commercially (Santa Cruz Biotechnology). Following incubation with the primary antibody, membranes were washed three times for 10 minutes each in TBST, and incubated in TBST with 1% powdered milk with HRP-conjugated secondary antibodies (1/5000) (Amersham). An anti-rabbit secondary was used for the β-dystrobrevin and dystrophin Westerns and an anti-mouse secondary was used for the GST and Gal4 Binding Domain Westerns. Membranes were again washed three times for 10 minutes in TBST. Proteins were visualized using Enhanced Chemiluminescence (Amersham).

Yeast Two-Hybrid Library Screen

To determine the appropriate concentration of 3-aminotriazole for the library screen, competent Y190 cells were transformed with the pGBT9-β-dystrobrevin bait and pTD1, a non-specific activation domain construct. Transformed cells were plated on Trp, Leu and His deficient SD selection plates supplemented with 25, 35, 45 or 55mM of 3-aminotriazole and incubated at 30°C for five days. Since β-dystrobrevin and pTD1 do not interact, this served as a control to observe the background levels of colony growth caused by the leaky His expression endogenous to the Y190 yeast strain. A concentration of 30mM 3-aminotriazole was selected for the screen.
A small-scale transformation was performed to obtain yeast cells containing the β-dystrobrevin bait construct. 100μl of competent Y190 cells were transformed with 0.5μg of pGBT9-β-dystrobrevin plasmid DNA. For the library screen, 5.5ml of competent Y190/pGBT9-β-dystrobrevin yeast cells were transformed with 110μg of yeast two-hybrid brain plasmid DNA (Clontech). Cells were plated on Trp, Leu and His deficient SD selection plates and incubated at 30°C in the presence of 30mM 3-aminotriazole. His+ colonies were picked after 3, 4 or 5 days of growth and tested for β-galactosidase expression using the β-gal filter assay. DNA from His+ lacZ+ colonies was purified using the following protocol. 2ml yeast cultures were grown to saturation in SD selection media. Cells were pelleted and resuspended in 200μl lysis buffer (2% Triton X-100, 1% SDS, 100mM NaCl, 10mM Tris-HCl pH 8.0, 1.0mM EDTA), 0.3g of acid washed glass beads and 200μl phenol:chloroform:isoamyl alcohol (25:24:1). Mixtures were vigourously vortexed for 5 minutes and centrifuged at 14,000 rpm for 10 minutes. The supernatant was collected and the DNA was precipitated with 2.5 volumes of ethanol and 1/10 volume of NaOAc. Following a wash in 70% ethanol, the DNA was resuspended in 10mM Tris pH 8.0. Plasmid purification was performed using a QIAEX extraction kit (Qiagen).

To purify the activation domain vector from each plasmid mixture, competent Escherichia coli MH6 (Leu') cells were transformed with each plasmid mixture and plated on LB-ampicillin plates to obtain ampicillin resistant clones. Individual colonies were then streaked onto M9 plates, which lack leucine, to select for the pACT2 Leu+ library activation domain plasmid. Colonies containing the activation domain plasmids were then grown up in liquid LB and DNA was isolated using the QIAprep Spin
Miniprep Kit (Qiagen). Activation domain inserts were then sequenced. To verify the specific interaction between β-dystrobrevin and each interacting protein, each target clone was reintroduced into yeast alone, with the β-dystrobrevin bait or with control Gal4 binding domain plasmids pVA3 (murine p53) and pLAM (laminC) and subjected to a β-gal filter assay to eliminate false positives (see above).

**In vitro Binding Assay**

A full-length β-dystrobrevin GST fusion was created by subcloning the β-dystrobrevin clone insert from the yeast two-hybrid bait plasmid into the pGEX 4T1 vector (Pharmacia). Negative control plasmids included a GST-alone construct and a dystrophin hydrophobic C-terminal GST/pGEX 4T1 construct encoding dystrophin amino acids 3668-3698. Induced fusion proteins were batch purified on Glutathione Sepharose 4B (Pharmacia) and tested for expression using Western Blot analysis (described above).

The yeast two-hybrid type I regulatory subunit of cAMP-dependent protein kinase, α1-liprin and α2-liprin clones were used as templates for PCR amplification using Elongase (Gibco BRL) and primers allowing for *in vitro* transcription/translation. (kinase forward: 5'-gctaatacgactcactatatggaatgttaaagcaatgtggc-3', α1-liprin forward: 5'-gctaatacgactcactatatggaatgttaaagcaatgtggc-3', α2-liprin forward: 5'-gctaatacgactcactatatggaatgttaaagcaatgtggc-3', and pACT2 vector reverse: 5'-gttgaagtgttaaagc-3'). PCR conditions were as follows: 94°C x 30s followed by 30 cycles of 94°C x 30s, 55°C x 30s, 68°C x 3min 30s. PCR products were
used to generate $[^{35}S]$methionine-labeled proteins using *in vitro* T7-coupled transcription/translation following the manufacturer's instructions (TnT kit, Promega).

50µl aliquots of the bead-conjugated GST-fusion slurries (~25µg of protein) in TBST (10mM Tris pH 8.0, 0.1% v/v Tween-20, 150mM NaCl) were incubated for 2 hours at 4°C with 5µl of each radiolabeled translation reaction. Mixtures were gently centrifuged at 2000rpm for 5 minutes and the unbound supernatant was removed and kept for analysis. The pellet was washed 5 times with 200µl of TBST, resuspended in SDS-sample buffer and boiled in water for 5 minutes to elute the bound protein. Equivalent amounts of unbound and bound fractions were separated by SDS-PAGE and examined by autoradiography.
Chapter V

General Discussion and Future Directions
The goal of this project was to analyze the function of dystrophin through the identification and characterization of dystrophin associated proteins. This approach was selected since the presently known functions of dystrophin are mediated by its interaction with a specialized extended complex of proteins. A more complete understanding of dystrophin function is impossible without information regarding the environment in which it is functioning. The retina was used as the model tissue for this investigation for several reasons. First, the retina is a well-studied, beautifully layered structure that expresses three unique dystrophin isoforms (Pillers et al., 1993, D'Souza et al., 1995, Howard et al., 1998a). One of these isoforms, Dp260 is predominantly expressed in the retina and is not found in muscle. Second, in depth immunofluorescence and electron microscopy studies have shown that these isoforms are differentially localized in the retina. Dp71 lines the endfeet of retinal Müller glial cells at the inner limiting membrane (ILM), outer limiting membrane (OLM) and surrounding the microvasculature, while Dp427 and Dp260 function at the pre-synaptic face of the outer plexiform layer (OPL) (Pillers et al., 1993, D'Souza et al., 1995, Howard et al., 1998a, Chapter II). Third, the dystrophin complexes at the synaptic OPL versus the non-synaptic Müller cell endfeet are further specialized based upon a regulated alternative splicing event that alters the C-terminus of the dystrophin protein. Dp71 utilizes the exon 79-encoded hydrophobic C-terminus, while Dp427 and Dp260 possess the shorter hydrophilic C-terminus (Howard et al., 1998a). Finally, in the absence of dystrophin, many DMD patients and mice display abnormal neurotransmission across the retina (Pillers et al., 1993, Cibis et al., 1993, Sigesmund et al., 1994, Fitzgerald et al., 1994, Pillers et al., 1999). This phenotype is measured using electroretinogram (ERG) analysis, providing a valuable tool for the study.
of dystrophin function in the retina. Of interest was the previous observation that Dp260 and Dp71 each has a unique, yet essential function in normal retinal neurotransmission, while Dp427 does not appear to be essential for the generation of a normal ERG (Cibis et al., 1993, Pillers et al., 1995, Pillers et al., 1999). In summary, Dp260 and Dp71 have unique amino and carboxy terminal structures, differential localization patterns and individual contributions to the ERG. The retina, therefore, was selected as the ideal tissue in which to study the molecular basis for the functional differences between these two dystrophin isoforms. Our hypothesis is that Dp260 and Dp71 form specialized extended protein complexes which serve to dictate their individual functions. Our results support this hypothesis, raising a number of new questions and suggesting several experimental approaches to further improve our understanding of dystrophin function.

V.1 Dystrophin Hydrophobic C-terminus

The existence of a highly conserved, alternatively spliced C-terminus that displays a unique localization pattern in the retina, strongly suggested a functional purpose for this extended C-terminal region. Prior to this study, however, the significance of this specialized hydrophobic C-terminus was unknown. A yeast two-hybrid approach was taken to determine whether the extended hydrophobic C-terminus, exposed a new protein binding site. This approach successfully identified α-catulin as a new member of the dystrophin protein complex that binds specifically to the hydrophobic C-terminus. Immunofluorescence experiments using an α-catulin specific antibody demonstrated colocalization with Dp71 at the ILM, OLM and surrounding retinal blood vessels. This uncovered a distinct difference in the protein composition of the Dp71
complex at these non-synaptic locations compared to the dystrophin complexes at the synaptic OPL. Although the function of α-catulin remains unknown, its predicted functional domains and its homology to proteins of known function suggest that it may associate with the integrin and/or cadherin complexes. α-catulin has the potential, therefore, to be involved in the structural and signaling functions of these transmembrane complexes. Through these interactions, α-catulin may be involved in maintaining the Müller cell membrane architecture required for the proper positioning of membrane molecules. The identification of α-catulin as a novel dystrophin-associated protein, and the finding that it serves to specialize dystrophin complexes, are critical advances towards our understanding of dystrophin function at these locations in the retina.

V.2 α-catulin Interacting Proteins

In the past, the characterization of dystrophin-associated proteins has produced important information regarding the function of the dystrophin complex. To unravel the function of the Dp71 complex in the retina, new insight can be obtained through a close examination of α-catulin's protein partners and predicted protein structure. Currently, the only known α-catulin interacting protein is dystrophin. It is crucial, however, that other α-catulin protein partners are identified. The identification of novel α-catulin interacting proteins could be accomplished through the use of a yeast two-hybrid analysis. Candidate interacting proteins obtained from the library screen could be analyzed in a manner similar to the approach taken with α-catulin in Chapter II, including *in vitro* binding assays and *in vivo* colocalization experiments. The binding interaction
between α-catulin and the hydrophobic C-terminus of dystrophin would provide a valuable internal control for the yeast two-hybrid library screen.

This broad-based library screening approach could also be modified to directly test for potential interactions between α-catulin and specific predicted protein partners. For instance, amino acid sequence analysis and homology modelling predict that α-catulin contains actin, talin and β-catenin binding sites (Janssens et al., 1999). Gal4 activation domain vectors containing actin, talin or β-catenin could be cotransformed into yeast with a Gal4 binding domain/α-catulin construct. Positive protein-protein interactions could be rapidly identified using a β-gal filter assay. If α-catulin is found to bind to talin and/or β-catenin, it would link the dystrophin transmembrane complex to other well-characterized transmembrane signaling protein complexes such as the integrin and cadherin pathways. As discussed in Chapter II, members of the integrin and/or cadherin families of proteins are localized to the ILM, OLM and surrounding the microvasculature providing the colocalization required for an interaction with α-catulin (Brem et al., 1994, Honjo et al., 2000). Because the integrin and cadherin pathways are well characterized, a link through α-catulin would immediately shed light on the function of the Dp71 complex.

V.3 Knockout Mouse Models

The localization of α-catulin in the retina also provides clues to its potential function. Along with Dp71 and the extended dystrophin complex, it lines the endfeet of Müller glial cells where they contact specific structures: the ILM, the OLM and the retinal blood vessels. This immediately indicates that α-catulin is not a synaptic protein
in this neural tissue. Instead, it functions within the primary glial cell of the retina where it may indirectly regulate synaptic function. This proposed function is based on the observation that in the absence of Dp71, α-catalin localization is severely disrupted and the ERG b-wave is abnormal. It remains to be determined whether this retinal phenotype is solely due to the deficiency of dystrophin or whether the concomitant absence of α-catalin is also a key factor. The creation of an α-catalin knockout mouse model would address this as well as a number of other related questions. Initially, immunofluorescence analysis of retinal sections from the α-catalin knockout mice would provide an opportunity to determine whether dystrophin requires α-catalin for proper localization at the ILM, OLM and microvasculature. Based on these results, an electroretinogram analysis of the knockout mice could then be interpreted to determine whether α-catalin plays any role in the generation of the ERG, or whether it is simply implicated due to its association with dystrophin. If dystrophin is expressed normally in the α-catalin knockout mice, and the ERG b-wave is abnormal, then α-catalin plays a direct role in the generation of a normal ERG. If, however, dystrophin localization is disrupted in the α-catalin knockout retinas, and the b-wave is abnormal, it will remain unknown as to whether the phenotype is a result of the absence of dystrophin and/or α-catalin. It is equally possible that the expression of Dp71 will be unaltered and the ERG will be normal in the α-catalin knockout mice. This would indicate that α-catalin does not play a role in the generation of the ERG. This knockout would also provide a model in which to analyze any potential disruption of novel α-catalin interacting proteins identified through the yeast two-hybrid screen (discussed in section V.2).
To parallel the creation of the α-catulin knockout, Dan Stevens, a graduate student in our laboratory, is currently creating two dystrophin isoform-specific knockout mouse mutants in which only one or the other of the dystrophin C-termini will be expressed. This will allow for significant progress in the determination of the functional significance of the dystrophin C-terminal splicing event. Since we have identified the only protein known to bind solely to the dystrophin hydrophobic C-terminus, these knockout mice will provide ideal models for the extension of our current findings. Since α-catulin localization was severely disrupted in the dystrophin-deficient retinas of mdx<sup>3Cv</sup> mice (Chapter II), immunofluorescence analysis on retinal sections from these knockout mice would be expected to show mislocalized α-catulin when the hydrophobic C-terminus is absent. In chapter 2 we discussed the possibility that α-catulin and the dystrophin complex perform a structural role stabilizing the Müller cell membrane, perhaps maintaining the membrane morphology required for the clustering of K<sup>+</sup> channels. Therefore, in addition to immunofluorescence analysis of retinal sections, electron microscopy could be utilized to examine the morphology of the Müller cell endfeet. Finally, electroretinogram analysis of these isoform-specific knockouts will help to determine the role the C-terminal region of the dystrophin protein plays in the generation of a normal ERG b-wave. If the hydrophobic C-terminus is essential to the formation of a fully functional dystrophin complex at the ILM, OLM and microvasculature, then the ERG b-wave would be expected to be abnormal. The ERG results could also be compared to those obtained from the α-catulin knockout mice (discussed above).
V.4 \( \alpha \)-catulin in Other Tissues

The characterization of a protein or protein complex requires an in-depth analysis of expression and localization patterns in multiple tissues at various stages of development. Western blot analysis and immunofluorescence data indicate that both \( \alpha \)-catulin and the dystrophin hydrophobic C-terminus are fairly ubiquitously expressed, suggesting that the dystrophin/\( \alpha \)-catulin complex is functional in several tissues. Our investigation was focussed primarily on the retina, but the study of \( \alpha \)-catulin localization in other tissues would greatly add to our understanding of its function. Polyclonal antibodies are available for both \( \alpha \)-catulin and the dystrophin hydrophobic C-terminus allowing for multi-tissue immunofluorescence colocalization experiments. Of particular interest is the situation in cardiac muscle. Preliminary immunofluorescence studies show that, similar to the scenario observed in the retina, the two dystrophin C-termini have different localization patterns in the heart (Figure V.1) (P. Demacio, personal observation). The hydrophilic C-terminus lines the cardiomyocyte membrane in a continuous fashion; whereas the hydrophobic C-terminus displays a more interrupted expression pattern along the plasma membrane with additional definitive staining along the transverse tubular system. An association between dystrophin and \( \alpha \)-catulin at the T-tubule level would help us to understand the function of the dystrophin complex at this specialized location. This could be determined using immunofluorescence analysis of longitudinal heart sections.

T-tubules are tubular invaginations of the cardiomyocyte sarcolemma that extend deep into the cell and are essential for the rapid propagation of the excitation impulse throughout the muscle fibre. Two major molecular linkage systems exist at the T-tubule
**Figure V.1:** Differential Localization of Dystrophin C-termini in the Heart. Longitudinal mouse heart sections depicting the differential localization of the two dystrophin C-termini. Panel a shows the continuous membrane staining pattern of the dystrophin hydrophilic C-terminus using antibody 1583, which is specific for the hydrophilic C-terminus. Panels b and c (high power) show the non-continuous membrane staining and the additional specific staining of the T-tubules using an antibody specific for the dystrophin hydrophobic C-terminus (ACT-1). Scale bar: 35μm.
membranes: the vinculin-talin-integrin link and the dystrophin-dystroglycan-laminin link (Kostin et al., 1998). These protein complexes are thought to be involved in cellular stability, the transduction of signals from the cell membrane to the nucleus and the positioning of proteins/channels along the T-tubular sarcolemma (Kostin et al., 1998).

Since α-catulin is known to bind to the dystrophin hydrophobic C-terminus and since it contains a predicted talin binding site, it is possible that α-catulin is the component that links these two systems together to form a stable multimolecular structural/signaling network. This emphasizes the importance of identifying α-catulin protein binding partners (see section V.2).

In the absence of dystrophin, many patients display cardiac abnormalities involving ventricular dysfunction (Emery, 1993). One of the most striking differences between atria and ventricles is the virtual absence of a T-tubular network in atrial muscle cells. The absence of dystrophin at the T-tubule level, may therefore be a key to understanding the DMD cardiac phenotype. Since dystrophin expresses the hydrophobic C-terminus at this location, and α-catulin binds to the hydrophobic C-terminus, α-catulin may dictate the function of the dystrophin complex at T-tubules. If α-catulin does indeed colocalize with the dystrophin hydrophobic C-terminus along the T-system, and if it is disrupted in dystrophin-deficient hearts, similar to the situation observed in the retina, α-catulin would be immediately implicated in normal cardiac function. It is therefore necessary to examine the localization of α-catulin in heart sections of dystrophin deficient mdx mice. Finally, to investigate the role of the dystrophin hydrophobic C-terminus and the α-catulin protein in cardiac conduction, high resolution echo- and
electrocardiogram studies can be performed on the dystrophin and α-catenin knockout mice discussed in section V.3.

V.5 Analysis of Proteins in the Extended Dystrophin Protein Complex

The diversity of the dystrophin protein complex is extensive. We have shown that the extended Dp71/α-catenin complex in the retina is further specialized through an association with α-dystrobrevin-1. This dystrobrevin isoform has an unknown function in the central nervous system, but in muscle, is thought to be important for the positioning of signaling molecules at the neuromuscular junction (Grady et al., 1999). In the absence of Dp71 in the retina, both α-catenin and α-dystrobrevin-1 are disrupted and there is a severe reduction in b-wave amplitude (Pillers et al., 1993, Cibis et al., 1993, Sigesmund et al., 1994, Fitzgerald et al., 1994, Pillers et al., 1999). As discussed in Chapters 2 and 3, this is thought to correspond to altered potassium flux through the retinal Müller cells. Kir4.1 is the principal potassium channel found in retinal Müller cells and is thought to be responsible for the potassium siphoning mechanism in the retina (Ishii et al., 1997, Kofuji et al., 2000). Its enrichment at Müller cell endfeet overlaps the observed localization patterns of Dp71, α-dystrobrevin-1 and α-catenin (Nagelhus et al., 1999). The abnormal ERG b-wave associated with the absence of this dystrophin complex is consistent with a role in localizing these channels at potassium conductance hotspots. An analysis of Kir4.1 localization in dystrophin deficient (mdx3Cy) retinas would indicate whether the ERG phenotype observed in these mice is associated with a deficiency in the clustering of this potassium channel. Dan Stevens is undertaking this project.
The vast number of dystrophin-associated proteins allows for the generation of countless specialized complexes. In contrast to the Dp71/β-dystrobrevin-1 complexes at the inner limiting membrane and surrounding retinal blood vessels, β-dystrobrevin colocalizes with Dp260 and Dp427 at the synaptic OPL. When both dystrophin isoforms are absent from the OPL, β-dystrobrevin localization is disrupted and the implicit time of the ERG b-wave is increased (Pillers et al., 1999, Chapter 3). The localization of these proteins at the pre-synaptic face of the OPL, coupled with the abnormal ERG recordings implicate these dystrophin complexes in retinal synapse function. We and others have suggested that the dystrophin/dystrobrevin complexes at the OPL position/anchor certain signaling molecules at the presynaptic face of the photoreceptors. Syntrophin is a member of the extended dystrophin complex that binds to both dystrophin and dystrobrevin. Syntrophin also recruits several signaling proteins such as nNOS, voltage-gated sodium channels and microtubule-associated kinases to the dystrophin complex (Lumeng et al., 1999, Gee et al., 1998, Schultz et al., 1998, Brenman et al., 1996). Immunofluorescence experiments examining the localization of the three syntrophin isoforms in the retina would further add to our understanding of dystrophin complex structure and function at this synaptic layer. The unique dystrophin/dystrobrevin complexes in the retina may associate with specific syntrophin isoforms, which in turn would recruit specific signaling proteins to each unique complex.

To further our understanding of dystrophin and β-dystrobrevin at the synaptic OPL, we used a yeast two-hybrid strategy to identify β-dystrobrevin interacting proteins. Several potential interacting proteins were identified including a regulatory subunit of cAMP-dependent protein kinase and two α-liprin homologues. The α-liprin/β-
dystrobrevin interaction was a major focus in Chapter IV. As discussed in this chapter, much of the functional data describing liprin function comes from studies on *C. elegans* mutants of the liprin ortholog, *syd-2* (Zhen and Jin, 1999). In addition to this liprin homologue, the *C. elegans* genome also contains orthologs of dystrophin (*dys-1*), dystrobrevin (*dyb-1*) and syntrophin (F30A10.8) (Gieseler et al., 1999a, Gieseler et al., 1999b, Giugia et al., 1999). This provides a unique opportunity to study the function of the extended dystrophin complex in a primitive, well-established model organism. Although these nematodes do not contain retinal synapses, their synaptic network has been well-characterized and could be used as a model for the structure and function of dystrophin complexes at the mammalian retinal synapse. The existence of *C. elegans* DYS-1 (dystrophin), DYB-1 (dystrobrevin) and SYD-2 (liprin) proteins would allow for in vivo co-localization and binding studies. These studies could be performed in parallel with the generation of mouse isoform-specific liprin antibodies for use in in vivo colocalization experiments using the murine retina as a model tissue. These antibodies will reveal the localization patterns of both α1 and α2 liprin, to determine if one or both of the liprin homologues colocalize with β-dystrobrevin in the retina. Since β-dystrobrevin localization in the retina is disrupted in the absence of dystrophin, these studies could be extended to examine the localization of liprin in the series of DMD mice lacking specific dystrophin isoforms. Likewise, the available *C. elegans dyb-1* and *syd-2* mutants could be used to further analyze the proposed binding interaction between β-dystrobrevin and liprin. During the course of these studies it would be necessary to determine whether the α-liprin homologues also bind to α-dystrobrevin. Just as β-
dystrobrevin is capable of binding two liprin homologues, it is equally possible for liprin to bind to both \( \alpha \) and \( \beta \)-dystrobrevin.

The liprin and kinase clones obtained in the yeast two-hybrid screen represent interesting candidates for further research but do require additional evidence such as co-immunoprecipitation and co-localization experiments to verify the potential interactions with \( \beta \)-dystrobrevin.

**V.6 Dp260-specific Knockout**

We have successfully shown that the dystrophin complexes differ in the synaptic and non-synaptic regions of the retina. This was facilitated by the fact that Dp71 has a different localization than Dp427 and Dp260. However, the expression of two dystrophin isoforms at the synaptic OPL that have unique contributions to the ERG poses an interesting question. The ERG results of DMD patients and existing \( m\text{dx} \) mouse models indicate that Dp427 is not required for the generation of a normal b-wave (Cibis et al., 1993, Pillers et al., 1995, Pillers et al., 1999). However, when both Dp427 and Dp260 are absent from the OPL, there is a significant delay in b-wave implicit time (Kameya et al., 1997, Pillers et al., 1999). It is unclear, however, whether Dp427 and Dp260 have redundant roles at the OPL of the retina. The overlapping nature of dystrophin transcripts creates a situation in which multiple isoforms are affected by a single \( m\text{dx} \) mutation. Consequently, it is difficult to distinguish between the functional contribution of each individual isoform. The absence of Dp427 in the \( m\text{dx} \) mouse allows for the characterization of the Dp260 complex at the synaptic OPL. However, there is at present
no naturally occurring mutation that disrupts Dp260 without altering the expression of Dp427.

To study the functional contribution of Dp260 and to examine the extended Dp427 protein complex, homologous recombination techniques can be used to target Dp260 to create an isoform-specific knockout. I have created a targeting vector such that homologous recombination in embryonic stem cells will eliminate expression of Dp260 while leaving unaltered expression of all other dystrophin isoforms (Figure V.2). Dp260 has a unique first exon that is spliced in-frame to exon 30 of full-length dystrophin (D'Souza et al., 1995). The targeting vector specifically targets this region of intron 29 to avoid inadvertent disruption of any of the other isoforms. This Dp260-specific knockout would allow for a detailed functional analysis of Dp260's contribution to the synaptic function of the retina. At the molecular level, immunostaining techniques can then be used to examine the expression of dystrophin-associated proteins including β-dystrobrevin and the syntrophin isoforms (see section V.5). This will allow us to characterize the components of the Dp427 complex at the OPL in an attempt to distinguish between the functional contributions of Dp427 and Dp260 at the OPL of the retina. The physiological consequences of Dp260's absence can also be examined through detailed ERG analyses of the Dp260 knockout mice. These studies are required in order to understand the genotype/phenotype relationships that exist in the retina.

V.7 Concluding Notes

In our effort to determine the functional differences between the dystrophin isoforms expressed in the retina, we have shown that unique extended dystrophin
Figure V.2: Strategy for the Development of a Dp260-specific Knockout Mouse. The knockout construct was generated to specifically target the unique first exon of Dp260 to avoid the disruption of any other dystrophin isoform. A neomycin positive selection cassette was inserted in such a way that a 113bp deletion is created, eliminating the Dp260 exon 1 and the initiator ATG. Because the neomycin cassette will be inserted into the middle of the DMD gene, it could affect the expression of other dystrophin isoforms. To ensure that components of the neo gene (promoter, polyA site, cryptic splice sites) will not interfere with transcription of the other overlapping isoforms, LoxP sites were included on either side of the neo gene to allow for the removal of this positive selection marker using the cre-recombinase system. ES cell clones will be screened by polymerase chain reaction (primers shown as arrows in figure V.2) and Southern analysis to identify homologous recombinant clones. The resulting Dp260 knockout will be null for Dp260 while maintaining full, unaltered expression of the other dystrophin isoforms.
complexes exist at the synaptic versus non-synaptic locations in the retina (Figure V.3). Dp71, containing the hydrophobic C-terminus, associates with α-dystrobrevin-1 and α-catenin at the non-synaptic Müller glial cell endfeet; whereas the extended dystrophin complexes at the synaptic outer plexiform layer include β-dystrobrevin, the type 1α regulatory subunit of cAMP-dependent protein kinase and α-liprin. The characterization of these complex components has permitted a greater understanding of dystrophin function in the retina. These findings support the functional hypothesis that the dystrophin complex serves as a scaffold for signaling molecules. α-catenin may link the dystrophin complex to integrin and/or cadherin signaling complexes, while the type 1α regulatory subunit of cAMP-dependent protein kinase and liprin may link the dystrophin complex to kinase and phosphatase signaling pathways, respectively. Further studies will uncover the mechanism by which these proteins and their associated complexes impact the function of the dystrophin complex. Our findings support the notion that a functional analysis of dystrophin in specific tissues requires the characterization of the extended dystrophin complex. In conclusion, we provide evidence that the unique extended protein complexes associated with the individual dystrophin isoforms mediate the function of dystrophin at the different layers of the retina. This demonstrates the functional significance for the existence of distinct dystrophin isoforms.
Figure V.3: Distinct Dystrophin Protein Complexes in the Retina. The Dp71 extended protein complexes at the non-synaptic Müller cell endfeet differ significantly from the Dp427/Dp260 complexes at the synaptic OPL. a) Dp71 possesses the hydrophobic C-terminus (HCT) which binds to α-catulin, forming another subcomplex of proteins associated with dystrophin. α-catulin contains predicted talin and β-catenin binding sites that could link dystrophin to integrin and/or cadherin signaling complexes. α-dystrobrevin-1 (α-db-1) is also associated with Dp71 at the inner limiting membrane and microvasculature. b) The Dp427/Dp260 complexes at the outer plexiform layer (OPL) include β-dystrobrevin (β-db) and its associated proteins liprin and cAMP-dependent protein kinase. Liprin is known to bind to transmembrane LAR phosphatases (LAR).
Appendix

α-catulin Maps to the Familial Dysautonomia Region on 9q31

This appendix formed the following publication:
Paula C. Demacio and Peter N. Ray. Genome, in press.
The work in this chapter is my own.
A.1 Abstract

Familial Dysautonomia is a severe autosomal recessive neurodegenerative disease that primarily affects the Ashkenazi Jewish population. We present the mapping of α-catulin and show that it maps precisely to the Familial Dysautonomia candidate region on 9q31. Patient sequence analysis identified two new sequence variants, which show linkage disequilibrium with this disease. A G to A transition at nucleotide 423 in exon 3 is a silent base change that does not alter the valine residue at position 141. A G to C transversion at nucleotide 1579 changes the Glu at position 527 to Gln. These base changes were analyzed in several patients, unaffected Ashkenazi Jewish controls and non-Jewish controls. Due to the presence of these sequence variants in several unaffected individuals, α-catulin is unlikely to be the causative gene in this disease.
A.2 Introduction

Familial Dysautonomia (FD), or Riley-Day Syndrome, is a rare congenital neuropathy that is inherited in an autosomal recessive manner (Riley et al., 1949, Axelrod et al., 1974). This disease is characterized by progressive neuronal degeneration primarily affecting sensory, sympathetic and some parasympathetic neurons resulting in a reduced quality of life and premature death (Riley et al., 1949, Axelrod and Abularrage, 1982, Axelrod et al., 1974). FD is almost exclusively restricted to the Ashkenazi Jewish population where it has a carrier frequency of 1 in 30 and an incidence of 1 in 3600 live births (Brunt and McKusick, 1970, Maayan et al., 1987). The FD "DYS" gene has been mapped to a 0.5cM region on 9q31-33 between markers 43B1GAGT and 157A3 (Blumenfeld et al., 1999). Strong linkage disequilibrium observed with linked markers in this candidate region suggests that one founder mutation is present on 98% of FD chromosomes in the Ashkenazi population (Blumenfeld et al., 1999).

Clinical features of FD include a decreased perception of temperature and pain, absence of fungiform papillae, diminished or absent patellar reflexes, excessive sweating, skin blotching and a diminished production of tears (Riley et al., 1949, Brunt and McKusick, 1970, Axelrod et al., 1974). Since these symptoms are the result of progressive neuronal depletion, it was originally hypothesized that the DYS gene may encode a gene required for the development and survival of neurons (Schwartz and Breakefield, 1980, Axelrod et al., 1981, Wrathall, 1986). Many neurotrophic factors/receptors have been examined and excluded as potential disease-causing genes (Breakefield et al., 1984, 1986, Blumenfeld et al., 1993). However, downstream partners in related signal-transduction pathways are still likely candidates.
α-catulin, an α-catenin related gene, has been localized to 9q31.2 by FISH analysis (Zhang et al., 1998, Janssens et al., 1999). Its homology to the catenin family and its potential role in the cadherin and/or integrin signaling pathways, along with its map location made α-catulin a candidate gene for FD. In this paper we describe the mapping of the α-catulin gene within the candidate region for FD and the results of mutation screening to determine whether α-catulin is associated with the pathogenesis of the disease.

A.3 Results and Discussion

A.3.1 α-catulin mapping in the 9q31 region

To determine if the α-catulin gene falls within the defined critical region for FD, a yeast artificial chromosome (YAC) contig of approximately 4.5Mb, spanning the candidate region was constructed using the CEPH YAC library and information from the Whitehead Human Physical Map (http://carbon.wi.mit.edu:8000/cgi-bin/contig/phys_map) (Figure A.1) (Albertsen et al., 1990). PCR reactions on YAC DNA using primer sets from exon 1 and the 3’ UTR of the α-catulin gene were performed to map the gene within the 9q31 region (Figure A.2). PCR products were obtained with DNA from YACs 757g8, 776g7 and 709b3 with both primer sets indicating that the entire gene lies on each of these YAC clones. Since the DYS gene has been mapped to a region on 9q between markers 43B1GAGT and 157A3, we performed PCR on our YAC DNA clones using the published primer sequences to place these markers on our YAC contig (Blumenfeld et al., 1999). 43B1GAGT is present on YAC clones 757g8, 776g7 and 709b3; marker 157A3 is found on clones 757g8, 750c6, 926h11, 776g7 and 709b3 (PCR data not shown). These markers were placed on our YAC contig and define
Figure A.1: A YAC Contig of the 9q31 Region. This contig was constructed using the CEPH YAC library and information from the Whitehead Human Physical Map. α-catulin PCR products were obtained with DNA from YACs 757g8, 776g7 and 709b3 with primer sets from both exon 1 and the 3' UTR indicating that the entire gene lies on each of these YAC clones (see Figure A.2). YACs displayed in white contain the α-catulin gene. Markers 43B1GAGT and 157A3 were placed on this map and define a maximal interval for the FD gene (horizontal lines) which contains α-catulin. The YACs are drawn approximately to scale using published YAC sizes and placed on the diagram adjacent to markers known to be contained within each YAC (http://carbon.wi.mit.edu:8000/cgi-bin/contig/yac_info).
Figure A.2: PCR of α-catulin on YAC Clones. PCR products were obtained in reactions using exon 1 (top) and 3' UTR (bottom) primers from YACs 757g8, 776g7 and 709b3 indicating that the entire α-catulin gene lies on each of these YAC clones. No PCR product was obtained with YACs 850h10 or 750c6, establishing centromeric and telomeric limits for the interval containing α-catulin. No PCR product was obtained from YACs 950a10, 926h11, 962c6, 810f2, 748g9 and 776h3 (data not shown).
a maximal interval to contain the FD gene (Figure A.1). The α-catulin gene falls within this 800kb region, but is not restricted to this interval.

A.3.2 Mutation analysis of α-catulin in Familial Dysautonomia patients

Since the α-catulin gene lies within the FD critical region, we sequenced the gene in several FD patients to determine whether α-catulin plays a role in the pathogenesis of FD. RNA was purified from cultured fibroblast cells from one FD patient (GM02342) and the coding region was fully amplified using nested RT-PCR. The full-length gene product was then sequenced to screen for mutations. In addition, each of the 19 α-catulin exons from two FD patients (NA008894 and NA09296) as well as one Ashkenazi Jewish control individual with no family history of FD was amplified and sequenced.

All three of the FD patients were found to be homozygous for two sequence alterations that were not observed in the control sample. A G to A transition was found at nucleotide 423 in exon 3. This is a silent base change not altering the valine residue at position 141. The second change was a G to C transversion at nucleotide 1579. This changes the Glu at position 527 to Gln.

Both sequence alterations were further examined in 4 additional FD patients (NA05042, NA05106, NA09295 and NA09789) and 4 additional unaffected Ashkenazi Jewish controls. Sequence analysis indicated that all FD patient DNA samples were homozygous for both base changes. One of the four unaffected Jewish control samples was heterozygous for both base changes, and the other three did not contain either base change. To further investigate whether the G1579C change in exon 11 is responsible for FD, exon 11 from 30 unaffected non-Jewish samples was sequenced. Of these controls,
10 were heterozygous and 3 were homozygous for both base changes. Since the G1579C mutation was the only base change found in the coding region of the gene in FD patients corresponding to an amino acid change in the protein, and since this mutation was found in the homozygous state in three unaffected individuals, it seems unlikely that α-catulin is the gene responsible for Familial Dysautonomia.

A.3.3 Linkage in Familial Dysautonomia Families

Although the G423A and G1579C variants are not responsible for FD, they are very closely linked to the disease. Haplotype analysis with these markers was done in two families segregating FD (Figure A.3). In both families the FD mutation segregated with the G423A and G1579C allele on both chromosomes. However, in family 810, two unaffected individuals were homozygous for G423A and G1579C, suggesting that the FD mutation arose in this preexisting haplotype.

In summary, we have mapped the α-catulin gene to YACs 757g8, 776g7 and 709b3 in the 9q31.2 Familial Dysautonomia region. Two base changes were found within the coding region of this gene that showed strong linkage disequilibrium with Familial Dysautonomia. However, analysis of several affected and unaffected individuals suggests that these base changes are unlikely to be a causative factor in the disease.
Figure A.3: Polymorphism Analysis in Two Familial Dysautonomia Families. Exon 3 and exon 11 polymorphism sequence results are depicted. Affected individuals are denoted by shading. * denotes the two unaffected individuals who are homozygous for both base changes.
A.4 Materials and Methods

Human DNA Sources

All Familial Dysautonomia patient and family DNA sources were obtained from Coriell Cell Repositories. Family 810 consists of samples NA05041, NA05042, NA05043, NA05044, NA05045 and NA05046. Family 816 consists of samples NA05105, NA05106, NA05107, NA05108, NA05109, NA05110, NA05111 and NA09662. Other FD patient samples included fibroblast cell line GM02342 and DNA samples NA09295, NA09296, NA008894 and NA09789. All control samples were obtained from the DNA Diagnostic Laboratory at the Hospital for Sick Children in Toronto.

α-catulin Mapping

Yeast artificial chromosomes (YACs) spanning the Familial Dysautonomia candidate region on 9q31 were selected and grown at 30°C in liquid YPD media. YAC DNA was then purified and subjected to PCR using α-catulin exon 1 primers (for: 5’-agtcctggtgtctggaagacat-3’ and rev: 5’-gcgcactttacctgagaaacc-3’) and 3’UTR primers (for 5’-ggcagcgatctggaacacc-3’ and rev: 5’-gaatcgacacttgaacc-3’). PCR reactions were performed with Elongase (Gibco BRL) under the following amplification conditions: 94°C for 30s, and 30 cycles of 94°C for 30s, 54°C for 30s and 68°C for 45s. PCR reactions for markers 43B1GAGT and 157A3 were performed using Advantaq Plus (Clontech), the published primer pair sequences and the following amplification conditions: 95°C x 60s and 30 cycles of 95°C x 30s, 55°C x 30s and 68°C x 45s (Blumenfeld et al., 1999).
α-catenin Amplification and Sequencing

RNA was purified from patient fibroblast cells (GM02342) using Trizol and a standard extraction protocol (Gibco BRL). Oligo dT primed cDNA was amplified using Elongase (Gibco BRL) and the following primers: for: 5'-agtcccgctgcggcatagacc-3' and rev: 5'-gaatctagcaattaccaagac-3' nested with for: 5'-gatagaccgaagccatgg-3' and rev: 5'-acaatcagtctctgatcag-3'. Amplification conditions for both rounds of PCR were as follows: 95°C x 1min, and 30 cycles of 95°C x 30s, 58°C x 30s, 68°C x 2min. The full-length gene product was then sequenced using primers: 5'-gatagaccgaagccatgg-3', 5'-ctgttgttgttagttctcaatac-3', 5'-ggagaagctatagaatgaatagc-3', 5'-gactagacattcataatgaatggc-3', 5'-gacggaacatgtccagtatggc-3', 5'-gactgacattcataatgaatggc-3', 5'-aagcaccagtctctgaattcgtgac-3', 5'-aagtgtgtgttggaagctctgtgac-3', 5'-caagcaattcagcgtgtagg-3' (USB cycle sequencing kit).

Primers used for the amplification and sequencing of the individual α-catenin exons are listed in Table A.1.
Table A.1: Primer sequences used for α-catulin gene amplification and sequencing.

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Chapter VI

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