Limb Girdle Muscular Dystrophy in the Hutterite Population of North America

by

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A Thesis Submitted to the Faculty of Graduate

Studies in Fulfilment of the Requirements for the

Degree of Doctor of Philosophy

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Abstract

Limb girdle muscular dystrophies (LGMDs) are a clinically and genetically heterogeneous group of myopathies characterized by weakness and wasting of the proximal musculature. There are currently seventeen loci associated with different LGMDs, seven with an autosomal dominant mode of inheritance (LGMD1A–1G) and 10 with an autosomal recessive mode of inheritance (LGMD2A– 2J). The cumulative worldwide prevalence of LGMD is thought to be ~1/15,000.[191] In the Hutterite population of North America there is an over-representation of autosomal recessive LGMD with a prevalence estimated to be >1/400. The objective of this work was to delineate the genetic basis of LGMD in this large genetically isolated population.

A genome-wide scan was performed on Hutterite LGMD patients and their families in order to locate the mutant gene. This allowed us to identify a novel locus at chromosome region 9q31-33 that was named *LGMD2H*.[279] Extensive haplotyping and mutation screening led to the discovery of c.1459G>A in *TRIM32* as the causative mutation of LGMD2H.[91] We then found that this same mutation was the cause of another previously described myopathy in the Hutterites, sarcotubular myopathy (STM). Analysis of the *TRIM32* gene product revealed that it is a potential E3-ubiquitin ligase, is expressed in many human tissues including muscle and brain, and has a punctate cytoplasmic distribution.

During the analysis of the LGMD2H region, it became apparent that there were Hutterite LGMD patients not linked to the *LGMD2H* locus. In order to identify the causative gene(s) in the remaining families, we performed a genome-wide scan. A locus at chromosome 19q13 was found to correspond to disease inheritance, the site of a previously

described LGMD locus, *LGMD2I*.[66] No causative gene had yet been identified at this locus so haplotyping and mutation screening was performed. We were able to identify c.826C>A in *FKRP* as the causative mutation in our remaining cohort of LGMD patients. The same mutation has since been found in many other populations, and is apparently a relatively common cause of LGMD. We obtained DNA from 19 non-Hutterite LGMD2I patients of diverse origins with c.826C>A and determined that it is an old founder mutation.[90]

There is no further evidence of any other loci causing autosomal recessive myopathy in the Hutterites. With the identification of c.1459G>A in *TRIM32* and c.826C>A in *FKRP* we appear to have delineated the genetic cause of all myopathies of increased prevalence in the Hutterite population. To date, we have been able to provide accurate, non-invasive diagnosis to over 70 patients and have provided carrier testing to approximately 120 at-risk family members. This kind of DNA-based approach is not feasible in the general population due the enormous amount of locus, allelic, and clinical heterogeneity among myopathy patients.

Aknowledgements

First and foremost I would like to thank the patients and their families for participating in our studies. The many Hutterite communities that we have visited have always been gracious and helpful. Without their constant support we would not have been able to do any of the work presented in this thesis.

I am grateful to my co-supervisors Drs. Klaus Wrogemann and Cheryl Greenberg for providing me with this very fruitful opportunity, not just the project but the lab environment as well. I have been given an enormous amount of independence and responsibility and I hope that I have repaid that trust by providing our group with solid meaningful results. I don't think that I could have found a laboratory better suited to my personality and interests. In addition, I am indebted to the many individuals that have been part of the lab at one time or another that have contributed to the work discussed in this thesis. Specific contributions will be mentioned prior to each chapter.

I have had the pleasure of working with quite a few investigators in the course of my work and would specifically like to thank: Dr. Andrew Halayko & Gerald Stelmack (immunocytochemistry / microscopy), Drs. Andrew Engel & Marc Del Bigio (muscle pathology), Drs. Benedikt Schoser & Hanns Lochmuller (STM manuscript), Dr. Ken Morgan & Mary Fujiwara (linkage analysis and manuscript preparation), Drs. Mayana Zatz, Kate Bushby, & Volker Straub (non-Hutterite LGMD2I samples), Dr. Molly Kulesz-Martin (discussions involving the role of TRIM32 in skin cancer), Dr. Jody Berry (production of anti-TRIM32 antiserum), Dr. Richard Hammond (teaching me cell culture technique and immunoblotting), and Dr. Eric Shoubridge & Timothy Johns (production of human myoblast

lines). In addition, the investigators in our department have always been available for questions and/or intriguing discussions, particularly Drs. Barb Triggs-Raine, Steven Pind, and Theresa Zelinski.

I would also like to thank my examining committee (Drs. Peter Ray, Mary Lynn Duckworth, Barb Triggs-Raine, Cheryl Greenberg, and Klaus Wrogemann) for thoroughly reading this thesis and providing such useful comments. Special thanks to Dr. Peter Ray as the external examiner for making the effort to come to Winnipeg in order to attend my thesis defense.

Finally I would like to thank my family for all their support and encouragement. The ten years (ouch!!) that I have spent in university would not have been bearable without them. Heartfelt thanks go to my partner Dan who has put up with a considerable amount of boring science talk and the occasional depression due to failed experiments for many years. Hopefully I will have a real job soon so I can support you for once!

This work was supported by many funding agencies including Manitoba Health Research Council (MHRC), Manitoba Institute of Child Health (MICH), University of Manitoba Graduate Fellowship (UMGF), Muscular Dystrophy Association (MDA), and Canadian Institutes of Health Research (CIHR).

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List of Abbreviations

The following is a list of abbreviations used throughout this thesis.

aa	amino acid
BHK	baby hamster kidney
BLAST	basic local alignment search tool
BMD	Becker muscular dystrophy
BSA	bovine serum albumin
B2H	bacterial two hybrid
CB	cytoskeletal buffer
CCD	central core disease
cDNA	complementary deoxyribonucleic acid
CEPH	Centre d'Etude du Polymorphisme Humain
CMD	dilated cardiomyopathy
CMH	hypertrophic cardiomyopathy
DA1	Distal arthrogryposis type 1
DAG	dystrophin-associated glycoprotein
DGC	dystrophin-associated glycoprotein complex
DM	distal myopathy
DMD	Duchenne muscular dystrophy
DMEM	Dulbecco modified eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDMD	Emery-Dreifus muscular dystrophy
EDTA	disodium ethylenediaminetetraacetic acid
EMG	electromyography
ER	endoplasmic reticulum
ERAD	endoplasmic reticulum associated degradation
EST	expressed sequence tag
FBS	fetal bovine serum
FCMD	Fukuyama congenital muscular dystrophy
FDC-CDM	Familial dilated cadiomyopathy, conduction defect and myopathy
FPLD	Familial partial lipodystrophy, Dunnigan type
FSHD	facio-scapulo-humeral muscular dystrophy
HGPS	Hutchinson-Gilford progeria sysndrome
IGF	insulin-like growth factor
LDB	Location database
LGMD	limb girdle muscular dystrophy
LOD	log of the odds
MADA	mandibuloacral dysplasia
MD	muscular dystrophy
MDC	congenital muscular dystrophy
MEB	muscle-eye-brain disease

MEM	modified eagle medium
MES	2-(N-Morphilino)ethanesulfonic Acid
MFM	myofibrillar myopathy
MHS	malignant hyperthermia sensitivity
MM	Miyoshi myopathy
mTBE	modified Tris-Boric Acid-EDTA buffer
OPMD	oculopharyngeal muscular dystrophy
PAGE	polyacrylamide gel electrophoesis
PCR	polymerase chain reaction
PFA	paraformaldehyde
RD	restrictive dermopathy
RMD	rippling muscle disease
SCARMD	severe childhood autosomal recessive muscular dystrophy
SCK	serum creatine kinase
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate - polyacrylamide gel electrophoresis
SM	skim milk
SNP	single nucleotide polymorphism
SR	sarcoplasmic reticulum
SSCP	single stranded conformational polymorphism
STM	sarcotubular myopathy
TBS	Tris buffered saline
TBST	Tris buffered saline with Tween 20
TCAG	The Centre of Applied Genomics
TE	Tris-EDTA buffer
Tris	Tris(hydroxymethyl)aminomethane
UCSC	University of California Santa Cruz
WRN	Werner syndrome
WWS	Walker-Warburg syndrome

List of Manufacturers

The following table lists manufacturers of products used during the course of this research and their cities of origin. All further reference to thesemanufacturers in the text will consist solely of the company name and/or abbreviation for the sake of space.

Name	Short form	City, Prov/State	Country	Website
Alpha Innotech	Alpha Innotech	San Leandro, CA	USA	www.alphainnotech.com
Amersham Pharmacia Biotech	Amersham	Baie d'Urfe, QC	CA	www4.amershambiosciences.com
Beckman Coulter	Beckman	Palo Alto, CA	USA	www.beckman.com
Becton Dickinson	BD Biosciences	Franklin Lakes, NJ	USA	www.beckman.com
Bio-Rad Laboratories	Bio-Rad	Hercules, CA	USA	www.bio-rad.com
Boekel Scientific	Boekel	Feasterville, PA	USA	www.boekelsci.com
Cambrex	Cambrex	East Rutherford,NJ	USA	www.cambrex.com
Coriell Cell Repositories	Coriell	Camden, NJ	USA	locus.umdnj.edu/ccr
Corning Lifesciences	Corning	Acton, MA	USA	www.corning.com/lifesciences
Eastman Kodak	Kodak	Rochester, NY	USA	www.kodak.com
EMD Biosciences	EMD	San Diego, CA	USA	www.emdbiosciences.com
Fisher Scientific	Fisher	Nepean, ON	CA	www.fishersci.ca
BioWhittaker Molecular Applications	BioWhittaker	Walkersville, ME	USA	www.bmaproducts.com
MP Biomedicals	ICN	Costa Mesa, CA	USA	www.mpbio.com
Integrated DNA Technologies	IDT	Coralville, IA	USA	www.idtdna.com
Interscience Incorporated	Interscience	Markham, ON	CA	www.interscience.com
Invitrogen Corporation	Invitrogen	Carlsbad, CA	USA	www.invitrogen.com
Jackson ImmunoResearch Laboratories	Jackson	West Grove, PA	USA	www.jacksonimmuno.com
Kendro	Kendro	Norwalk, CN	USA	www.kendro.com
Mandel Scientific	Mandel	Guelph, ON	CA	www.mandel.ca
MBI Fermentas	Fermentas	Burlington, ON	CA	www.fermentas.com
MJ Research	MJ research	Waltham, MA	USA	www.mjr.com
New England Biolabs	NEB	Beverly, MA	USA	www.neb.com
Origene Technologies	Origene	Rockville, MD	USA	www.origene.com
Pierce Biotechnology Incorporated	Pierce	Rockford, IL	USA	www.piercenet.com
Promega	Promega	Madison, WI	USA	www.promega.com
Qiagen	Qiagen	Mississauga, ON	CA	www1.qiagen.com
Roche Applied Sciences	Roche	Laval, QC	CA	www.roche-applied-science.com
Sigma-Aldrich	Sigma	St. Louis, MO	USA	www.sigmaaldrich.com
Stratagene	Stratagene	La Jolla, CA	USA	www.stratagene.com
Techne	Techne	Burlington, NJ	USA	www.techne.com
Thermo Electron Corporation	Thermo Electron	Waltham, MA	USA	www.thermo.com
VWR Canlab	VWR	Edmonton, AB	CA	www.vwrcanlab.com

1. Introduction

The muscular dystrophies are disorders with a primary defect in muscle that causes premature myocyte death and/or defective regeneration. As a group they show an extreme amount of allelic, locus, and clinical heterogeneity. They are usually classified by their pattern of muscle involvement and mode of inheritance. One subgroup is the limb girdle muscular dystrophies (LGMD), a group of autosomal disorders with primary involvement of the pelvic and shoulder muscles. Even within this one group there is extensive heterogeneity with seven dominant forms (LGMD1A– 1G) and ten recessive forms (LGMD2A– 2J). This has made the identification of novel LGMD loci very difficult and most of the successes in this area have been through mapping studies in large families or inbred populations. Once the causative genes are found muscle biopsies can be used as a diagnostic method to show defective or absent protein in isolated patients. This has made the use of large kindreds segregating for LGMD mutations essential to our success in identifying the genes responsible and providing diagnostic tools for all patients with an unknown myopathy.

The aim of my project has been to identify the genetic cause of LGMD in the Hutterite population. The Hutterites have a limited number of founders and very little influx of new alleles so it was originally hypothesized that there was a single mutation responsible for the high frequency of LGMD in this population. Prior to my joining the laboratory a genome scan using pooled affected and unaffected genomic DNA was performed and a large locus now know to be ~6.4 Mb in size was identified and named *LGMD2H*.[279] I began my studies by narrowing this region extensively and eventually discovering the disease-causing

mutation. Through the ascertainment of new patients it became apparent that our original hypothesis was not correct, as there were patient families that did not show linkage to the *LGMD2H* locus. We were able to perform a genome scan on a subset of these unlinked families and discovered another locus, *LGMD2I*.[90] In addition to these two main projects I have also worked on limited functional characterization of the LGMD2H gene product, the natural history of the most common LGMD2I mutation, and the discovery of sarcotubular myopathy (STM) as a phenotypic variant of LGMD2H.

In the following chapters, I review the current state of knowledge regarding LGMDs (chapter 2), describe the materials and methods used during my research (chapter 3), and detail the actual work as follows:

Chapter 4: Identification and characterization of the LGMD2H gene

Chapter 5: Identification of the LGMD2I gene

Chapter 6: Identification of the STM gene

The final chapter will discuss the results and how they relate to muscular dystrophy research in general as well as outlining potential future research efforts. Please note that many of the methods discussed in chapter 3 are related to fine details of the LGMD2H gene product, particularly sections 3.6 - 3.11. It may be advantageous to read chapter 4 prior to these sections within chapter 3.

2. Review of Limb Girdle Muscular Dystrophies

2.1 General Features of LGMD

Muscular dystrophies (MDs) are extremely heterogeneous, both genetically and clinically. They are inherited disorders with a primary defect in skeletal muscle which leads to progressive weakness and wasting in the affected individuals. The defect being in muscle differentiates them from neuropathies where the defect is in the nerves supplying the muscle. Historically, disorders of muscle were separated into two categories, dystrophies and myopathies. MD was defined as a disease resulting in a breakdown of muscle integrity (sarcolemmal defect) whereas myopathy referred to a defect in the sarcomere or another internal component of muscle.[31] Recent evidence has made this distinction somewhat meaningless as the following review will show. Further mention of myopathy will simply refer to a disorder of muscle in a general sense.

MDs have been classified on the basis of observable characteristics such as: pattern of muscle involvement; severity, age at onset, and mode of inheritance. Each disorder has its own characteristic signs and symptoms, often with considerable variability and overlap with other disorders.[222] The following list outlines the more common of these clinical entities.

DMD - Duchenne muscular dystrophy is a common X-linked disorder with severe and rapidly progressive proximal weakness and wasting, onset in the first few years of life, hypertrophy of the calf muscles, mild intellectual impairment, and late cardiac involvement. Patients usually die in their 2nd or 3rd decade.[5,211]

BMD - Becker muscular dystrophy is a milder variant of DMD with a later onset and slower

progression (now known to be allelic to DMD).[5,211]

EDMD - Emery-Dreifuss muscular dystrophy is also a proximal myopathy with an age at onset in the 2nd or 3rd decade of life and variable mode of inheritance (X-linked,[28,29] autosomal dominant,[30] or autosomal recessive[209] [see LGMD1B]). It is distinguished from DMD and BMD by the presence of early contractures of the elbows, spine and ankles as well as severe cardiac conduction defects.[288]

FSHD - Facioscapulohumeral muscular dystrophy is an autosomal dominant disorder with a characteristic pattern of facial, scapular, and peroneal weakness. There is usually marked scapular winging with sparing of the deltoids and age at onset is variable.[253]

OPMD - Oculopharyngeal muscular dystrophy is an autosomal dominant disorder with onset in middle age. Weakness in the extraocular and pharyngeal muscles leads to ptosis, dysphagia, and dysarthria. Weakness in proximal and/or distal limb muscles may also be present.[33-35]

MDC - Congenital muscular dystrophy is actually a group of disorders with weakness and wasting apparent right from birth. Inheritance is autosomal and many patients show severe neurological involvement.[142,187]

Distal - Distal myopathies are another group of disorders that are characterized by a primary involvement of distal muscles. This group is unusual in that distal weakness is often associated with neuropathy rather than myopathy.[222]

There are quite a number of proximal myopathies with autosomal inheritance that do not fit within these classifications. This led to the very loose definition of limb girdle muscular dystrophy (LGMD) as an autosomal proximal myopathy that does not show any of the

Table 1: Seventeen currently defined LGMD loci

Locus	MIM #	Inheritance	Chromosomal localization	Gene	Protein	Associated Disorders
LGMD1A	159000	AD	5q22-34	*TTID*	Myotilin	Myofibrillar myopathy
LGMD1B	159001	AD	1q11-21	*LMNA*	Lamin A/C	EDMD, CMD1A, CMT2B1, FPLD2, MADA, HGPS, RD, WRN2
LGMD1C	607801	AD	3p25	*CAV3*	Caveolin 3	RMD, DM, HyperCKemia
LGMD1D	602067	AD	6q23	--	--	--
LGMD1E	603511	AD	7q36	--	--	--
LGMD1F	608423	AD	7q32	--	--	--
LGMD1G	--	AD	4p21	--	--	--
LGMD2A	253600	AR	15q15.1	*CAPN3*	Calpain 3	--
LGMD2B	253601	AR	2p13	*DYSF*	Dysferlin	MM, DAM
LGMD2C	253700	AR	13q12	*SGCG*	γ-sarcoglycan	--
LGMD2D	608099	AR	17q21	*SGCA*	α-sarcoglycan	--
LGMD2E	600900	AR	4q12	*SGCB*	β-sarcoglycan	--
LGMD2F	601287	AR	5q33-34	*SGCD*	δ-sarcoglycan	--
LGMD2G	601954	AR	17q11-12	*TCAP*	Telethonin	CMD1N
LGMD2H	254110	AR	See Chapter 4			
LGMD2I	607155	AR	See Chapter 5			
LGMD2J	608807	AR	2q31	*TTN*	Titin	TMD, CMH9, CMD1G

distinctive characteristics of the aforementioned groups (Table 1).[40-42,156,191,265]

As more causative genes for muscle disorders have been identified it is becoming clear that the distinctions between many of these groups are somewhat arbitrary. It has been suggested that the whole phenotypic classification scheme be replaced by one based on the underlying genes (eg/ DMD, BMD, and isolated X-linked cardiomyopathy are all caused by mutations in the dystrophin gene and would therefore define a group termed dystrophinopathy). However, from a clinical perspective these classifications still have utility. The following is an attempt to review the current knowledge regarding the LGMDs, highlighting the heterogeneity of the group and the numerous places where there is a blurring of the distinctions separating this group from other groups of neuromuscular disorders.

2.2 Dominant Forms

(A) LGMD1A

Dominant forms of LGMD are relatively rare, accounting for an estimated 10% of all LGMDs.[40] LGMD1A was the first dominant form attributed to a specific chromosomal locus under the current nomenclature guidelines.[42] It was originally described in a single kindred from West Virginia which included 16 affected individuals.[100] In these individuals, there was a 'bottom up' distribution of proximal weakness with pelvic preceding shoulder involvement, variable distal and facial involvement, onset in the 2nd or 3rd decade, and elevated serum creatine kinase (SCK) levels of 1.6-9X normal. The two most characteristic features of the disorder were tight calcaneal tendon (inhibits dorsiflexion of the foot) and a dysarthric speech (indicative of pharyngeal involvement). Patient muscle biopsies showed increased variation in fibre size, central nuclei, fibre splitting and a distinct overabundance of Z-line streaming. The gene was mapped to chromosome region 5q31 and a mutation in

the myotilin gene (T57I) was subsequently identified in this family.[117,285] Enormous efforts to identify other patients with LGMD1A led to the identification of only one other family of Argentinian origin. This family had similar clinical findings and an S55F mutation in the myotilin gene.[116]

The gene encoding myotilin (*TTID*) has 10 exons and is 20 kb in size.[101] The protein is 498 aa (MW ~ 55 kDa) and contains a unique serine-rich N-terminal domain and two repeats with similarity to the titin immunoglobulin-like domain (see LGMD2J for a discussion on titin). Expression is widespread during development but becomes restricted to mainly skeletal muscle and bone marrow with small amounts in salivary gland, mammary gland, and prostate.[101,179] The protein exists as a dimer and localizes to the Z-line, the structure delineating the boundaries of each sarcomere that distributes mechanical stresses from the contractile apparatus through to the cytoskeletal network (Figure 1). It is known to bind "-actinin and filamin c (two important components of the Z-line) and cross-links actin filaments, especially in the presence of its two binding partners.[217] These characteristics suggest that it plays an important role in the stabilization and anchorage of thin filaments (actin) in muscle and may have a role in the formation of the sarcomere.

Recently a group trying to define the molecular pathology underlying 'myofibrillar myopathy' (MFM), a loose term referring to any chronic progressive disease of skeletal muscle with myofibrillar disorganization, studied myotilin as one of five candidate genes.[140,225] This was mainly due to the potential role of myotilin in the assembly of the sarcomere and its presence in related pathological lesions of muscle such as nemaline rods and central cores.[224] They found six patients out of a cohort of 63 unrelated idiopathic MFM

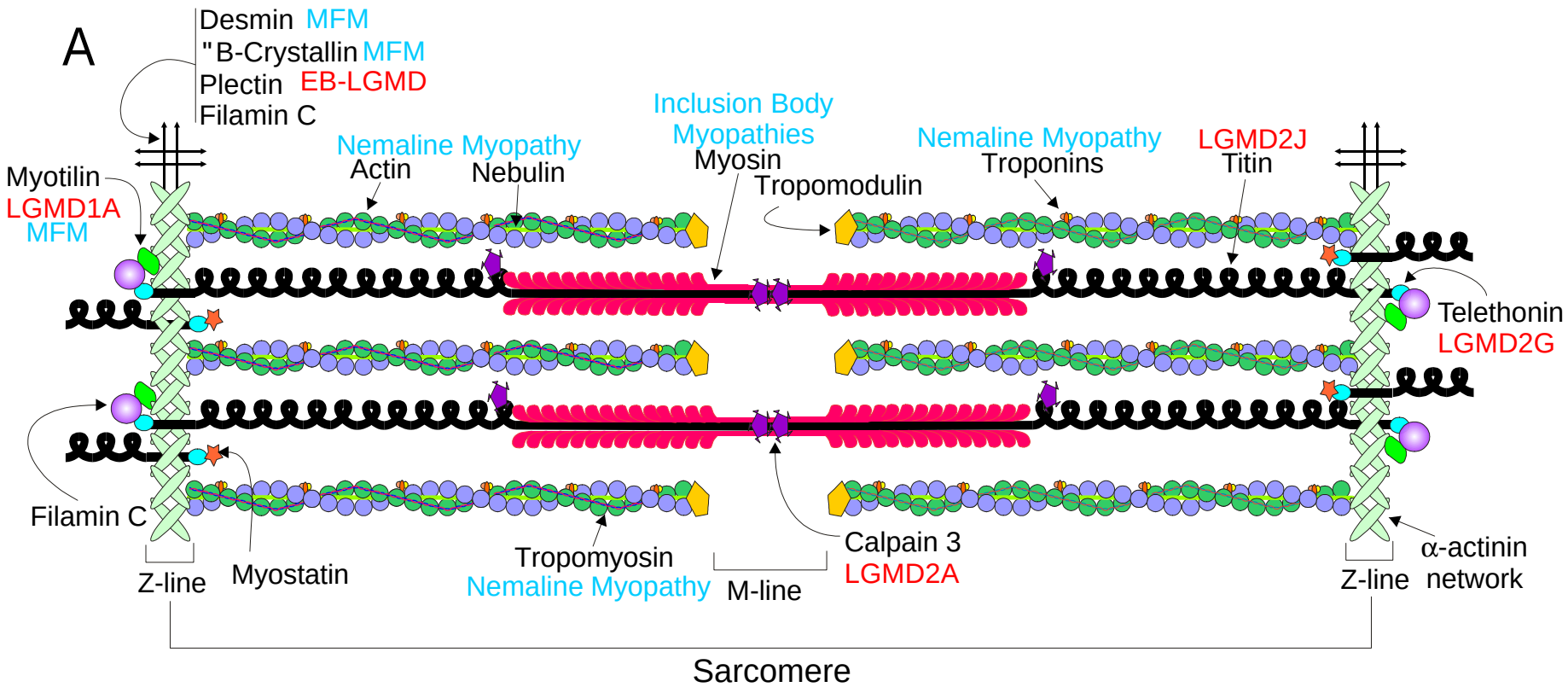

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Figure 1: Muscular dystrophy proteins found in the sarcomere. A) Diagram representing one sarcomere from Z-line to Z-line. Proteins with associated human disease are shown, with classic myopathies in blue and muscular dystrophies in red. B) Electron micrograph of a longitudinal section of human muscle showing the sarcomeric organization. Modified from *Pathology of Skeletal Muscle*, Carpenter and Karpati (1984).[47]

patients to have mutations in the myotilin gene. Mutations were all localized within the N-terminal serine-rich domain in which the previous two mutations had been found. Three patients had a S40C mutation, one had a S40F, one had a S95I, and one had the identical mutation as in the previously described Argentinian LGMD1A family, S55F. In these six patients there was primarily distal involvement with minor proximal weakness, cardiomyopathy, peripheral neuropathy, and a later age at onset in the 6th to 8th decade.

The clinical features of the six MFM patients did not mirror those of the previously described LGMD1A families (even the individual with the S55F mutation). In addition, there was no mention of a distinctive myofibrillar disorganization other than an increase in Z-line streaming in the previously described LGMD1A biopsies. While there does appear to be some overlap, clearly there is unpredicted variability in the phenotype associated with myotilinopathy.

(B) LGMD1B

LGMD1B was originally described in three large kindreds of diverse origins (Netherlands, Surinam, and Carribean) with 35 patients in total.[269] Affected individuals showed a slowly progressive proximal weakness (predominantly in the lower limbs), age at onset was in the 1st to 4th decade, SCK was variably elevated from 1-25X normal, deep tendon reflexes were reduced or absent, sensation was unimpaired, and most notably, more than half of the patients had a dilated cardiomyopathy with conduction disturbance. Muscle pathology was unremarkable with clear but mild signs of myopathy including increased fibre size variation, central nuclei, and fibre splitting. A genome scan was performed on these families and a locus at chromosome 1q11-21 was identified.[270] Mutations in the *LMNA* gene

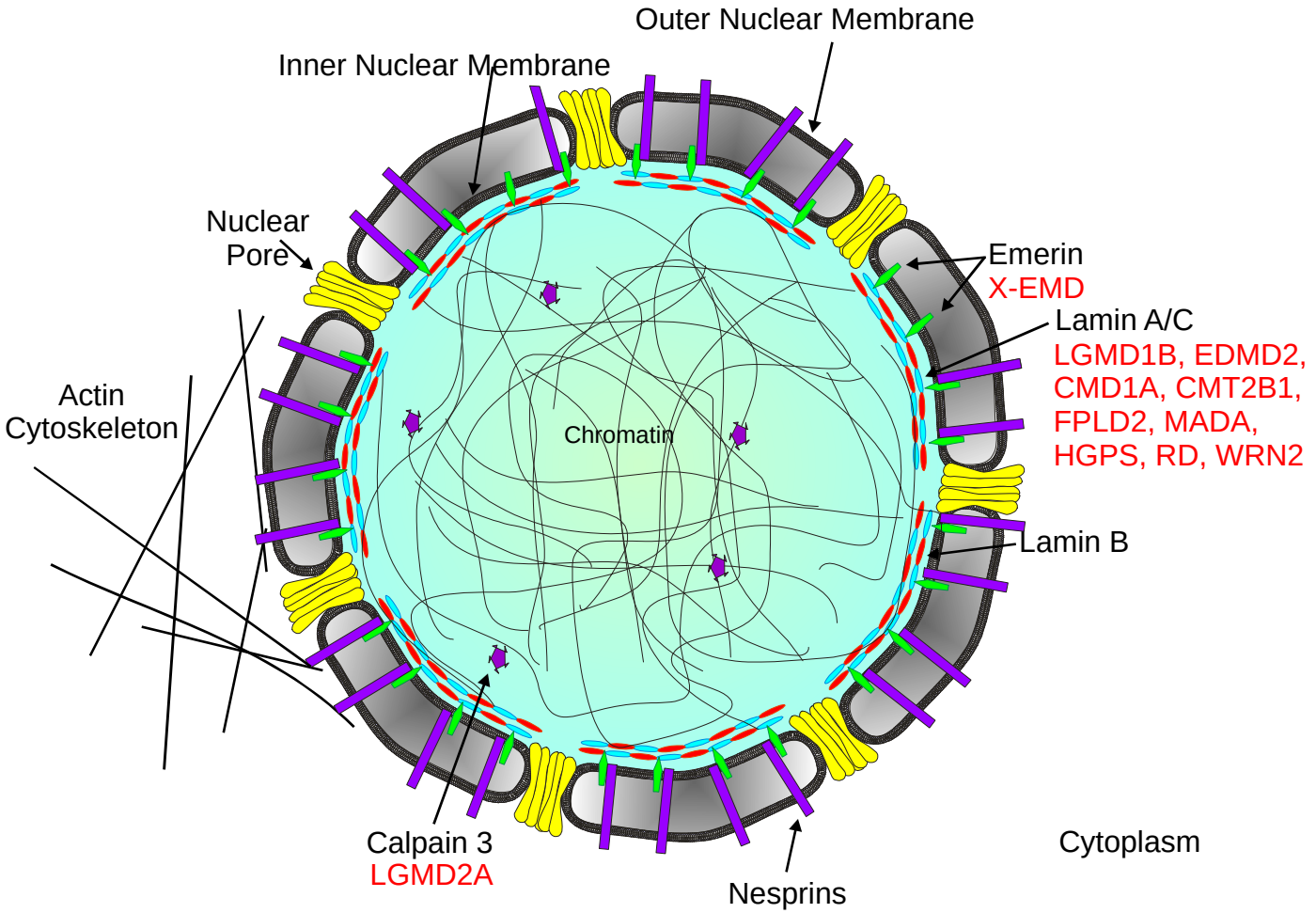


Figure 2: Muscular dystrophy proteins found in the nucleus. Associated diseases are in red. Note the nuclear lamina is connected through to the actin cytoskeleton via interactions between emerin and nesprins.

encoding the lamin A and C proteins were concluded to be the ultimate cause of this disorder.[183]

The lamins are intermediate-filament proteins that polymerize to produce the nuclear lamina, the principle structure maintaining the integrity of the nucleus by forming a lattice-like network lining the inside of the nuclear envelope (Figure 2).[130] The lamin family is divided into two groups, B-type lamins that are essential for cell viability and are expressed in every cell, and A-type lamins that are only expressed in differentiated somatic cells.[107] Lamins consist of a small N-terminal globular domain, a large elongated rod domain, a C-terminal globular domain, and a terminal tail region. All four A-type lamins are produced from the *LMNA* gene by alternate splicing (A, AΔ10, C, and C_2). The two major species are A (664 aa) and C (572 aa), that are identical except for unique C-terminal tails, and the two minor species, AΔ10 and C_2, are variants of the initial two that are only found in testes. Lamin C is translated into it's functional form directly whereas lamin A is proteolytically cleaved in its unique C-terminal tail region and farnesylated (presumably for association with the inner nuclear membrane).[188]

Interestingly, in addition to LGMD1B, the *LMNA* gene has been found to be mutated in eight other disorders. While some of them are related to LGMD1B there are definitely distinct characteristics attributable to each disease. The following lists each of these other laminopathies along with a short clinical description:

Emery-Dreifuss muscular dystrophy (EDMD2, MIM:181350) - The autosomal forms (both dominant and recessive) are caused by mutations in *LMNA*.[30,209] Interestingly the more common X-linked form is caused by mutations in emerin, a binding partner of lamin A/C.

This MD is differentiated from LGMD1B by the presence of early contractures of the elbow and spine, a characteristic humeroperoneal pattern of weakness, and cardiac defects usually manifest earlier.[288]

Dilated cardiomyopathy with conduction defects (CMD1A, MIM:115200) - This dominant disorder is very similar to the more severe forms of cardiac defects found in LGMD1B and EDMD but with no associated skeletal muscle involvement.[79,86]

Charcot-Marie-Tooth disease type 2B1 (CMT2B1, MIM:605588) - A recessive peripheral neuropathy of an axonal type with an age at onset in the 2nd or 3rd decade. Patients show symmetrical weakness and wasting, sensory deficits, predominantly in the distal lower limbs, foot deformities, and slight or absent reduction in nerve conduction velocities (non-demyelinating). Muscle biopsy and SCK levels are normal suggesting little to no muscular involvement.[50,220]

Dunnigan familial partial lipodystrophy (FPLD2, MIM:151660) - A dominant disorder that begins manifesting at puberty, characterized by the marked loss of fat deposits in the extremities, excess fat in the head, neck and trunk, profound insulin resistance, dyslipidemia, diabetes, acanthosis nigricans, and atherosclerotic vascular disease. No signs of myopathy or cardiac defects are present in these patients.[226,244]

Mandibuloacral dysplasia type A (MADA, MIM:248370) - A recessive progeria-like disorder with an age at onset of 5-6 yrs and consisting of growth retardation, bony abnormalities (mandibular and clavicular hypoplasia, delayed closure of cranial sutures, acro-osteolysis, and dental overcrowding), lipodystrophy, insulin resistance, partial alopecia (in males), and skin abnormalities (acanthosis nigricans, mottled hyperpigmentation on lower

trunk and extremities). Patients generally have a normal life span although it may be somewhat shortened due to complications of the lipodystrophy (dyslipidemia and atherosclerosis).[196,205]

Hutchinson-Gilford progeria syndrome (HGPS, MIM:176670) - A dominant disorder characterized by premature aging. Age at onset is in the first year of life with a failure to thrive. Over time patients show bony abnormalities similar to MADA, alopecia, sclerodermatous skin changes, variable lipodystrophy, joint contractures, and usually die in their early teens due to atherosclerosis and coronary artery disease.[44,73,219]

Restrictive dermopathy (RD, MIM:275210) - An apparent recessive disorder characterized by intrauterine growth retardation, tight and rigid skin with erosions, hyperkeratosis, bony abnormalities (clavicular hypoplasia, mineralization defects in the skull, and micrognathia) pulmonary hypoplasia, joint contractures, increased adiposity, and patients usually die within the first week of life. Dominant *LMNA* mutations (*de novo*) were found in a small subset of patients with this disorder but the bulk of the cases of RD are caused by autosomal recessive mutations in *ZMPSTE24*, the enzyme responsible for the proteolytic processing of prelamin A to produce functional protein.[188] Interestingly this is the same gene that is mutated in Mandibuloacral dysplasia type B (MADB).[1]

Atypical Werner syndrome (WRN2, MIM:277700) - Werner syndrome is a recessive progeroid disorder with an age at onset in the 3rd decade caused by mutations in the DNA helicase gene, *RECQL2.* A subset of patients that did not have *RECQL2* mutations, and had a more severe disease with an earlier age at onset in the 2nd decade, were found to have novel dominant mutations in the *LMNA* gene. The atypical Werner syndrome patients were

Table 2: List of *LMNA* mutations

Disease	Mode	Head (1-30)[a]	Rod (31-429)	Globular Domain (430-545)	A Tail (567-664)	C Tail (567-572)
LGMD1B	D		ΔK208, Y259X, R377H	Y481H, IVS9+5G>C		
EDMD	D	Q6X[b], R25P	ΔK32, E33G, A43T, Y45C, R50P/S, I63S, ΔE112, R133P, T150P, ΔLQT197-199, H222P, G232E, R249Q, ΔK261, Q294P, R336Q, R343Q, E358K, M371K, R366Q, R386K[c]	V442A, V452F, R453W, N456K/I, I469T, I497T, W520S/C, R527P, T528K, L530P, R541H		
	R		H222Y		R624H	
CMD1A	D		R60G, L85R, N195K, E203G/K	R541C	R571S, R644C	
CMT2B1	R		R298C			
FPLD	D			G465D, R482Q/L/W, K486N	R582H, R584H	
MADA	R			R471C, R527H, R527C, K542N		
HGPS	D		E145K		G608G(GGC>GGT), G608S(GGC>AGC)	
RD	D				G608G(GGC>GGT), IVS11+1G>A	
WRN2	D		A57P, R133L, L140R			
Combination	All D	R28W (FPLD, MD, CM)	E33D (CMT, MD, DCM, Skin), R60G + R62G (FPLD, MD, CM), R133L (Lipoatrophy, HCM, Skin), Δc.959T (CMD1A W/ var MD), S143F (MD w/ progeria)	R527P (FPLD, MD, CM)		

[a] Mutations are named according to the effect on protein where possible

[b] Blue signifies nonsense or frameshift mutations

[c] Green signifies splicing mutations

characterized by cataracts (variable), sclerodermatous skin changes, short stature, premature greying/thinning of hair, increased urinary hyaluronic acid (variable), diabetes, atherosclerosis, osteoporosis, and cardiomyopathy (only in one patient).[51]

Numerous mutations, both dominant and recessive, have been described and each one is usually associated with only one of the above nine phenotypes (Table 2). The different disorders can be roughly grouped into two categories, neuromuscular diseases and progeroid diseases (Table 3). LGMD1B, EDMD2, CMD1A, and CMT2B1 belong to the neuromuscular group that includes muscle, heart, and nervous tissue involvement. Peripheral nervous involvement is only definitively involved in CMT2B1 although there may be minor effects in LGMD1B and EDMD2. The progeroid group appears to involve adipose tissue, metabolic changes, and skin, hair, and bony findings. They appear to form a spectrum from least to most severe being FPLD, WRN2, MADA, HGPS, and RD. Strictly speaking, FPLD and RD are not progeroid disorders, however, one can see how they fit at either end of the spectrum. FPLD is mild with only the adipose and metabolic findings and onset doesn't occur until puberty whereas RD is so severe (particularly the skin phenotype) that the babies never live long enough to manifest some of the metabolic disturbances such as diabetes, hypertriglyceridemia, and atherosclerosis.

In addition to mutations that consistently manifest as one of the nine defined phenotypes there are a number of case reports describing mutations that are associated with 'mixed' phenotypes. Some of these mutations are unique and include R28W, R60G, and R62G (muscular dystrophy, dilated cardiomyopathy with conduction defect, and lipodystrophy),[98,268] R133L (hypertrophic cardiomyopathy, diabetes, liver steatosis,

Table 3: Tissues affected in each of the laminopathies

Clinical Classification	Muscle	Heart	Nerve	Adipose	Metabolic	Skin	Hair	Bone
LGMD1B			?					
EDMD			?					
CMD1A								
CMT2B1								
FPLD2								
MADA								
HGPS								
RD								
WRN2		?						
Combination								
E33D								
R60G, R527P								
R133L								
R28W, R62G								
S143F								

lipoatrophy, and whitish papules),[48] and S143F (muscular dystrophy and HGPS-like progeria).[143] Only two mutations, both of which were originally described as dominant EDMD mutations, have been found to be also associated with mixed phenotypes in certain families. The first is E33D (neuropathy, muscular dystrophy, and dilated cardiomyopathy with conduction defect)[102] and the second is R527P (muscular dystrophy, dilated cardiomyopathy with conduction defect, and lipodystrophy).[268] With the exception of these two cases it appears that the specific allele at the *LMNA* locus determines the phenotype with little effect of modifiers, either environmental or genetic. However, there are very few conclusions that can be made by reviewing mutation position in relation to disease phenotype (Table 2). Most of the identified mutations cause dominant EDMD and are spread throughout the domains common to lamins A and C. There is an apparent over-representation of mutations causing the progeroid diseases in the C-terminal regions of the proteins including the globular domain and the A-specific tail. The most consistent finding is that HGPS is almost exclusively associated with mutations at one specific codon (608) that activate a cryptic splice site, leading to the deletion of 50 aa and defective proteolytic processing of lamin A.[44,73,219] This same recurrent mutation is also associated with one case of atypical restrictive dermopathy.[188] Interestingly recent studies in a *ZMPSTE24*-/- mouse, with a total inability to produce mature lamin A, suggest that prelamin A is toxic and is ultimately responsible for the more severe phenotypes associated with *LMNA* mutations.[85]

Two hypotheses have been proposed to explain the pathophysiology underlying this bewildering array of phenotypes, one is structural and the other is based on gene expression.[130] The structural hypothesis is based on the observation that the nuclear lamina

is connected to the actin cytoskeleton, and therefore indirectly to the extracellular matrix, via a link between A-type lamins and emerin/nesprins embedded in the nuclear envelop. Cells with mutations at the *LMNA* locus show nuclear fragmentation and are much easier to deform using compression.[38,184] Presumably certain cells, particularly those that undergo constant mechanical stress like muscle and heart, may be more likely to become damaged and die due to this disturbed link. The gene expression hypothesis is based on the interaction of the nuclear lamina with specific transcription factors or with chromatin itself to affect or modulate gene expression. A-type lamins are known to bind to and indirectly regulate the activities of the transcription factors Rb, SREB1 and MOK1. Interestingly Rb activity is involved in myocyte and adipocyte differentiation and SREB1 is involved in adipocyte differentiation.[130] The tissue specificity of *LMNA* mutations may be based on regulation of these or other transcription factors therefore indirectly affecting gene expression. In addition, lamins have been shown to bind directly to chromatin and negatively regulate transcription.[170] Neither hypothesis provides all the answers so potentially it is a combination of both that leads to such complex effects when *LMNA* is mutated.

(C) LGMD1C

LGMD1C is unique amongst the dominant LGMD loci in that it was identified by a biochemical / candidate gene method rather than by positional cloning. Caveolin-3 was found to be a novel protein interacting with dystrophin and the dystrophin-associated glycoprotein complex (DGC) in muscle (Figure 3).[238] Muscle biopsies were screened by immunofluorescence microscopy using a caveolin-3 antibody and a sarcolemmal staining was found, identical to the localization of the DGC.[172,178] Patients from two separate families

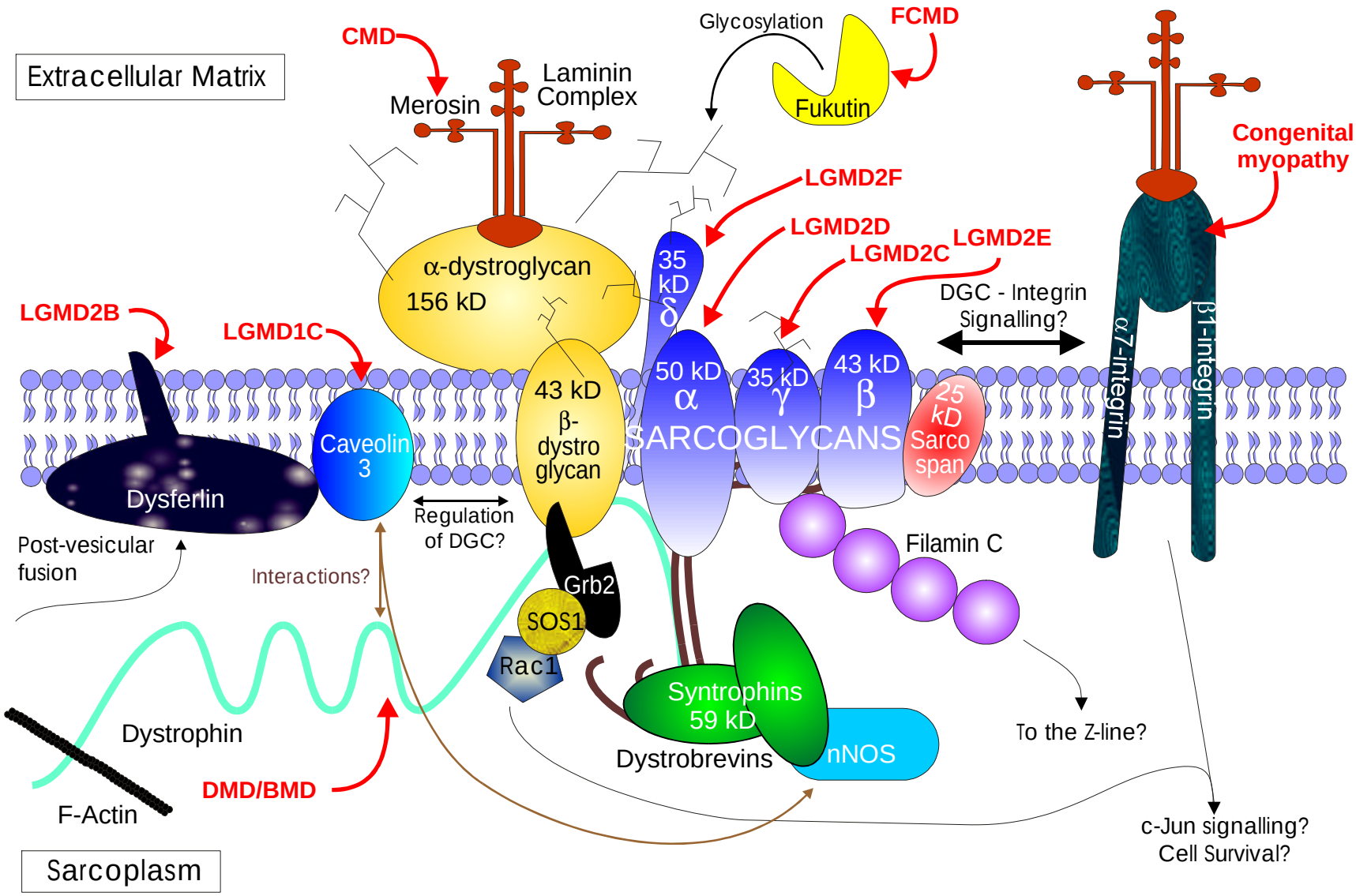


Figure 3: Muscular dystrophy proteins found at the sarcolemmal membrane. Associated diseases are in red. The central complex is the dystrophin-associated glycoprotein complex (DGC).

with an unknown myopathy were shown to have markedly reduced staining for caveolin-3 and this was confirmed by immunoblotting. Sequencing of patient DNA revealed heterozygous mutations in *CAV3*, the gene at chromosome region 3p25 encoding caveolin-3. Subsequently other patients were identified and this locus was named *LGMD1C*.[83,121,169]

The LGMD1C patients were clinically unremarkable. They had mild to moderate proximal weakness with an age at onset in the 1st decade, SCK elevation of 4-25X normal, exercise-induced cramping / myalgia, and calf hypertrophy. Other than the lack of caveolin-3, patient muscle showed mild non-specific myopathic changes with scattered necrotic fibres and increased connective tissue.[282]

In addition to LGMD1C, *CAV3* mutations are also associated with idiopathic hyperCKemia (HCK, MIM:123320),[46] rippling muscle disease (RMD2, MIM:606072),[26] and a distal myopathy (DM).[252] Idiopathic hyperCKemia is simply an elevated CK in the absence of an apparent cause (alcoholism, strenuous exercise, muscle strain, hypothyroidism, etc.) and with no signs of an underlying myopathy. RMD is an unusual non-dystrophic disorder with mechanically induced muscle contractions, swelling, and rippling (undulating muscle contractions in the absence of an action potential). The distal myopathy was only described in one case and is exceptional in that it only affected the intrinsic hand muscles and sternocleidomastoid, sparing all the proximal muscles and those in the forearm and leg.

Caveolins are intrinsic membrane proteins that are the main component of caveolae, small invaginations in the plasma membrane of most cells. These structures are formed through the self association of large numbers of caveolin monomers within 'lipid rafts' or microdomains composed of glycosphingolipids and cholesterol.[148] Caveolae are thought to

concentrate certain membrane components for the purposes of facilitating membrane trafficking and signal transduction. The caveolin family consists of three members, caveolin-1 and -2 having a wide tissue distribution whereas caveolin-3 is only found in heart and skeletal muscle.[27] Caveolin-3 is 151 aa in size and is post-translationally modified by the addition of palmitoyl groups (result is 21-24 kDa). It contains an N-terminal homo-oligimerization domain, part of which is referred to as a caveolin scaffolding domain (known to bind to signalling molecules), and a membrane-embedded region.[282] A portion of membrane caveolin-3 is associated with the DGC,[55] a complex which contains numerous proteins that are mutated in muscular dystrophies and is thought to maintain muscle membrane stability (Figure 3). This association is thought to occur via direct interaction with $-dystroglycan although interaction with other components such as dystrophin itself cannot be ruled out.[172,243] Caveolin-3 has also been found to interact with neuronal nitric oxide synthase (nNOS), an important signalling molecule transiently associated to the DGC, and dysferlin (see LGMD2B), a protein involved in muscle membrane repair.[169,272]

To date, twelve mutations have been identified in *CAV3* that cause disease (Table 4).[251,282] Most of these mutations are in well conserved residues and act via a dominant negative mechanism, largely explaining the marked absence of caveolin-3 in patient muscle. The mutant proteins have a greater propensity toward very high molecular weight oligimerization (with other mutant as well as wild type proteins) prior to their recruitment into detergent-insoluble lipid rafts.[94] The result is an induction of the endoplasmic reticulum-associated protein degradation pathway (ERAD or ER stress response) and the aggregates get ubiquitinated and degraded by the 26S proteasome.[95] Virtually none of the caveolin-3,

either mutant or wild type, that gets translated makes it to the sarcolemma to produce caveolae. Interestingly the phenotypic consequences of this process are not the same even when the underlying mutation is identical (Table 4). One mutation can be associated with all four phenotypes in different families (R26Q)[82] or can cause combinations of dystrophy (LGMD or DM) and muscle irritability (RMD2) in different branches of one family (D27E).[84] Unlike the situation with LGMD1B this is clear evidence that modifying factors have a major influence on the phenotypic expression of these mutations.

How the lack of caveolin-3 and/or caveolae in muscle leads to myopathy is unknown.[123] An LGMD1C phenotype has been recapitulated in a mouse model by complete knockout of the *CAV3* gene.[93] No caveolae were present in muscle of *CAV3*-/- mice despite the expression of caveolins-1 and -2 and they showed a mild muscular dystrophy with hypertrophic cardiomyopathy. Heterozygous mice did not show any defect and had normal caveolae confirming the detrimental dominant negative effects of mutant caveolin-3 protein found in human disease. Interestingly, a transgenic mouse over-expressing caveolin-3 had a severe DMD-like phenotype.[96] This suggests muscle is sensitive to both increased and decreased levels of caveolin-3. The binding site of caveolin-3 on $-dystroglycan is identical to that of dystrophin and it is possible that disruption of the DGC-dystrophin link is responsible for the dystrophy in these over-expressing mice.[243] Another possibility may be the fact that caveolin-3 negatively regulates nNOS and nitric oxide is an important activation factor in the regeneration process.[106,272,283] How either of these links relates to caveolin-3 deficiency is obscure. It is possible that there is a signalling defect in caveolin-3 deficiency but if so, it remains to be elucidated.

Table 4: List of *CAV3* mutations

Mutation[a]	LGMD1C	RMD	DM	HCK
R26Q	■	■	■	■
D27E	■	■	■	
P28L				■
N32K	■			
V43E	■			
A45T	■	■		
A45V		■		
T63P	■			
ΔTFT 63-65	■			
L86P[b]		■		
A92T[b]	■	■		
P104L	■	■		

[a]Modified from Woodman, Neurol 2004; 62:538-543

[b]Recessive mutations reported in Kubisch, Ann Neurol 2003; 53:512-520

(D) LGMD1D

LGMD1D was originally described in one large French-Canadian family and is alternatively referred to as FDC-CDM (familial dilated cardiomyopathy with conduction defects and myopathy).[175] The most prominent feature of this disorder was a dilated cardiomyopathy with atrioventricular conduction defects resulting in brachycardia. Age at onset was generally in the 3rd decade with slowly progressive proximal weakness appearing subsequent to cardiac symptoms. Muscle biopsy shows a classic dystrophic pattern with an increased fibre size variation, central nuclei, and increased connective tissue and fat. All patients remained ambulatory but most developed symptoms of congestive heart failure and arrhythmia, requiring pace maker and in some cases heart transplant. Symptoms were not dissimilar to those found in LGMD1B caused by mutations in the *LMNA* gene.

Linkage studies led to the identification of the *LGMD1D* locus at chromosome region 6q22-23 flanked by markers *D6S1705* and *D6S1656*.[175] To date, no causative gene has been reported at this locus although many genes linked to familial cardiomyopathy have appeared in the literature recently. In addition to mutations in lamin A/C (*LGMD1B*) and emerin (*EDMD*), both of which have an associated cardiomyopathy, many of the familial cardiomyopathies are caused by mutations in proteins in the sarcomere or at the sarcolemma. Sarcomeric proteins such as cardiac myosin heavy chain $, cardiac troponin T, cardiac troponin I, cardiac myosin binding protein C, regulatory myosin light chain, essential myosin light chain, and cardiac "-actin appear to be involved in hypertrophic forms of cardiomyopathy (heart is enlarged due to compensatory myocyte hypertrophy secondary to a contractile defect). A mixture of proteins of sarcomeric or sarcolemmal localization are

associated with dilated cardiomyopathies (heart becomes enlarged by dilation of chambers) such as cardiac "-actin, dystrophin, titin (see LGMD2J), vinculin, and phospholamban.[138,190] The LGMD1D region is roughly 1.8 Mb in size and contains only 11 known or predicted genes with the potential for 3 or 4 more on the basis of sequence analysis.[139,141] Interestingly a few very good candidates, including phospholamban, are found near to the region but have been excluded by recombination. No other genes within the region are known sarcolemmal/sarcomeric genes so there is the potential at this locus for the identification of a novel mechanism leading to dilated cardiomyopathy.

(E) LGMD1E

The group responsible for the identification of the LGMD1A gene had five families not linked to the chromosome 5 *LGMD1A* locus.[246] They performed a 430 marker genome-wide scan to identify additional dominant LGMD loci. The two largest families (comprised of 11 and 22 affected individuals) showed definite linkage to the telomeric end of the long arm of chromosome 7 and this locus was named *LGMD1E*. The remaining three families showed definite exclusion of the *LGMD1E* locus but did not have any further significant findings due to lack of power. The two American families that showed linkage were originally described in two previous publications.[223,245] The phenotype in the individuals affected with LGMD1E is unremarkable with progressive proximal weakness, pelvic preceding shoulder involvement, age at onset in the 3rd or 4th decade, and mildly elevated SCK levels of 1.5-10X normal. Muscle biopsies revealed mild non-specific myopathic changes.

Through haplotyping, the *LGMD1E* locus was defined as the region between the

markers *D7S2546* and *D7S2423* at chromosome region 7q36. Interestingly, even though the two families linked to this locus are unrelated they have identical haplotypes, suggesting that the same mutation is responsible for all known cases of LGMD1E. The candidate interval is 3.5 Mb in size and contains 20 known or predicted genes with the potential for at least two more due to the presence of novel spliced expressed sequence tags (ESTs).[139,141] Some of the genes present in the interval include two prominent developmental genes (*SHH*, sonic hedgehog and *EN2*, engrailed 2), a serotonin receptor (*HTR5A*), a regulator of intracellular cholesterol (*INSIG1*), and a ring-finger protein potentially involved in spermatogenesis (*RNF32*). No causative gene has yet been reported for this locus and there are no obvious candidates based on expression and similarity to previously characterized MD genes.

(F) LGMD1F

LGMD1F has only been described in one large Spanish kindred with 32 affected individuals.[97] There was progressive proximal weakness with pelvic preceding shoulder involvement and elevated SCK levels were only present in approximately 60% of cases (average of ~4X normal). The disorder shows a clear anticipation phenomena over the three generations studied, resulting in two patient groups, one with onset prior to 15 yrs and the other with an adult onset in the 3rd or 4th decade. Those individuals with an adult onset were less severe in respect to weakness and showed a slower progression. In those with a juvenile onset, there were individuals with additional facial and respiratory weakness, lordosis, and most had marked muscle wasting. Muscle biopsies showed increased fibre size variation, central nuclei, increased connective tissue, and autophagic rimmed vacuoles.

A genome-wide scan led to the identification of the *LGMD1F* locus at chromosome

region 7q32 flanked by markers *D7S680* and *D7S2544*.[200] The locus is distinct from the LGMD1E locus on the basis of observed recombinations but the proximity of the two loci has caused some problems in nomenclature. The interval spans 3.2 Mb and contains 46 potential genes. No causative gene has yet been described in this disorder despite screening efforts. The most attractive candidate within the interval is filamin C (*FLNC*), a binding partner of myotilin, the gene mutated in LGMD1A, and an important component of the Z-line in muscle. The investigators thoroughly scanned *FLNC* and its entire surrounding region and were unable to find any potential mutations.[200] Other good candidates include two actin bundling/binding proteins (*FSCN3*, fascin 3 and KIAA0265), an E2 ubiquitin conjugating enzyme (*UBE2H*, ubiquitin conjugating enzyme E2H), and a peripheral membrane protein involved in the proper functioning of the *trans*-golgi network (*GCC1*, golgi coiled-coil 1). In addition to these three genes it would be valuable to search for potential pathogenic repeat expansions because of the observation of anticipation.

(G) LGMD1G

LGMD1G is the most recently described LGMD and has only been found in one Brazilian kindred with twelve patients. There is pelvic and shoulder involvement, muscle cramping, onset in the 4th or 5th decade, and SCK levels of 1-10X normal. The most striking feature of this myopathy is a progressive digital flexion limitation but with no associated distal weakness. Biopsy shows a mixed myopathic/neuropathic pattern with an increased variation in fibre size, central nuclei, groups of atrophic angulated fibres, and rimmed vacuoles. The disorder has been mapped to chromosome region 4q21 between markers *D4S2947* and *D4S2409* but a causative gene has not yet been found.[247]

2.3 Recessive Forms

(A) LGMD2A

LGMD2A was the first LGMD to be attributed to a specific locus. It was originally described in a founder population living on the French island of Reunion located in the Indian Ocean 800 km east of Madagascar.[18] Affected individuals showed proximal weakness and wasting of varying severity often with shoulder involvement occurring prior to pelvic involvement, onset in the first to third decade, heel-cord contractures, mild lordosis, variable calf hypertrophy, and elevated SCK of 2-10X normal (higher in presymptomatic individuals).[77,78] Progression was slow with loss of ambulation usually occurring 20-30 yrs after onset. The causative gene was mapped to chromosome region 15q15.1 by linkage analysis and eventually the causative gene was identified as *CAPN3*, encoding the sarcoplasmic protease calpain-3.[18,87,213] LGMD2A has been found in many different populations and is now thought to be the most prevalent LGMD world-wide.[76] Well over 100 mutations have been described leading to LGMD2A since its discovery.[248]

Calpains are calcium-dependant neutral cysteine proteases found in the cytoplasm of all cells. The calpain family consists of fourteen members, twelve are large subunits encoded by *CAPN* 1-3 and 5-13 (73 - 117 kDa) and two are small subunits encoded by *CAPN* 4 and 14 (30 kDa).[129] Most active calpains exist as heterodimeric holoenzymes containing one large subunit (which confers most of the enzymes' substrate specificity and calcium concentration dependance) and one small subunit. Most of the information relating to the structure and function of calpains has been obtained by studying calpains -1, -2, and to a lesser extent, calpain-3.[240] There are four distinct regions in classic calpains, from N to C terminus, I, II,

III, and IV. Region I is a potential prodomain that is autocatalytically cleaved upon exposure to calcium to produce a functional protease. Region II contains the active site of the enzyme and is homologous to many cysteine proteases like cathepsin B.[103] Region III has no known homologues outside of the calpain family and is thought to be a linker domain. Region IV contains five EF-hand calcium-binding domains that mediate the calcium dependancy of these enzymes (along with another five EF-hands that are present on the small subunit).[125,129] Calpains do not have a general degradative protease activity but rather perform limited proteolysis at specific sites for the purposes of cell signalling, particularly in relation to cell death.[276] Different calpains have been associated with a variety of complex diseases including stroke, traumatic brain injury, Alzheimer's, diabetes, and gastric cancer[129]. However, calpain-3 has been the only family member shown to be the direct cause of a specific phenotype upon mutation.

The *CAPN3* gene consists of ~24 exons spanning 64 kb and encodes a protein of 821 aa (94 kDa). Calpain-3 is somewhat longer than the classical calpains (~80 kDa) due to an additional N-terminal sequence (NS) and two unique sequences, IS1 and IS2, inserted into region II and III respectively. IS2 contains a nuclear localization signal which gives calpain-3 the unique ability to enter the nucleus although it is not exclusive to the nucleus. Calpain-3 has a limited tissue distribution with high expression in muscle and low expression in heart and liver.[103] Interestingly calpain-3 does not appear to associate with a small subunit like the other calpains and may act alone. Studies on its proteolytic properties have been limited due to the enzyme's extreme instability. Little to no 94 kDa protein has been isolated and when it was, no activity could be detected. Not only is calpain-3 exquisitely sensitive to

intracellular proteases but was able to autolytically cleave itself regardless of the inhibitors present (calpastatin, calcium chelators, and leupeptin).[103] However, it has recently been discovered that the IS1 sequence actually acts as an inactivating prodomain and it must be cleaved out of the enzyme for it to be active against exogenous substrates. The remaining fragments appear to associate to form an active enzyme.[60] With this new information there will undoubtedly be an explosion of information regarding *in vivo* substrates of this elusive enzyme.

Due to the difficulties in studying calpain-3 there is very little hard evidence on how it causes a muscular dystrophy when mutated. The most prevalent theory is based on the observation of small numbers of apoptotic myonuclei in LGMD2A muscle biopsies (0.4% in LGMD2A, 0.007% in DMD, 0% in normal controls).[11] When these biopsies were studied further, there appeared to be an increased level of I6B" and decreased nuclear localization of NF6B. NF6B is known to protect cells against apoptosis when present in the nucleus but is inactive when bound to I6B" in the cytoplasm. If calpain-3 was necessary for getting rid of I6B" in muscle then in LGMD2A, when calpain-3 is inactive,[199,242] there would be a decrease in NF6B signalling and the muscle may have a sensitivity to apoptosis.[12] The problem with this hypothesis are that it depends on the assumption that muscle tissue is constantly receiving pro-apoptotic signals and it is not known what an apoptotic nucleus can do in the context of a gigantic multinucleated muscle fibre. Also it does not address the localization of calpain-3 in the sarcomere (calpain-3 has been shown to bind to titin) nor its potential role in the disassembly of the myofibril.[128] It is difficult to understand why a member of a protein family that normally promotes cell death actually causes cell death when

it is inactivated.[276] Further work is clearly needed to define how calpain-3 is different from the other calpains and what its *in vivo* substrates are in muscle.

(B) LGMD2B

LGMD2B was the second recessive LGMD described prior to the reclassification of the severe childhood autosomal recessive muscular dystrophies (SCARMDs) as LGMDs.[17] The disorder was originally described in only two families but the numerous families that have been identified since show a very similar clinical picture.[167,198,204,264] LGMD2B patients show a proximal weakness and wasting with pelvic preceding shoulder involvement, variable mild distal weakness (posterior compartment), onset usually in the second or third decade, variable calf hypertrophy, and highly elevated SCK of 10-60X normal. Muscle pathology showed signs of active dystrophy (increased variation in fibre size, central nuclei, regenerating and degenerating fibres), inflammation, and macrophage invasion.[75] The gene was localized to chromosome region 2p13 by linkage analysis and subsequently identified as *DYSF*, a novel gene encoding a protein similar to the *C. elegans* spermatogenesis factor fer-1. It was given the name dysferlin to reflect this homology and the fact that it causes a muscular dystrophy when mutated.[16] Over 40 mutations have been identified in dysferlin that lead to myopathy.[248]

A second, very similar disorder prevalent in Japan, called Myoshi Myopathy (MM) was mapped to the same interval on chromosome 2.[19,20] It is distinct from LGMD2B in that weakness was initially confined to the posterior compartment of the leg, leading to the patient's characteristic inability to walk on their toes. Many of the other features are similar to LGMD2B but the initial presentation of the patients is very different. Researchers working

on MM identified *DYSF* as the causative gene of this disorder concurrent with the studies on LGMD2B.[162] Another distinct phenotype associated with *DYSF* mutation is a distal anterior compartment myopathy (DAM) very similar to MM, only weakness is initially confined to the anterior compartment, leading to impaired dorsiflexion rather than plantarflexion.[131] One might hypothesize that these three manifestations of dysferlin mutation are allelic. However, families have been identified that contain branches with LGMD2B or distal myopathy that are caused by the same mutation.[132,277] This is a strong indication that genetic modifiers are causing the differences in phenotype but while good candidates for modifiers have been identified, none have been shown to be responsible.

Dysferlin is the founding member of the ferlin protein family which consists of four members, all showing homology to the fer-1 protein in *C elegans* (dysferlin, otoferlin, myoferlin, and fer1L4).[13] The full-length forms of these proteins consist of a tandem array of C2 domains (usually six) and a C-terminal transmembrane region. C2 domains are the second most common calcium binding motif (EF hands being the most common) and are thought to mediate calcium dependent or independent interactions with phospholipids. The domain was originally described in protein kinase C (PKC) which has one copy and multiple copies are often found in proteins involved in membrane trafficking and fusion (eg/synaptotagmins).[231] In addition to LGMD2B another human disease has been attributed to this gene family, DFNB9, a form of autosomal recessive deafness caused by mutations in otoferlin.[286] Very little is known about the other two members of the ferlin family, particularly fer1L4, as it was only just recently described.[56,61]

The *DYSF* gene has 55 exons spanning 234 kb and encodes a protein of 2080 aa (230

kDa). Dysferlin is widely expressed although its levels are highest in muscle and heart. It is embedded in the sarcolemmal membrane via the C-terminal transmembrane domain with the N-terminal portion containing the C2 domains exposed to the sarcoplasm. The C2 domains of dysferlin mediate anionic phospholipid interaction and are strictly dependant on calcium.[13] One dysferlin mutant that has been studied did not respond to calcium and lost the ability to bind phopholipids.[57] Recent studies in two dysferlin knockout mouse lines firmly establish its main function as a membrane repair protein.[14,158] These mice have a progressive proximal dystrophy very similar to LGMD2B patients and show a clear defect in the ability to repair the sarcolemmal membrane if it is ruptured. Dysferlin is present on the outer surface of large numbers of subsarcolemmal vesicles. Upon membrane damage there is a massive influx of calcium due to the large concentration gradient between the inside and outside of the cell. The calcium induces a cascade mediated by dysferlin and its two binding partners (annexin A1 and A2, two other proteins involved in phospholipid binding) whereby these subsarcolemmal vesicles fuse to each other and the sarcolemmal membrane to form a patch over the disrupted area.[158]

LGMD2B/MM results from lack of functional dysferlin and therefore it is likely that membrane repair is an essential process, particularly in muscle. It appears that when dysferlin is mutated, membrane repairs cannot take place and local areas of the muscle fibre undergo necrosis, leading to inflammation and macrophage invasion. With time, regeneration probably cannot compensate for this constant focal necrosis and weakness ensues. While dysferlin has a wide tissue distribution this effect is mainly seen in the muscle due to the constant physical stresses this tissue is under, reflected in the high levels of dysferlin

normally present in muscle. It is interesting to speculate whether any of the other dystrophies, particularly those with ill-defined pathogenic mechanisms like LGMD1C, have defects in this process as well.

(C) LGMD2C–2F, Sarcoglycanopathies

LGMD2C, 2D, 2E, and 2F are all caused by mutations in proteins that are part of the same complex and, for the purposes of this review, will be treated together. The first of these disorders was described in Algerian and Tunisian children affected with a severe DMD/BMD type muscular dystrophy.[10,22] It was originally referred to as severe childhood autosomal recessive muscular dystrophy (SCARMD) and was mapped by linkage analysis to chromosome 13q12.[23] Subsequently it was found that these patients had a deficiency in a newly described member of the DGC called 50DAG (50 kDa dystrophin-associated glycoprotein, now called "-sarcoglycan).[9] Attempts were made to find the chromosomal localization of the 50DAG gene (*SGCA*) with the assumption that it was in the SCARMD interval. However, it was soon discovered that it was actually at chromosome region 17q21 and responsible for another SCARMD-like disorder found in French families.[214,215] Eventually another member of the same complex, 35DAG ((-sarcoglycan encoded by *SGCG*) was found to be mutated in the original SCARMD.[195] Two other forms of muscular dystrophy were mapped to novel loci around this same time, an LGMD in the Amish (4q12)[2] and another LGMD in Brazilian families (5q33-34).[203] These two disorders were subsequently found to be caused by mutations in distinct members of the sarcoglycan family, $-sarcoglycan and *-sarcoglycan respectively.[32,159,192] Since all four disorders are LGMD-like, just with variable severity, they were all classified as LGMDs and named accordingly

(Table 5). To date, over 100 mutations have been discovered in the sarcoglycan gene family that lead to muscular dystrophy.[248]

In general, the clinical course of all four sarcoglycanopathies is more severe than many of the other LGMDs. These disorders show a 'bottom-up' distribution of weakness and wasting with scapular winging and limited distal weakness appearing as later features. Some cases of late facial involvement have been reported but this appears to be uncommon.[21] Onset was usually in the first or second decade but progression could be either rapid or slow. Later onset in the 3rd or 4th decade has been reported, particularly associated with "-sarcoglycan mutations, and these patients usually had a much slower progression and residual levels of sarcoglycan staining upon muscle biopsy.[6] Most patients lost ambulation eventually and many of the rapidly progressive, early onset cases died by 20 yrs. Elevated SCK was present with a high degree of variability from 2-200X normal,[6] with LGMD2D patients often showing the highest elevations.[204] Muscle biopsy revealed an active dystrophic process with absence or marked reduction in sarcoglycan staining. Mild cardiac manifestations are estimated to occur in about 30-70% of patients although they are infrequently found in patients with "-sarcoglycan mutations.[173,206] Interestingly, rare cases of isolated dilated cardiomyopathy caused by *-sarcoglycan mutations have been reported.[260]

There are six members in the sarcoglycan family (Table 5); "-sarcoglycan (*SGCA*), $-sarcoglycan (*SGCB*), (-sarcoglycan (*SGCG*), *-sarcoglycan (*SGCD*), ,-sarcoglycan (*SGCE*), and .-sarcoglycan (*SGCZ*).[139,141] They are single-pass transmembrane proteins found in the plasma membrane. A small cytoplasmic domain is present at either the N or C-terminus followed by the transmembrane domain and a large N-glycosylated extracellular

Table 5: The sarcoglycan gene family

Name	Gene	Chromosome	Disorder	Reported Mutations	Exons	Gene Size	AA	Pedicted MW	Actual MW	Cytoplasmic End	Tissue Distribution
α-sarcoglycan	*SGCA*	17q21	LGMD2D	47	10	11 kb	387	43 kDa	50 kDa	N-Terminus	Muscle, Heart
β-sarcoglycan	*SGCB*	4q12	LGMD2E	25	6	15 kb	318	35 kDa	43 kDa	C-Terminus	Wide
γ-sarcoglycan	*SGCG*	13q12	LGMD2C	20	8	145 kb	291	32 kDa	35 kDa	C-Terminus	Muscle, Heart
δ-sarcoglycan	*SGCD*	5q33-34	LGMD2F	8	10	440 kb	290	32 kDa	35 kDa	C-Terminus	Wide
ε-sarcoglycan	*SGCE*	7q21-22	DYT11	17	12	72 kb	437	50 kDa	--	N-Terminus	Wide
ζ-sarcoglycan	*SGCZ*	8p22	--	--	7	466 kb	299	33 kDa	--	C-Terminus	Wide?

Table 6: Homology between the sarcoglycans

	α	β	γ	δ	ε	ζ
α	100%	0	0	0	45% / 63%	0
β	0	100%	21% / 39%	21% / 40%	0	22% / 40%
γ	0	21% / 39%	100%	52% / 70%	0	58% / 74%
δ	0	21% / 40%	52% / 70%	100%	0	56% / 75%
ε	45% / 63%[a]	0	0	0	100%	0
ζ	0	22% / 40%	58% / 74%	56% / 75%	0	100%

[a]First number is percent amino acid identity, second is percent amino acid similarity

domain.[109] There are two types of sarcoglycans (Table 5); " and , are type 1 transmembrane proteins with the N-terminus facing the extracellular surface (these two contain an extracellular dystroglycan-type cadherin domain) and $, (, *, and . are type 2 transmembrane proteins with the C-terminus facing the extracellular surface (these four contain a unique extracellular 'sarcoglycan domain').[109] Members of each group are homologous with other members within the group but there is little to no homology between groups (Table 6). In addition, while technically $-sarcoglycan belongs to the type 2 group it only shares weak homology with the other three and probably has a unique function.[152]

Sarcoglycans form heterotetrameric complexes (1:1:1:1) containing one type 1 sarcoglycan and three type 2 sarcoglycans. These complexes form in the endoplasmic reticulum and, when complete, they are sent to the plasma membrane, associating with the DGC in muscle and heart. $ and * associate first and appear to form an invariant core for the sarcoglycan complex (SGC) upon which, one type 1 (" or ,) and one type 2 ((or .) sarcoglycan can attach.[49,228] The expression of " and (is largely restricted to muscle and heart whereas the expression of the other four subunits is much wider. It is thought that ", $, (, and * form the main functioning SGC in muscle and heart whereas complexes containing , and . are found in other tissues.[163,250] Interestingly mutations in ,-sarcoglycan cause a neurological disorder called myoclonus dystonia (DYT11) suggesting that complexes containing , are important in the central nervous system.[194,290] In general, if one subunit is missing, the whole complex does not make it to the plasma membrane. This explains the originally unexpected finding in SCARMD muscle biopsies: if an LGMD patient has a mutation in one subunit they are found to be deficient in all four of the main muscle subunits.

There are exceptions when there are only reductions in the other members rather than complete absence.[110] This occasionally occurs in the case of " and (mutation, possibly due to the low frequency replacement of either of these subunits with , and ., which are also expressed in muscle.[163] However, these alternate complexes do not fully compensate for the mutation because muscular dystrophy still results.

There are still many uncertainties regarding the biochemistry of sarcoglycans. An additional non-sarcoglycan protein, called sarcospan (a tetraspanin-like protein), is part of the SGC in muscle. The SGC can form without it, but sarcospan cannot make it to the membrane in the absence of the SGC.[53,54] In addition, .-sarcoglycan is newly discovered and little is known about its function or tissue distribution. There is some evidence that . may form a heteropentameric structure with the four main sarcoglycans in muscle and is reduced along with the others in LGMD patient muscle.[280] There are also indications that four members are not always necessary for a functioning SGC, particularly in non-muscle tissue.[69,250] Most puzzling is that when the supposed invariant members of the complex, $ and *, are mutated, the phenotype is limited to muscle and heart. This suggests that either some of the sarcoglycans can perform their functions outside of a complex, there are alternate ways for the complex to be formed, or there are uncharacterized members of the sarcoglycan family that have yet to be uncovered.

In addition to the uncertainty regarding the biochemistry of these proteins there is also some controversy regarding their function in muscle tissue. The DGC is thought to be an important link between the cytoskeleton and the extracellular matrix, preventing membrane damage that may occur during repeated cycles of contraction and relaxation. The main axis

of this link is through actin-dystrophin-dystroglycan-laminin (Figure 3) but the SGC does associate directly with dystroglycan and may be important for the functioning of the DGC.[49] The whole DGC is absent in dystrophinopathy but only the SGC is deficient in sarcoglycanopathy. Yet, most sarcoglycanopathies show the hallmark membrane fragility seen when the whole DGC is gone.[108] This suggests the role of the SGC is largely mechanical however there is some evidence that the SGC may have a role in signalling. It has been shown that "-sarcoglycan has an ecto-ATPase activity and may be involved in regulating extracellular ATP levels.[218] The increased ATP levels can activate passive channels in the sarcolemma such as the $P2X_7$ receptor that would allow intracellular calcium levels to rise, initiating cell death without actual physical disruption of the membrane.[25] In addition, the SGC, perhaps via sarcospan,[119] may have a role in integrin signalling although the current evidence is not very conclusive.[287] If either of these is true, a signalling deficit would also contribute to the pathogenesis of dystrophinopathies and it may be a combination of mechanical and signalling defects that are responsible for MD resulting from any disruption of the DGC.

(D) LGMD2G

LGMD2G was originally described in one Italian and one Brazilian family with a total of nine affected individuals.[181] These individuals were characterized by marked weakness in the distal leg muscles, milder proximal involvement, onset in the 1st or 2nd decade, loss of ambulation in the 3rd or 4th decade (~20 yrs after onset), very minor SCK elevation, and minor cardiac manifestations (unspecified and found only in three patients). Upon mapping the causative gene to chromosomal region 17q11-22, another Brazilian family

with three patients was identified that was also linked to this locus.[182] This family showed a slightly different phenotype with pronounced calf hypertrophy and SCK elevation ranging from 10-30X normal. Muscle biopsies from all three families showed increased variation in fibre size, central nuclei, increased connective tissue, and an abundance of rimmed vacuoles. Sequencing of genes within the region led to the identification of *TCAP*, the gene encoding the sarcomeric protein telethonin, as the causative gene of LGMD2G. [182]

Two mutations in *TCAP* were found to cause LGMD2G and both produce premature termination codons in the N-terminal half of the protein. None of the LGMD2G families are known to be consanguinous but one mutation, Q53X, was found to be homozygous in two families and heterozygous in the third. The other mutation in this last family was c.)109-110GG leading to a frameshift and a nonsense codon 5 aa downstream.[182] In addition to these two LGMD-causing mutations a dominant missense mutation, R87Q, has been associated with a form of dilated cardiomyopathy (CMD1N).[144]

Telethonin or Titin-cap is small protein of 167 aa that is localized to the periphery of the sarcomere Z-line in muscle (Figure 1). It has no recognizable domains or significant homology to any other protein. It is highly expressed in heart and skeletal muscle and has been shown to bind to the extreme N-terminus of titin, the giant filamentous protein that acts as a scaffold for the assembly of the sarcomere (see LGMD2J for further information on titin).[266] It is thought to behave as an anchor, immobilizing one end of the titin molecule so that it remains fixed within the Z-line matrix (a complex of proteins consisting mainly of "-actinin and accessory proteins like filamin c and myotilin) (Figure 1).[104] In addition, telethonin becomes phosphorylated by the C-terminal kinase domain of titin during

myofibrillogenesis but the significance of this observation is unknown.[171]

A number of different functions for telethonin have been proposed based on protein-protein interaction data that may have relevance on these human MD and CMD phenotypes. Besides titin, telethonin has been shown to bind to FATZ, myostatin, MinK, ankrd2, and CSRP3. FATZ is an intrinsic Z-line component that binds telethonin, "-actinins, and filamins, presumably functioning to enmesh telethonin into the Z-line matrix.[81] Myostatin is a well known paracrine growth factor that inhibits the proliferation and differentiation of myoblasts. Telethonin has been shown to bind to intracellular myostatin and prevent its secretion, thereby increasing muscle growth and regeneration.[189] MinK is a subunit of the I(Ks) potassium channel in cardiac T-tubules suggesting a role for telethonin in stretch-dependant ion flux.[92] Ankrd2 is a ankryrin-repeat containing transcription factor that shuttles between the sarcomere and the nucleus, implicating telethonin in stretch-dependant gene regulation.[146] CSRP3 or muscle LIM protein in a complex with telethonin is thought to be the key component in the cardiac stretch sensor machinery (mutations in either partner leading to decreased binding results in human dilated cardiomyopathies CMD1M [*CSRP3*] and CMD1N [*TCAP*]).[144] Many of these binding partners are found in both muscle and heart and yet there are no major cardiac phenotypes in LGMD2G and no skeletal muscle dysfunction in CMD1N (or CMD1M).

Clearly telethonin has a major role in signalling, both intracellular and intercellular, and in maintaining the structural integrity of the sarcomere. Severe disruptions in telethonin seem to be associated with muscle phenotypes and may be more dependant on the structural importance of this molecule. The only cardiac mutation found was a dominant missense

mutation affecting signalling functions but not localization or titin binding. While this provides a conceptual framework for understanding telethonin mutation it does not explain why stretch-signalling is not a major factor in LGMD2G. Perhaps there are other mechanisms to compensate for the lack of functional telethonin when it is absent but not if it is present and non-functional. More studies will be necessary before this becomes clear.

(E) LGMD2H

This form of LGMD was originally described by Shokeir and Kobrinsky in the Manitoba Hutterite population.[232] The mapping and identification of the LGMD2H gene is discussed in chapter 4.

(F) LGMD2I

LGMD2I was originally described in a Tunisian family and localized to chromosome region 19q13.3.[66] It is also likely to be the cause of the myopathy described by Shokeir and Rozdilsky in Saskatchewan Hutterites.[233] Further discussion on this myopathy can be found in chapter 5.

(G) LGMD2J

LGMD2J is the severe childhood form of tibial muscular dystrophy (TMD, MIM:600334), a distal myopathy found at high frequency in the Finnish population.[261] TMD is a relatively benign late onset dominant disorder, usually manifesting in the 4th to 6th decade, mainly affecting the anterior compartment of the leg, and with little to no SCK elevation.[262,263] Patients have impaired dorsiflexion and tend to stumble. In its homozygous form it causes LGMD2J, a more severe proximal myopathy very similar to LGMD2A. The causative gene was mapped to chromosome 2q31 and identified as *TTN*, the gene encoding

the largest known protein titin, a component of the sarcomere (Figure 1).[112,114] In addition to the Finnish, other populations have been identified with distal myopathies caused by novel mutations in *TTN*.[59,262,267] No other homozygous patients have been identified however, so it is not known whether LGMD2J can result from any homozygous *TTN* distal myopathy mutation or whether it is specific to the one found in the Finnish.

Mutations in *TTN* are also known to cause two other dominant disorders, hypertrophic cardiomyopathy 9 (CMH9, MIM:188840) and dilated cardiomyopathy type 1G (CMD1G, MIM:604145). CMH9 has only been reported in one patient and is not thought to be a major cause of hypertrophic forms of cardiomyopathy.[221] CMD1G has been described in both large multigenerational kindreds[99] and sporadic patients,[134] with a total of 6 different mutations identified. Both of these disorders lead to congestive heart failure with a variable age at onset and an apparent lack of muscular symptoms.

TTN is an extremely large and complex gene of 294 kb and composed of 363 exons. It is alternatively spliced to produce a variety of tissue and developmentally specific isoforms, producing titin molecules ranging in size from 0.7 mDa to 3.7 mDa (maximum potential size is 4.2 mDa / 38,138 aa).[111] Titan assumes a large rod-like structure of ~1 ：m which spans an entire half sarcomere from the Z-line to the M-line (Figure 1). From its N-terminus it contains a Z-disk binding region, a long tandem array of Ig-like repeats (spanning half of the light coloured I-band from the Z-disk to the A-I interface), a PEVK domain (a large region rich in proline, glutamate, valine and lysine), another long tandem array of Ig-like repeats interspersed with fibronectin type III repeats (spanning half of the dark coloured A-band from the A-I interface to the M-line), and a C-terminal kinase domain found right

at the M-line. It is thought to associate with the actin thin filaments as it passes through the I-band and to the myosin thick filaments as it passes through the A-band, acting as a molecular ruler that determines the even spacing of the sarcomere and as a molecular scaffold for a variety of sarcomeric proteins. The repetitive Ig-like domains and the large flexible PEVK domain allows titin to act like a spring during cycles of contraction and relaxation in the muscle.[111,259]

In addition to interaction with the major structural components of the sarcomere, actin and myosin, titin binds to numerous other muscle proteins. Examples include telethonin (binds at the Z-disk / see LGMD2G), "-actinin (binds at the Z-disk), nebulin (binds at the PEVK domain), myomesin (binds at the C-terminus), MURF-1 (binds at the C-terminus), and calpain- 3 (binds at an area found in the I-band, termed N2A, and at the M-line right near the C-terminal kinase domain / see LGMD2A). In addition to its structural functions titin potentially forms a signalling complex consisting of the C-terminal kinase domain, calpain-3, and MURF-1 (an E3-ubiquitin ligase) that relays information regarding the status of the contractile apparatus to the nucleus, however, this is purely speculation.[111] Titin may also have a role in regulation of myofibrillogenesis conferred by its kinase domain via interaction and phosphorylation of telethonin.[258]

Mutations causing human disease are spread throughout the titin molecule. The mutation causing the Finnish TMD (and LGMD2J) is a unique replacement of 11 nucelotides in the last exon of *TTN* (363rd), presumably an insertion plus a deletion, affecting four amino acids in the skeletal muscle form of titin (AAGTAACATGG>TGAAAGAAAAA causing EVTW>VKEK)[112]. The only other known mutation causing a distal myopathy is in the very

same exon (C>T transition causing L>P).[112] Neither of these mutations affect the kinase domain but they are both predicted to disrupt the terminal Ig-like repeat(s) of titin and may affect the second binding site of calpain-3. It is thought that calpain-3, due to its extreme lability, is quickly degraded if it is not associated to titin. In addition, it has been found that TMD / LGMD2J patients have a reduction in caplain-3 levels by immunoblot, perhaps as a result of a loss of calpain-3 stabilization.[115] This reduction in calpain-3 may contribute to the pathogenesis of TMD / LGMD2J and would explain the similarities between LGMD2A and LGMD2J. The mutations associated with cardiomyopathies are as follows: an exon 3 G>A transition (V>M), 2 exon 14 mutations three codons apart (C>T transition causing A>V and G>T transversion causing R>L), a T>C transition in exon 18 (W>R), 2 mutations in exon 49 (C>T transition causing Q>X and G>A transition causing S>D), and a 2 bp insertion in exon 326 (induces a frameshift with a stop codon produced 5 codons downstream).[99,134,221] Surprisingly only the mutations in exon 49 (termed N2B) are specific to cardiac isoforms, all other mutations are expressed in skeletal muscle as well even though there is no discernible myopathic phenotype in these patients. To date, no functional studies have shed light on the mechanism of any of these mutations other than the potential for calpain-3 binding being involved in skeletal muscle phenotypes. All currently described mutations allow for the proper assembly of the sarcomere. Further studies will be needed to decipher the bewildering complexity present at this locus before it becomes clear how such tissue specific phenotypes can result from mutations in such an important sarcomeric gene.

2.4 Pathogenic Themes

The study of neuromuscular disorders is extremely complex. There is a confusing array of different genes and phenotypes all associated with muscle weakness in humans. Even when concentrating on only a small aspect of muscular dystrophies like LGMD there is still a plethora of different genes involved. Many of these genes can also cause other phenotypes, putting into question the whole classification scheme for neuromuscular disorders. Despite this, by studying small parts it is hoped that a comprehensive theory behind the whole will become apparent.

The pathogenic mechanisms underlying LGMDs are intricate but there are certain themes that are emerging from studies on each new disorder. Each protein that has been shown to cause an LGMD (or numerous other types of myopathies) when mutated can be localized to either the sarcolemma, the sarcomere, or the nucleus. There is also the potential for connections or commonalities between these three sites.

The sarcolemma is the site of the most MD gene products known to date (Figure 3). The discovery of dystrophin as the DMD/BMD gene opened the floodgates for the identification of a huge number of other MD-associated genes. As mentioned previously, dystrophin along with the DGC forms a continuous link from the cytoskeleton to the extracellular matrix. The DGC is concentrated in costameric regions of the sarcolemma (areas overlying the Z-line of the sarcomere)[177] and prevents membrane damage from occurring during contraction.[152] The core of the DGC is the dystroglycan complex, formed by the transmembrane protein, $-dystroglycan, and the heavily glycosylated membrane-associated protein, "-dystroglycan. The dystroglycans are created by the proteolytic

processing of a single polypeptide, are widely expressed, and are known to be involved in the formation of the basement membrane.[118] Through the cytoplasmic portion of $-dystroglycan this complex is bound to dystrophin[120] (or alternatively caveolin-3)[243] which in turn acts as the connection with cytoskeletal actin.[216] Through the O-linked glycosylation of "-dystroglycan, the complex is bound to laminin, an important component of the extracellular matrix.[120] This is the main axis of the link, however numerous other proteins are associated including the sarcoglycans, syntrophins, and dystrobrevins.[152]

It is many of these associated proteins that give the DGC a potential signalling role. Associated with the complex are known signalling molecules like nNOS, Grb2, Sos1, and Rac1.[197] In addition to the aforementioned ATPase activity of "-sarcoglycan, the SGC is thought to act in bidirectional signalling between the DGC and integrins that are also associated with muscular dystrophies.[287] All the current data seem to point to the DGC as being an important mechanosignalling complex in muscle and heart, not only protecting the cell against major membrane damage but also regulating gene expression dependant on what is mechanically occurring to the cell membrane.

Other important sarcolemmal MD-associated proteins that are not strictly part of the DGC are caveolin-3 and dysferlin. Caveolin-3 may have a function in signalling although it is not clear whether it is part of the same pathway as the DGC. In addition, it has a potential role in the formation of the DGC via its interaction with $-dystroglycan. Dysferlin appears to be involved in a different mechanism than the DGC even though the result is the same. Dysferlin acts by closing up tears in the membrane that have already occurred rather than preventing them from occurring in the first place.[14] Due to the reported interaction between

dysferlin and caveolin-3, it is also possible that caveolin-3 may even be directly involved in this same repair mechanism.[169] Either way, it is necessary for both of these mechanisms to be in place to escape muscular dystrophy.

The second site of MD gene products is within the sarcomere, particularly at the Z-line (Figure 1). The sarcomere is delineated on each side by a Z-line which anchors the actin thin filaments that extend toward the centre. In the middle of each sarcomere is an M-line from which myosin thick filaments extend, slightly overlapping the thin filaments in a relaxed state. Huge increases in calcium concentration caused by nerve impulse induce the myosin to slide along the actin, thereby decreasing the total length of the sarcomere (controlled by regulatory proteins like tropomyosin and troponins). In addition to the two main filamentous components, enormous titin molecules extend from the Z-line to the M-line and provide a spring-like support mechanism and a defined sarcomere spacing. Numerous other proteins are involved in a functioning sarcomere including nebulin (involved in thin filament length) and a host of Z-line proteins, accessory/regulatory proteins, and transiently associated enzymes and transcription factors.[31] The Z-line is important, not only for anchoring filamentous components of the sarcomere but for transmitting stresses created by contraction through to the cytoskeleton as well.[80] In addition, evidence is accumulating that the Z-line is also part of a stretch sensor that controls gene expression on the basis of mechanical signals from the contractile apparatus.[144,146]

It is not entirely clear how mutations in genes associated with the sarcomere can lead to muscular dystrophy. Most of the sarcomeric genes associated with muscle disorders lead to what would historically been referred to as myopathies. Myopathies tend to have a distinct

pathological manifestation such as nemaline rods, inclusion bodies, central cores, vacuoles or tubular aggregates.[47] Often this pathology is a result of a metabolic defect, a defect in myofibrillogenesis, or abnormal accumulations of a product. However, there are numerous examples such as LGMD1A, 2G, and 2J where there appears to be a dystrophic pathology and yet the mutated protein is an integral component of the sarcomere. Titin is arguably the most important component of the sarcomere after actin and myosin and yet recessive mutations in its gene do not lead to catastrophic sarcomeric disorganization but to a relatively benign progressive MD.[112] Mutations in the essential Z-line components, myotilin and telethonin, also lead to similar pathologies.[117,182] Again, it appears signalling and gene expression must be invoked to explain this phenomenon particularly in light of recent evidence linking Z-line components to transcription factors in heart and muscle.[144,146] It may be that if purely mechanical functions of the sarcomere are affected, a 'myopathy' results, whereas if signalling capabilities are affected, a more dystrophic pathology results.

The third site is the nucleus, particularly the nuclear lamina (Figure 2). As previously mentioned the nuclear lamina is an intricate network of proteins, consisting mainly of lamins, that lines the inner nuclear membrane.[107] This structure maintains the integrity of the nucleus and disassociates and reforms (bringing the nuclear membrane along with it) during cell division.[130] A-type lamins and emerin are the two main gene products mutated in muscular dystrophy and both are part of the nuclear lamina in differentiated cells. The precise mechanisms behind the disorders caused by lamin and emerin mutation are not known. As with the other three sites of MD gene products, there are two potential pathogenic mechanisms, one structural and one based on gene expression.

It is difficult to understand how calpain-3, a proteolytic enzyme, fits into this grand scheme. There is evidence linking this enzyme to all three of the key compartments in muscle cells. Calpain-3 has a nuclear localization signal and by immunohistochemistry is largely nuclear.[213] This suggests a potential role related to the nuclear lamina or directly involving the breakdown or activation of transcriptional modulators. The most prevalent theory on its mode of action is based on its potential regulation of the NF6B transcriptional activation through breakdown of I6B.[11] Calpain-3 also appears to have a role in the sarcomere as there are two well defined binding sites on titin for this enzyme.[241] Binding to titin appears to stabilize calpain-3, either simply preventing its action or holding it in reserve for a specific purpose. Patients with titin mutations often have a secondary reduction in total levels of calpain-3.[115] It may also be connected to the sarcolemma as there appears to be secondary reductions in calpain-3 in patients with dysferlin mutations.[4] This suggests either a direct or indirect connection to sarcolemmal repair mechanisms. How any of these potential mechanisms relate to one another is completely open to speculation.

Within the field of muscle disorders one thing is clear, there are no easy answers. This conceptual framework that I have presented only answers the most superficial of questions and does not address the intricacies of what is actually going on during the myopathic process. I have no doubt that the more genes we find, the more examples we will have that do not fit into these tight little categories. There are certainly important mechanisms within muscle, such as the maintenance of an intact membrane, that are often disrupted in MDs. However, it is likely that other minor processes in muscle can also result in an MD if they are disturbed. In addition, I did not even mention some of the more

complicated muscle disorders like myotonic dystrophy. We have not even begun to scratch the surface on the mechanism behind the huge amount of phenotypic and tissue-specific variability present in many of these disorders. Our current approach of finding individual genes in large families and then attempting to extrapolate to the general population has gotten us this far but it does have major limitations. Perhaps a more large scale approach using microarrays, either for determining signature expression profiles or for screening some of the massive genes involved in muscle disorders (eg/*TTID*, *DYSF*, and *RYR1*), will be necessary to provide comprehensive diagnostic capabilities.

3. Materials and Methods

Short names and abbreviations will be used for manufacturers; refer to the list of manufacturers (page xii) for full names, locations, and websites. When not specifically stated, equipment and general chemicals were obtained from Fisher Scientific. General methodologies used during this research are as follows:

3.1 Patient Resources

Affected individuals were largely identified by clinical geneticists in the Department of Pediatrics and Child Health, Health Sciences Centre, Winnipeg, MB. Patients and their families were asked to participate in the study by Dr. Cheryl Greenberg and signed informed consent was obtained (Appendix 1). Whole blood was drawn for serum creatine kinase (SCK) determination (Health Science Centre) and genomic DNA isolation. Neuromuscular examination was performed by Dr. Greenberg for assessment of any weakness or wasting, muscle hypertrophy, and cardiac difficulties. A subset of patients underwent muscle biopsy, electromyography (EMG), and full cardiac workup or some combination of these.

Patients referred to us from other provinces or countries were assessed locally by various physicians and results, including pedigreees, were provided to us along with isolated genomic DNA or whole blood. All non-Hutterite subjects used in our study, with the exception of one, were anonymous and were identified by number alone.

Anonymous control samples from healthy individuals not known to have LGMD, were used to determine allele frequencies. Samples were largely from Manitobans and were obtained from the Rh Laboratory, University of Manitoba. 90% of the individuals from which samples were obtained are Caucasian with the remainder being Aboriginal, Asian, and

African. All aspects of this study were approved by the Health Research Ethics Board of the University of Manitoba (Appendix 2).

3.2 Genomic DNA Isolation

Whole blood was collected in a 5 or 10 ml Vacutainer EDTA tube (BD Biosciences) either directly by our group or sent via courier from a referral centre. Blood was added to enough NH_4Cl:Tris (0.14 M, 0.017 M Tris, pH 7.7) to make up the volume to 45 ml in a 50 ml conical centrifuge tube. The tube was incubated for 10 min to lyse the red blood cells and white blood cells were collected by centrifugation at 1100 g for 10 min using a Sorvall SS34 fixed angle rotor (Kendro). The pelleted white blood cells were resuspended in 0.85% NaCl and centrifuged again at 1100 g for 10 min. The pellet was resuspended in 2 ml High TE (100 mM Tris, 40 mM EDTA, pH 8.0). 2.5 ml of blood lysis solution (100 mM Tris, 10 mM EDTA, 0.2% SDS, 1M NaCl, pH 8.0) was added using a syringe fitted with an 18 gauge needle. Theresulting viscous solution was drawn up and down into the syringe 3-4 times. An equal volume of Tris-saturated phenol (Invitrogen) was added and the mixture was rotated on an orbital mixer (Boekel) for 10 min. The aqueous and organic phases were separated by centrifugation fro 5 min at 1500 g. The aqueous phase was transferred to another tube and 7 ml of Tris-saturated phenol along with 2 ml of High TE was added. The sample was mixed, centrifuged, and re-extracted with 7 ml of $CHCl_3$:isoamyl alcohol (24:1). The sample was again mixed, centrifuged, and collected and 150 ：l of 5 M NaCl was added. 7 ml of absolute ethanol was then added to begin precipitation of the DNA. The DNA can be easily visualized at this point. Liquid is removed without disturbing the partially precipitated DNA and small amounts of absolute ethanol are added until the DNA collapses into a small loose

precipitate (~ 70% ethanol). Precipitated DNA is then washed once in 70% ethanol and then again in absolute ethanol and placed in a 1.5 ml microcentrifuge tube and dried at room temperature. The pellet was then resuspended in 0.2-1 ml of Low TE (10 mM Tris, 1 mM EDTA) overnight at 4°C on an orbital mixer. The next day, DNA is quantitated by absorbance at 260/280 nm and 1∶1 is subjected to electrophoresis on a 1% horizontal submarine agarose gel, along with standards of known concentration, and intensity of ethidium bromide (EtBr) staining is used as a confirmation of the spectrophotometry results.

3.3 Polymerase Chain Reaction (PCR)

(A) Primer design

Oligonucleotide primers were designed mainly by eye rather than through the use of the numerous primer design programs available on the world wide web. Target sequences were input into GeneRunner, a simple, freely available sequence manipulation program (www.generunner.com). Stretches of sequence 18-24 bp in size, with GC content between 40 and 60 %, were selected in the desired positions. These sequences were then tested for physical characteristics such as melting temperature, hairpinning, and duplex formation both with itself and with potential reaction partners at the Integrated DNA Technologies website (IDT, www.idtdna.com). Sequences with low potential for duplex formation, melting temperatures appropriate to the application, and no matches other than the target (BLAST, www.ncbi.nlm.nih.gov/BLAST/) were then ordered from IDT. All primer sequences can be found in Appendix 3.

(B) General Method

PCR reactions were largely performed using Taq polymerase from Qiagen or

Invitrogen although under some circumstances, such as when proofreading was necessary, other brands were used. A typical reaction is as follows:

100 - 300 ng of genomic DNA

1X PCR buffer (generally supplied by manufacturer of polymerase as a 10X concentrate)

200 ：M dNTPs (Invitrogen)

200 nM each of the forward and reverse primer

2.5 U of polymerase

Water to 50 ：l

The resulting mixture was subjected to the following cycling conditions using either a Techne gradient thermocycler or one of two MJ Research thermocyclers: 3 min at 95ºC, 30 cycles of (1 min 95ºC, 1 min melting temperature, 1 min 72ºC), and 10 min at 72ºC. Minor modifications were made based on the nature of the primers, the length or GC content of the fragment, or the presence of non-specific products. A no-template reaction was always performed to control for PCR contamination; if any product was found in this control all samples were discarded and reamplified.

When working with new primers a PCR optimization was generally performed. This usually consisted of preparing a 125 ：l reaction mixture as outlined above as well as ones containing additives such as 15% glycerol, 5% dimethylsulfoxide (DMSO), and 1X Q-solution (provided as a 5X concentrate from Qiagen). The same template DNA was added to these four reaction mixtures and each one was split into five tubes (25 ：l). These tubes

were then subjected to the cycling conditions outlined above with five different melting temperatures spanning the predicted temperatures for the primers (Techne gradient thermocycler). The twenty different reactions were then analysed to identify the conditions which best amplify the fragment of interest.

(C) Fragment analysis

DNA fragments were separated on either agarose or polyacrylamide gels based on the size of the fragment and required resolution. Agarose gels ranging from 1-2% were used for most general purposes particularly when the DNA being analysed was >1 kb in size. Agarose gels were prepared with 1X mTBE buffer (130 mM Tris, 44 mM Borate, 2 mM EDTA) and supplemented with 1 ：g/ml EtBr. Horizontal gels were subjected to electrophoresis at 100-120 V for the required time period. Mini vertical polyacrylamide gels (Bio-Rad) ranging from 6-20% (29:1, acrylamide:N-N'methylenebisacrylamide) were used for applications that required precise resolution of small fragments (<1 kb). Polyacylamide gels were also prepared with 1X mTBE buffer, however, they were stained post-electrophoresis in 1 ：g/ml EtBr. All gels were then visualized and photographed using a gel documentation system from Alpha Innotech.

3.4 Genotyping and Analysis

Once obtained, all genotypes were stored in a Microsoft Access database accessible only from computers requiring password-based login to allow for security and confidentiality. In order to maintain the accuracy and integrity of the database, after being entered, data was proofread by another individual and any mistakes were corrected. Any subsequent analyses were done on data extracted from the database by query.

(A) Short tandem repeats (microsatellites)

Oligonucleotide primers for known microsatellites were obtained commercially (Invitrogen) and primers for novel microsatellites were designed as outlined previously. PCR reactions were performed as above but with minor modifications. Reactions were scaled down to 15 ：l. Prior to assembly of the reaction, the forward primer was radioactively end-labelled using T4 polynucleotide kinase (Invitrogen) and [(-^{32}P]ATP (ICN biomedicals), as per the manufacturer's instructions. After the PCR, 7.5 ：l of stop solution (98% formamide, 10 mM EDTA, 0.1% bromophenol blue) was added. Samples were denatured at 95°C for 5 min and snap cooled on ice. 5 ：l was then loaded on a 6% denaturing polyacrylamide gel (1X mTBE, 7M urea, 6% 19:1 acrylamide:N-N'methylenebisacrylamide) and subjected to electrophoresis at 70 W for 1.5-3.5 hrs (apparatus manufactured by Invitrogen). Gels were mounted on filter paper, dried on a slab gel dryer (Thermo Electron), and exposed to Biomax MR film (Kodak). Alleles were sized in relation to CEPH control individuals with known genotypes (Coriell).

(B) Single nucleotide polymorphisms (SNP)

Single nucleotide changes were generally assayed by one of two different methods. The first, and by far the most common method in our laboratory is by restriction analysis. The variants were amplified on fragments with a natural restriction site (or one created by intentional primer mismatch) created or abolished by the variant. Fragments were digested with the corresponding restriction enzyme (mainly NEB, Promega, and Roche) and separated by electrophoresis on an agarose or polyacrylamide gel. The second method was allele-specific PCR where one primer in the reaction has its 3'end at the site of the variation. Two

versions of this primer were used, one matching the first allele and the other matching to the second allele. The presence or absence of a PCR fragment with each of the given primers allows deduction of the genotype. In order to increase the potential differentiation between the two alleles an intentional mismatch was incorporated at the penultimate 3' position to further destabilize any incorrect duplexes.

(C) Linkage analysis

Two-point linkage analyses were performed using the LINKAGE suite of programs (version 5.2).[153-155,254] A fully penetrant autosomal recessive disease model was generally used and the Hutterite families were considered to be unrelated since consideration of all known genealogical relationships made the computation intractable. For most linkage calculations, we used a disease allele frequency of 0.01. Marker allele frequencies for the markers were estimated using all genotyped individuals. Multipoint linkage analyses were done using GENEHUNTER 2.1.[147,168]

(D) Haplotype analysis

Genotypes were extracted from our database and imported into Microsoft Excel. Marker positions were obtained from numerous sources (CEPH, UCSC, LDB, Marshfield) and markers were placed in order from telomere to centromere (p-arm) or centromere to telomere (q-arm). Alleles were then placed in haplotypes by minimizing recombinations and reviewed by an independent individual to identify errors.

3.5 Mutation screening

(A) Single stranded conformational polymorphism (SSCP) analysis

Oligonucleotide primers were designed to amplify exons of candidate genes from

genomic DNA for SSCP analysis and/or sequencing. For most exons longer than 350 bp, overlapping primers were designed. SSCP analysis was performed on PCR products from three individuals: one patient and two siblings (one with one disease-associated chromosome and one with no disease-associated chromosomes). The analysis was performed under three conditions (i) 6% acrylamide (19:1) with 10% glycerol at 20/ C, 8 mA for 20 hrs; (ii) 6% acrylamide (19:1) with 20% glycerol at 4/ C, 10 W for 20 hrs; and (iii) MDE gel as per manufacturer's instructions (BioWhittaker). Fragments with potential shifts were then sequenced according to the following protocol.

(B) Sequencing, alignments, and analysis

Fragments were sequenced through the Centre for Applied Genomics (TCAG) sequencing core (www.tcag.ca). Primers flanking the area to be sequenced were used in a large PCR reaction (200 - 400 ：1 split into 50 ：1 / tube) and the pooled amplicon was subjected to electrophoresis in a large well, 1% agarose gel. The resulting band was excised using a scalpel or razor blade and DNA was isolated using a Qiagen gel extraction kit as per the manufacturer's instructions. Alternatively PCR products were cloned into a TOPO-TA cloning vector (Invitrogen) and transformed into chemically competent *E.coli*. Cells were allowed to grow at 37°C on LB plates supplemented with 50 ：g/ml carbenicillin (BD Biosciences) for 18-24 hrs. Five to twenty resistant colonies were picked (depending on the application) and grown at 37°C in 3 ml of LB broth supplemented with 50 ：g/ml carbenicillin (BD Biosciences) for 18 hrs. Plasmid DNA was extracted using a Qiagen miniprep kit as per the manufacturer's instructions. DNAisolated using either method was quantitated by comparison with a mass standard (Invitrogen) on an agarose gel and samples

exceeding the minimum recommended concentration by TCAG were sent via courier to undergo automated sequencing. The minimum concentrations used were 50 ng/∶l for PCR fragments < 2 kb and 200 ng/∶l for plasmids or PCR fragments ≥ 2 kb. All sequences obtained had at least 2X coverage using two separate primers. Primers used for sequencing were either well known sequencing primers (T7, M13 reverse), nested primers designed for the specific fragment, or the same primers used for amplification.

Electropherograms, sent via email, were analysed using a combination of Chromas (an electropherogram viewer, www.technelysium.com.au/chromas.html), BLAST[3] (an alignment tool), and GeneDoc (an alignment viewer/editor, www.psc.edu/biomed/genedoc/). Sequences were compared to those obtained through the Human Genome Project (www.ncbi.nlm.nih.gov/)[151] and Celera Genomics (www.celera.com/).[273] Any ambiguities were resolved by direct viewing of the electropherograms, particularly in the cases of potential heterozygosity.

3.6 Northern Blot

A 12-tissue northern blot obtained from Origene was used to determine the normal distribution of the TRIM32 transcript. A TRIM32 probe was prepared by amplifying a 733 bp fragment of the gene using the primers HT2A_ex2(5)F and HT2A_ex2(6)R from the genomic DNA of an unaffected individual. The fragment was subjected to electrophoresis in a 1% agarose gel, purified as above and then labeled using the Random Primers Labeling kit (Invitrogen). A product with a specific activity of $3x10^9$ cpm/mg was obtained. The blot was hybridized using Ultrahyb hybridization solution (Ambion) as per the manufacturer's instructions. The hybridized membrane was visualized by autoradiography using Biomax MS

film (Kodak). Film exposure was at -80° C with intensifying screens for 100 hours.

3.7 Plasmid Production

Numerous plasmids were containing various parts of the TRIM32 coding sequence created for the characterization of TRIM32. The original plasmid from which all subsequent plasmids were made was created by PCR cloning (pCR2.1, Invitrogen) a 2.2 kb fragment of TRIM32 amplified from genomic DNA using HT2A_ex2(1)F and HT2A_ex2(6)R. The resulting plasmid was sequenced to preclude any PCR-induced errors. pCR_HN contains the entire coding sequence of TRIM32 as well as small amounts of 3' and 5' UTR. As mentioned previously, all primer sequences are available in appendix 3. Maps for plasmids discussed in this section including pCR_HN can be found in appendix 4. Maps were drawn using the web server PlasMapper (http://wishart.biology.ualberta.ca/PlasMapper), a freely accessible service hosted by the University of Alberta.[63]

pCR_HNF & pCR_HNT:

Stock plasmids for propagation of TRIM32 coding sequence

pCR_HN was converted into two plasmids (pCR_HNF and pCR_HNT) that contain in-frame restrictions sites, allowing for subsequent transfer into plasmids that require fusion to an N-terminal tag. A 208 bp PCR fragment amplified with HT2A_Nde1F and HT2A_ex2(1)R and then cut to 70 bp by *Bsm*I was used to replace the N-terminal end of TRIM32. This 70 bp fragment was ligated to a 5.8 kb *Eco*RV - *Bsm*I fragment of pCR_HN. The resulting plasmid was named pCR_HNF and contained in-frame *Eco*RI and *Nde*I sites at the N-terminal end of TRIM32 (created by an overhang on HT2A_Nde1F). For pCR_HNT, a 695 bp fragment amplified with HT2A_Nde2F and HT2A_ex2(5)R was

digested with *Eco*NI to obtain a 317 bp fragment. This 317 bp fragment was ligated to a 4.5 kb *Eco*RV - *Eco*NI fragment of pCR_HN to produce a truncated TRIM32 with only the six C-terminal NHL domains and an in frame *Nde*I site at the N-terminal end (created by a non-templated extension on HT2A_Nde2F).

pET14b_HNF:

Plasmid for production of bacterial-expressed 6XHis tagged TRIM32 protein

pET14b (EMD Biosciences) is a bacterial expression plasmid containing a 6XHis tag along with a thrombin site to cleave the 6XHis tag off the resulting protein. A 2.2 kb *Nde*I - *Bam*HI fragment of pCR_HNF was ligated to a 4.7 kb *Nde*I - *Bam*HI fragment of pET14b to create pET14b_HNF. Amino acids added N-terminal to the native initiator methionine of TRIM32 are as follows: N term-MGSSHHHHHHSSGLVPRGSH-C term. All subsequent manipulations were done according to the manufacturers instruction to produce recombinant *E. coli* expressed TRIM32.

pcDNA+HNF & pcDNA–HNF:

Plasmids for production of mammalian-expressed TRIM32 protein (with or without a tag)

TRIM32 was inserted into pcDNA3.1 (Invitrogen) with or without a 6XHis tag for expression in mammalian cells. For pcDNA+HNF, a 2.2 kb *Xba*I - *Bam*HI fragment of pET14b_HNF was ligated to a 5.3 kb *Nhe*I - *Bam*HI fragment of pcDNA3.1(-). The resulting plasmid still contains the 6XHis tag and thrombin site from the pET14b plasmid. For pcDNA–HNF, a 2.1 kb *Nhe*I - *Bam*HI fragment of pCR_HN was ligated to the same 5.3 kb *Nhe*I - *Bam*HI fragment of pcDNA3.1(-) as was used for the previous plasmid. This plasmid does not contain any tag attached to TRIM32 and uses the native start codon of TRIM32 for

expression.

pBT_HNRB & pBT_HNT:

Plasmids for use in the bacterial two hybrid system

TRIM32 was inserted into pBT (Stratagene) to create a bait plasmid for use in the Bacteriomatch system (see section 3.11). This plasmid contains the DNA binding domain of 8cI protein for fusion with the N-terminus of TRIM32. For pBT_HNRB, a 2.1 kb *Eco*RI / Klenow-filled fragment of pCR_HNF was ligated to *Sma*I digested pBT (3.2 kb). The resulting plasmids were then screened for orientation using *Hind*III and those that were in the correct orientation were named pBT_HNF. The C-terminus of TRIM32 was deleted by digesting with *Bsr*GI - *Bam*HI, filling with Klenow, and ligating the blunt-ended 3.6 kb fragment to produce pBT_HNRB. This plasmid only contains the RING finger and B-Box domains from the N-terminal portion of TRIM32. For pBT_HNT, a 1 kb *Eco*RI / Klenow-filled fragment of pCR_HNT was ligated to *Sma*I digested pBT (3.2 kb). The resulting plasmids were then screened for orientation using *Hind*III and those that were in the correct orientation were named pBT_HNT. This plasmid only contains the six NHL domains from the C-terminal portion of TRIM32.

3.8 Protein Electrophoresis

(A)Sodium dodecyl sulfate - polyacylamide gel electrophoresis (SDS-PAGE)

Samples were obtained from tissue or cell culture, homogenized in a lysis buffer compatible with downstream applications, and treated with a protease inhibitor cocktail (Sigma). After quantitation using an RC-DC protein assay kit (Bio-Rad) an appropriate amount of 4X sample buffer was added to achieve a final concentration of 1X (2% SDS, 62

mM Tris-HCl, 10% glycerol, 5% $-mercaptoethanol, 0.001% bromophenol blue, pH 6.8). Samples were diluted to the same concentration and equal amounts were loaded on a mini vertical polyacrylamide gel (Bio-Rad) made with 29:1, acrylamide:N-N'methylenebisacrylamide. Protein gels contained a 4% acrylamide stacking gel (125 mM Tris-HCl, 0.1% SDS, pH 6.8) and a separating gel ranging from 7.5-15% acrylamide or a gradient thereof (375 mM Tris-HCl, 0.1% SDS, pH 8.8). An unstained or prestained protein ladder (Fermentas) was loaded along-side the samples and empty wells were loaded with equal amounts of 1X loading buffer. Gels were subjected to electrophoresis at 150V for 0.75-1.5 hrs in SDS-PAGE running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS). After electrophoresis the apparatus was disassembled, the stacking gel was removed, and the separating gel was used for all further analysis.

(B) General protein stains

To view all proteins in the gel one of two methods was used, coomassie staining or silver staining (the first is low sensitivity and the second is high sensitivity). For coomassie staining, gels were soaked in staining solution (0.1% coomassie blue R-250, 40% methanol, 10% acetic acid) for 15 min. Gels were then soaked in destaining solution (40% methanol, 10% acetic acid) 5X for 30 min. Silver staining was accomplished using a kit from Bio-Rad, as per the manufacturer's instructions. Gels stained in either fashion were dried using a DryEase kit from Invitrogen.

(C) Immunoblot

For visualization of a specific protein(s), an immunoblot was performed. Proteins were transferred for 18 hrs onto supported nitrocellulose membranes in blotting buffer (25

mM Tris, 192 mM glycine, 20% methanol) at 4°C using an electroblotting apparatus set at 30V (Bio-Rad). Membranes were then washed 3X 5 min in water, stained with 0.1% Ponceau S in 5% acetic acid (Sigma) for 5 min, destained 3X 5 min in water, and pictures were taken using a digital camera (Alpha Innotech). This stain will allow visualization of all proteins in order to determine if samples were equally loaded and to assess transfer efficiency. Ponceau S was removed by soaking in TBST (10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20) for 10 min. Membranes were then blocked using 5% skim milk powder in TBST (SM-TBST) for 1 hr. Primary antibodies directed against the protein of interest were then applied in SM-TBST for 1-2 hrs at a concentration ranging from 1:100 - 1:5,000 depending on the strength and specificity of the original preparation. Membranes were then washed 3X 10 min in TBST and secondary antibodies (raised against the primary antibody) conjugated to horseradish peroxidase were applied in SM-TBST for 30 min at a concentration ranging from 1:5,000-1:15,000. Membranes were again washed 3X 10 min in TBST and chemiluminescent substrate was added (West Femto, Pierce). Membranes were wrapped in cellophane and exposed to Biomax light film (Kodak) from 1 sec - 10 min, depending on the intensity.

3.9 Cell Culture

Cells used in this study include BHK21 (baby hamster kidney, ATCC), C2C12D (mouse myoblasts, ATCC), adult human skin fibroblasts (primary cultures prepared by the Health Sciences Centre Cytogenetics department), and semi-transformed adult human myoblasts (prepared by collaborators at McGill University by published methods).[164,234] BHK21 and C2C12D were grown in DMEM containing 10% fetal bovine serum (FBS,

Invitrogen); all fibroblast cultures were grown in "MEM containing 10% FBS, and human myoblasts were grown in a complex medium called SkGM2 (Cambrex) containing 10% FBS and numerous supplemental growth factors optimized for the maintenance of myoblast lines.[234] All cells were grown in non-coated plastic dishes of various sizes (Corning). Cells were detached from plates by treating with 0.05% Trypsin and 0.5 mM Na_4EDTA in Hanks balanced salt solution (Invitrogen) for 1-5 min at 37°C. Trypsin activity was always terminated by addition of two volumes of growth media containing FBS. Established lines with high growth rates (BHK21, C2C12D) were passaged at 1:10 and allowed to reach near ~95% confluence before subsequent passaging. The remaining lines were passaged at a frequency to keep them between 30% and 90% confluence at all times. Cells were usually harvested in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) on ice, using a cell scraper. BHK21 transfections were done using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions (a standard seeding density of 5 $X10^5$ cells / 60 mm plate was used).

3.10 Immunofluorescence

Six-well culture dishes containing a round sterile coverslip in each well were seeded at a density of ~100,000 cells per well. Cells were allowed to attach and grow to desired confluence. Cover slips with attached cells were then rinsed 3X in cold CB (10 mM 2-morpholinoethanesulfonic acid [MES], 150 mM NaCl, 5 mM EGTA, 5 mM $MgCl_2$ 5 mM glucose, pH 6.1) and treated with 3% paraformaldehyde (PFA) in CB for 15 min at 4°C. Cells were then permeabilized in 0.3% Triton X100 and 3% PFA in CB for 5 min at 4°C. Cells were again rinsed 3X in cold CB and then stored in Cyto-TBS (20 mM Tris-HCl, 154 mM

NaCl, 2 mM EGTA, 2 mM $MgCl_2$, pH 7.2) at 4°C until use.

Detection of the protein of interest requires a protocol akin to that for the immunoblot procedure described above. All the following incubations occur in a humidified chamber to minimize evaporation. Coverslips are removed from the dish and inverted onto a 50 ：l drop of blocking solution (2% normal donkey serum and 1% IgG-free bovine serum albumin [BSA] in Cyto-TBST, Cyto-TBS as above with 0.1% Tween 20) on a sheet of parafilm and incubated for 2 hrs at room temperature. Coverslips are then transferred to 50 ：l drop containing an antibody directed against the protein of interest at a concentration ranging from 1:50 - 1:500 in Cyto-TBST and incubated overnight at 4°C. Coverslips were then washed in Cyto-TBST 4X 15 min and placed on a 50 ：l drop containing a secondary donkey antibody conjugated to a fluorophore (Invitrogen) at a concentration ranging from 1:100 - 1:300. Coverslips were incubated for 2-3 hrs at room temperature, washed again with Cyto-TBST 4X 15 min, and then a nuclear stain which does not fluoresce in the range of the secondary antibody was applied (Hoescht33342 / Blue [2 ：g/ml], Propidium Iodide / Near red [1 ：g/ml], or TOTO-3 / Far Red [0.6 ：M])(Invitrogen). Coverslips were washed in water 3X 5 min and mounted using Prolong mounting media (Invitrogen).

3.11 Bacterial Two Hybrid (B2H) screening

Interaction screening was used to identify novel binding partners of TRIM32 in an attempt to learn more about its native function. Bacteriomatch™ (Stratagene) is based on transcriptional activation in *E. coli* (Figure 4).[64,65] The bait protein (TRIM32) is fused to the DNA binding domain of 8cI protein and the target library is fused to the "-subunit of RNA-polymerase. Interaction between bait and target allows recruitment of transcriptionally

Figure 4: Stratagene Bacteriomatch[tm] bacterial two hybrid system. Schematic describing the principle behind the bacterial two hybrid system used to identify proteins that interact with TRIM32. The bait constructs used contained either an N-terminal or C-terminal portion of TRIM32 and the target library was derived from rat skeletal muscle. Figure was modified from a promotional figure obtained from the Stratagene websitewww.stratagene.com/homepage/.

active complexes to promoters containing 8cI binding sites. A reporter episome is present with both $-galactosidase and $-lactamase under the control of 8cI sites and interaction can be detected by breakdown of X-gal and resistance to carbenicillin. Selection for the presence of the two plasmids and the reporter episome is accomplished by antibiotic resistance: chloramphenicol on the bait plasmid (pBT), tetracycline on the library plasmid (pTRG XR), and kanamycin on the reporter episome. A human cDNA library for BacteriomatchTM was unavailable so a rat library was used. It is composed of pooled Sprague-Dawley rat skeletal muscle cDNA cloned unidirectionally into pTRG XR.

Screening constructs included two TRIM32 fusions consisting of the N-terminal portion (amino acids 1-144, RING finger and B-Box Zn finger) or the C-terminal portion of TRIM32 (amino acids 364-653, consists of six NHL domains)(see section 3.7 for creation of these two plasmids). These areas of the protein were chosen due to preliminary results with whole TRIM32 constructs suggesting a high degree of non-specific interactions, possibly via the central coiled-coil domain. Two screens were performed, one for each TRIM32 construct, using both carbenicillin resistance and X-Gal breakdown due to the high background levels. Once putative positives were grown and plasmid isolated (Qiagen miniprep) they were sequenced as previously described and re-introduced into the B2H system for confirmation of interaction. Unfortunately due to the nature of Bacteriomatch,™ the differences between control positives and control negatives were too small to use this system for reliable two protein interaction analysis.

4. Identification and characterization of the LGMD2H gene

The data related to the identification of the LGMD2H gene have been published and forms the basis of ongoing work involving the characterization of the LGMD2H gene product:

- ***P Frosk**, T Weiler, E Nylen, T Sudha, CR Greenberg, K Morgan, TM Fujiwara, K Wrogemann. Limb-girdle muscular dystrophy type 2H associated with mutation in TRIM32, a putative E3-ubiquitin-ligase gene. Am J Hum Genet 2002; 70(3):663-672.*

The subjects discussed within this chapter have also been the topic of two oral presentations, the first was a platform session at the 10th International Congress on Neuromuscular Disorders (Vancouver, BC) and the second was an invited lecture at the 7th International Congress of the World Muscle Society (Rotterdam, The Netherlands). The abstracts can be found in the following journals:

- ***P Frosk**, T Weiler, E Nylen, T Sudha, K Morgan, TM Fujiwara, CR Greenberg and K Wrogemann. Limb Girdle Muscular Dystrophy Type 2H (LGMD2H) is Associated with Mutation in TRIM32. (2002) J Neurol Sci 199, Suppl.1:S90-S90 (**Speaker, PF**).*
- *K Wrogemann, **P Frosk**, R Lamont, L Brunham, A Hoke, T Weiler, E Nylen, D Frappier, N Roslin, T Hudson, K Morgan, TM Fujiwara, and CR Greenberg. TRIM 32, another new gene in limb girdle muscular dystrophy, codes for a putative E3 ubiquitin ligase. (2002) Neuromuscular Disorders 12, 718 (**Speaker, KW**).*

I would like to acknowledge the contributions of the following individuals: (1) Ted Nylen and Dr. Tracy Weiler for the localization of LGMD2H to chromosome 9 and determination of some genotypes, (2) Tess Laidlaw and Dr. Sudha Thangirala for determination of some genotypes, (3) Dr. Ken Morgan and Mary Fujiwara for help with the preparation of the American Journal of Human Genetics manuscript, and (4) Alyssa Tennese for help with the bacterial two hybrid system screening.

4.1 Introduction

The Hutterites, an anabaptist religious sect, live on farming colonies located predominantly in the Prairie Provinces and Great Plains of North America. They constitute a unique religious and genetic isolate. They immigrated to North America from Europe in the 1870s and formed three subdivisions; Schmiedeleut, Dariusleut, and Lehrerleut. There has been very little intermarriage between subdivisions.[126] The ancestry of the overwhelming majority of the Hutterites can be traced back to 89 ancestors.[193] The current population of Hutterites in Canada alone is approaching 30,000 (2001 Canadian Census; http://www.statcan.ca/) due to the high proportion of families with many children. Not unexpectedly, largely due to founder effect, there is an elevated prevalence of autosomal recessive disorders in the Hutterites.[127]

LGMD is one of the disorders that is over-represented in this population. Current estimates, based solely on the number of ascertained patients, exceed 1/400. LGMD in Manitoba Hutterites was originally described in 1976 by Shokeir and Kobrinsky[232] and then again in Saskatchewan Hutterites in 1985 by Shokeir and Rozdilsky.[233] Due to the nature of the population it was presumed this was one disorder caused by the high frequency of one particular allele. More recently, a study was undertaken by our laboratory to locate the mutant gene by linkage analysis using 18 patients and their families (a subset of which were described in the Shokeir and Kobrinsky paper).[278] A novel locus of 6.4 Mb[139] was identified at chromosome region 9q31-33, using a limited pooled-DNA approach to scan the genome, and was named LGMD2H.[279]

I joined the laboratory at this point and was given the task of identifying the causative gene of LGMD2H. We were able to identify additional affected Hutterite families that allowed us to reduce the size of the region substantially. A combination of bioinformatic and PCR-based approaches led to the localization of four genes within this region that we screened for potential disease-causing mutations. We were able to identify *TRIM32* as the most likely gene for LGMD2H. Characterization of this gene and its product suggests that it exists within cytoplasmic bodies and may function in the ubiquitin proteasome pathway.

4.2 Results

(A) Subjects

We studied 35 clinically affected Hutterite patients and members of their immediate families for the purposes of the work reported here (Figure 5). A further 17 affected individuals and their families have been studied since this work was completed for a total of 52 individuals affected with LGMD2H. Genotypes were obtained for 133 individuals, who comprise eleven interrelated families (one to four generations) with 1 to 20 family members affected with LGMD2H. The families are from two of the three subdivisions of the Hutterites; 9 families are Schmiedeleut (A-F, I-K) and 2 families are Lehrerleut (G and H). The Hutterites keep meticulous genealogical records going back to before they came to North America and these records are published for general use. Most individuals can be included in one large, complex pedigree that extends back to the mid-eighteenth century based on information from one of these genealogical records (Schmiedeleut).[105,281] Note that we do not have access to the Lehrerleut genealogical record and as such have not been able to include pedigrees G and H (Figure 5) into a comprehensive pedigree.

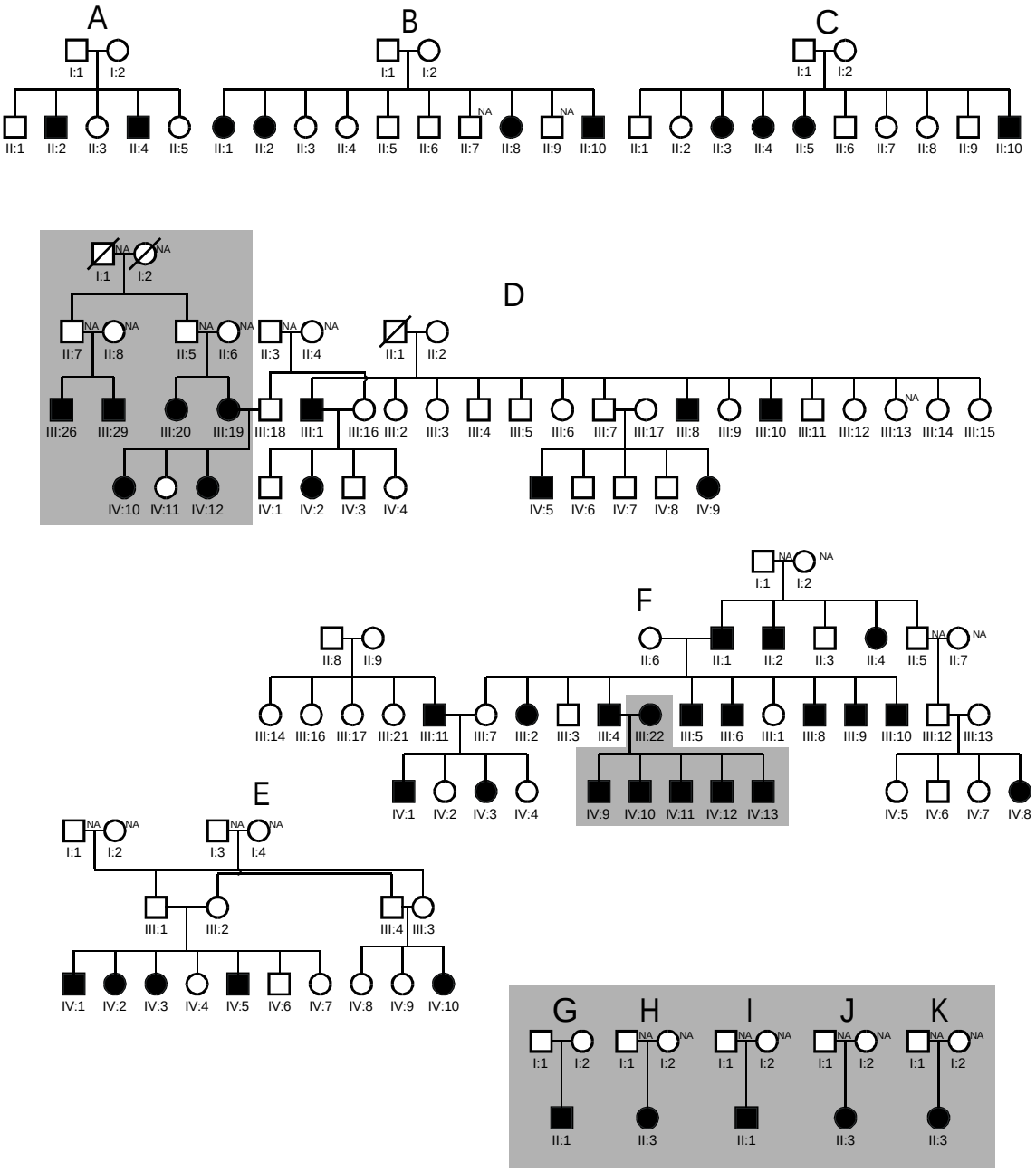


Figure 5: LGMD2H pedigrees. Each pedigree is assigned an identifier (A-K) and individuals are numbered according to generation. Birth order has been randomized to protect anonymity. Individuals denoted with an NA were not available for study. Individuals highlighted by a grey background had not yet been ascertained at the time of the identification of the LGMD2H gene. However, subsequent haplotyping did not reveal any new information.

Eight CEPH families with recombinations in chromosome region 9q3 (Dr. S. Povey, Personal Communication) were used to help define marker order. CEPH individuals 1331-01, 1331-02, 1347-01, and 1347-02 were used to size microsatellite alleles (CEPH website, http://www.cephb.fr/). In addition 100 healthy control individuals of diverse origin were used to determine allele frequencies.

(B) Haplotype analysis

Haplotype analysis was used to both define the order of markers used and fine map the candidate region. Approximately 6000 genotypes were obtained for markers on chromosome 9. Genotypes for our LGMD study population and selected CEPH individuals were used to construct haplotypes (only key recombinants are shown in Figure 6). Three recombination events were identified in individuals E-IV:4, E-IV:5 and D-III:9, reducing the candidate region from 6.5 Mb to an interval of 713 kb flanked by *D9S241* and *D9S931* (Figure 6). The region was further reduced to a 560 kb region flanked by *D9S1126* and *D9S737* based on two inferred ancestral recombination events (F-II:9 and D-III:17)(Figure 6).

(C) Physical mapping

Early physical mapping efforts used a combination of Human Genome Project sequences and radiation hybrid mapping.[151] The efforts reported here focussed on contiguous sequence obtained from the Celera Genomics web site (GA_x54KRCDB2PK: 3500001-4500000).[273] Four genes are present in the region, which spans 560 kb of DNA (Figure 7). *PAPPA* (NM_002581.1) encodes pregnancy-associated plasma protein A (also known as insulin-like growth factor binding protein 4 [IGFBP4] protease). It is found at a

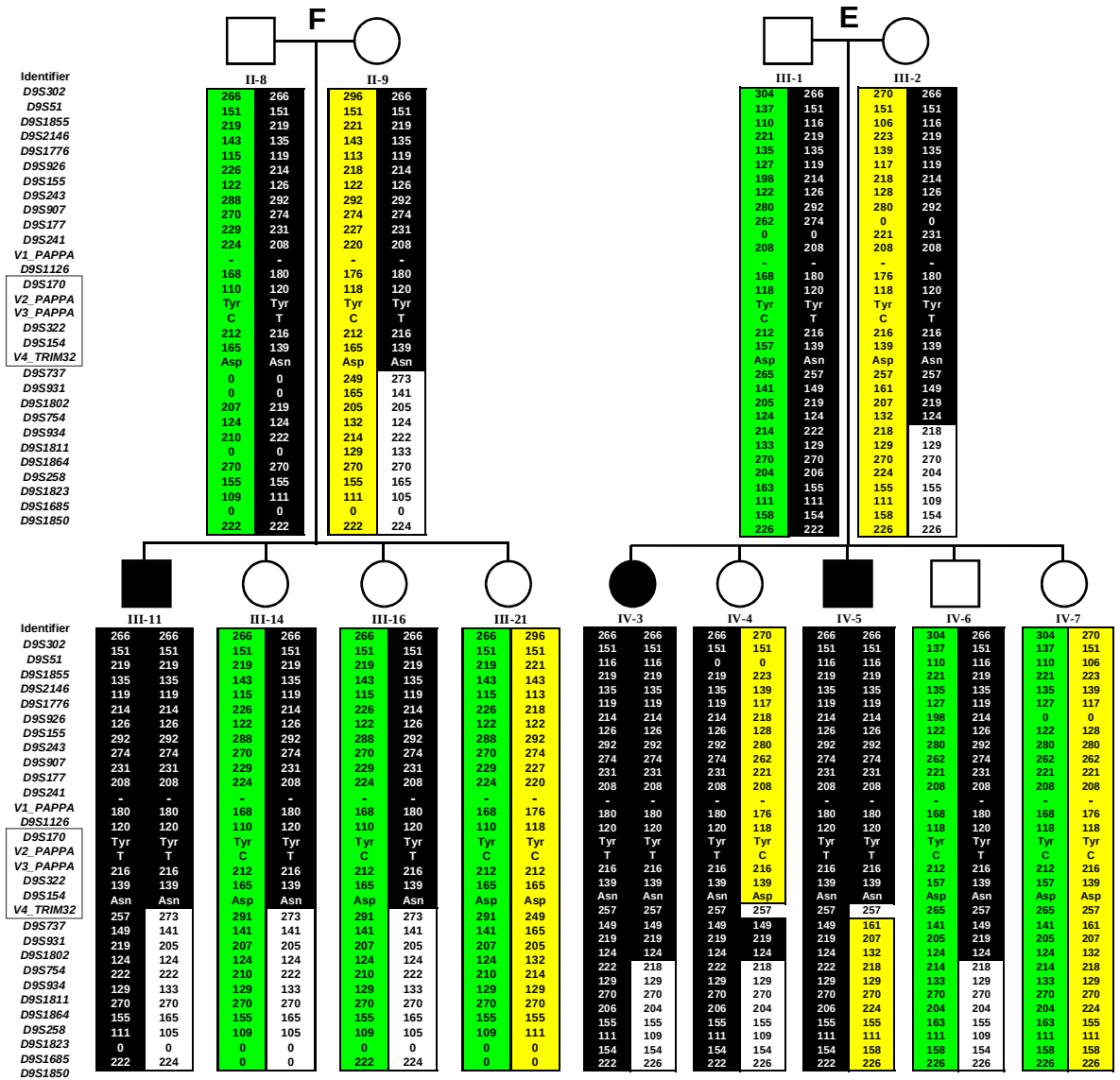
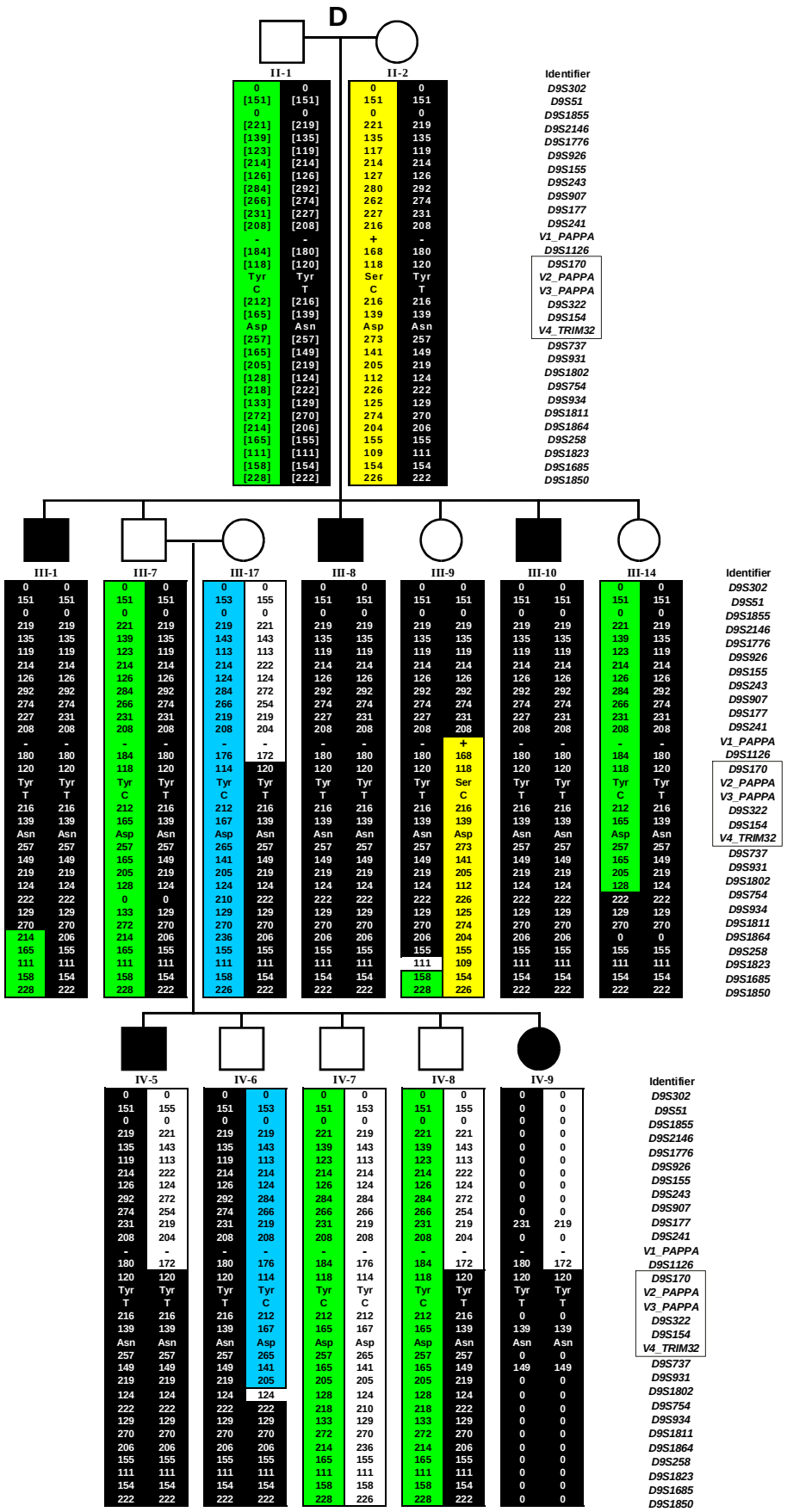


Figure 6: Chromosome region 9q31-33 haplotypes. Recombinations are evident (E IV:4, E IV: 5, and D III:9) that reduce the 6.5 Mb *LGMD2H* candidate region flanked by *D9S302* and *D9S1850* to a 713 kb region defined by *D9S241* and *D9S931*. Inferred ancestral recombinants in individuals F II:9 and D III:17 further reduce the region to an interval of 560 kb flanked by *D9S1126* and *D9S737* (region enclosed by the open rectangle). Alleles are given in bp, 0 indicates no data. Black background on haplotypes indicates consensus disease-associated alleles, white indicates non-consensus disease associated alleles, and yellow, green and blue indicate a normal parental chromosome. Alleles enclosed in square brackets are inferred, alleles without an outer vertical border indicate that the allele is uninformative for a crossover. See table 7 for the alleles of V1-V4. See next page for pedigree D.


D
II-1
II-2
Identifier
D9S302
D9S51
D9S1855
D9S2146
D9S1776
D9S926
D9S155
D9S243
D9S907
D9S177
D9S241
V1_PAPPA
D9S1126
D9S170
V2_PAPPA
V3_PAPPA
D9S322
D9S154
V4_TRIM32
D9S737
D9S931
D9S1802
D9S754
D9S934
D9S1811
D9S1864
D9S258
D9S1823
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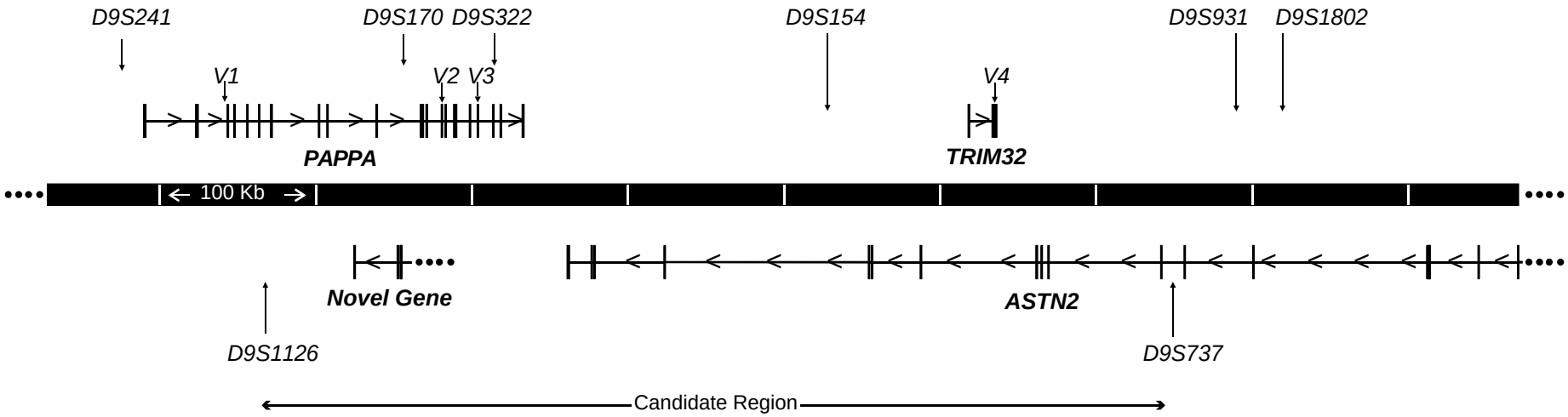


Figure 7: Physical map of the *LGMD2H* candidate region. The region is delineated by D9S1126 and D9S737. The four sequence variations identified in patient samples are labelled V1-V4 as in table 7. The map shows 1Mb and is based on contiguous sequences obtained from NCBI and Celera Genomics.

high concentration in the serum of pregnant women.[52,157,235] The IGFBP4 protease substrate, IGFBP4, is known to be a potent inhibitor of the IGF signaling system and PAPPA appears to prevent this inhibition. *ASTN2* (astrotactin 2, AF116574.1) encodes a paralog of astrotactin 1, which is a mediator of neuronal-glial contact.[72] *TRIM32* (NM_012210.2), previously known as *HT2A*[89] encodes a member of the tripartite motif family of proteins. It was originally discovered using a yeast two-hybrid assay with the human immunodeficiency virus type 1 Tat acting as bait.[89,212] The RING finger domain suggests that this protein is an E3 ubiquitin ligase. Sequencing of orphan expressed sequence tag (EST) clones revealed a putative fourth gene in the region. The 5' end of this gene is still undefined but the 3' end consists of at least four exons found in the cDNA clone IMAGE:2591170 (AF492356.1). Attempts to amplify and sequence the 5' end of this gene have been unsuccessful due to the apparent rarity of the transcript. RT-PCR suggests that this transcript is specific to lung and salivary gland (two tissues not used in the original attempts to clone the 5' end). However, this was not followed up due to the lack of expression in muscle and the mutation screening results of the other genes (data not shown).

(D) Mutation screening

All four genes were considered as potential candidates for LGMD2H based on their chromosomal position. Both SSCP analysis and DNA sequencing were used to search for disease-causing mutations. SSCP of exons located between the flanking microsatellite markers *D9S1126* and *D9S737* revealed only one silent substitution (V3) in *PAPPA*. DNA sequencing of the candidate gene exons shown in Figure 7 revealed another three sequence variations (V1, V2, and V4). We were unable to detect V2 and V4 when studied by SSCP

in our initial screen. However subsequent amplification in a smaller size PCR product allowed visualization of both alleles of V2 and V4 by SSCP. The four sequence variations, an 11 bp deletion and three point mutations, were identified on the basis of differences between sequences from an affected individual and published sequences (Table 7).

The four potential mutations were assayed for as follows (Figure 8): **V1**-Genomic DNA was amplified using primers PAPPA_ex3F and PAPPA_ex3R. The 11 bp deletion was readily visible as a shift in fragment size (291 bp to 280 bp) after radiolabelling (as per microsatellite protocol in chapter 3) and electrophoresis on a sequencing gel. **V2**-Genomic DNA was amplified using primers designed with a mismatch, P14_BsmA1F and P14_BsmA1R. P14_BsmA1R creates a *Bsm*A1 restriction site in all fragments while P14_BsmA1F creates a BsmA1 site only in the V2(Ser) allele. Restriction enzyme digestion of the V2(Ser) allele with BsmAI yields three fragments (94, 24 and 27 bp) whereas the V2(Tyr) allele has two fragments (118 and 27 bp). **V3**-Genomic DNA was amplified using primers PAPPA_ex18F and PAPPA_ex18R. *Hpa*II digestion of the V3(C) allele yields two fragments (162 and 63 bp) whereas the V3(T) allele migrates as a single fragment of 225 bp. **V4**-Genomic DNA was amplified using primers HT2A_ex2(5)F and H2_HpyR. H2_HpyR was designed with a mismatch that creates an *Hpy*99I restriction site only in the V4(Asp) allele. Digestion of the V4(Asp) allele yields two fragments (136 and 16 bp) whereas the V4(Asn) allele migrates as a single fragment of 152 bp.

V1, an 11 bp deletion in intron 2 of *PAPPA* can be ruled out as the disease-causing mutation because it does not segregate with LGMD2H (Figure 8) and is outside the candidate

Table 7: Sequence variations at the *LGMD2H* locus and control allele frequencies

Mutation	Gene (location)	Sequence variation[a]	Variation type	Controls	
				Genotype[b]	No.
V1	*PAPPA* (intron 2)	c.(1479-59)-(1479-49)del	Deletion	+/+	27
				+/-	50
				-/-	23
V2	*PAPPA* (exon 14)	c.3671C>A, p.S1224Y	Transversion, missense	Ser/Ser	8
				Ser/Tyr	33
				Tyr/Tyr	59
V3	*PAPPA* (exon 18)	c.4374C>T, p.C1458C	Transition, silent	C/C	96
				C/T	4
				T/T	0
V4	*TRIM32* (exon 2)	c.1459G>A, p.D487N	Transition missense	Asp/Asp	100
				Asp/Asn	0
				Asn/Asn	0

[a]Numbering of nucleotides and amino acids is based on GenBank NM_002581.1 for PAPPA and NM_012210.2 for TRIM32; the A of the initiator Met codon is considered as nucleotide +1.

[b]For V1, + and – denote the presence or deletion of 11 bp, respectively; for V2 and V4, alleles are denoted by amino acid codes; for V3, alleles are denoted by nucleotides.

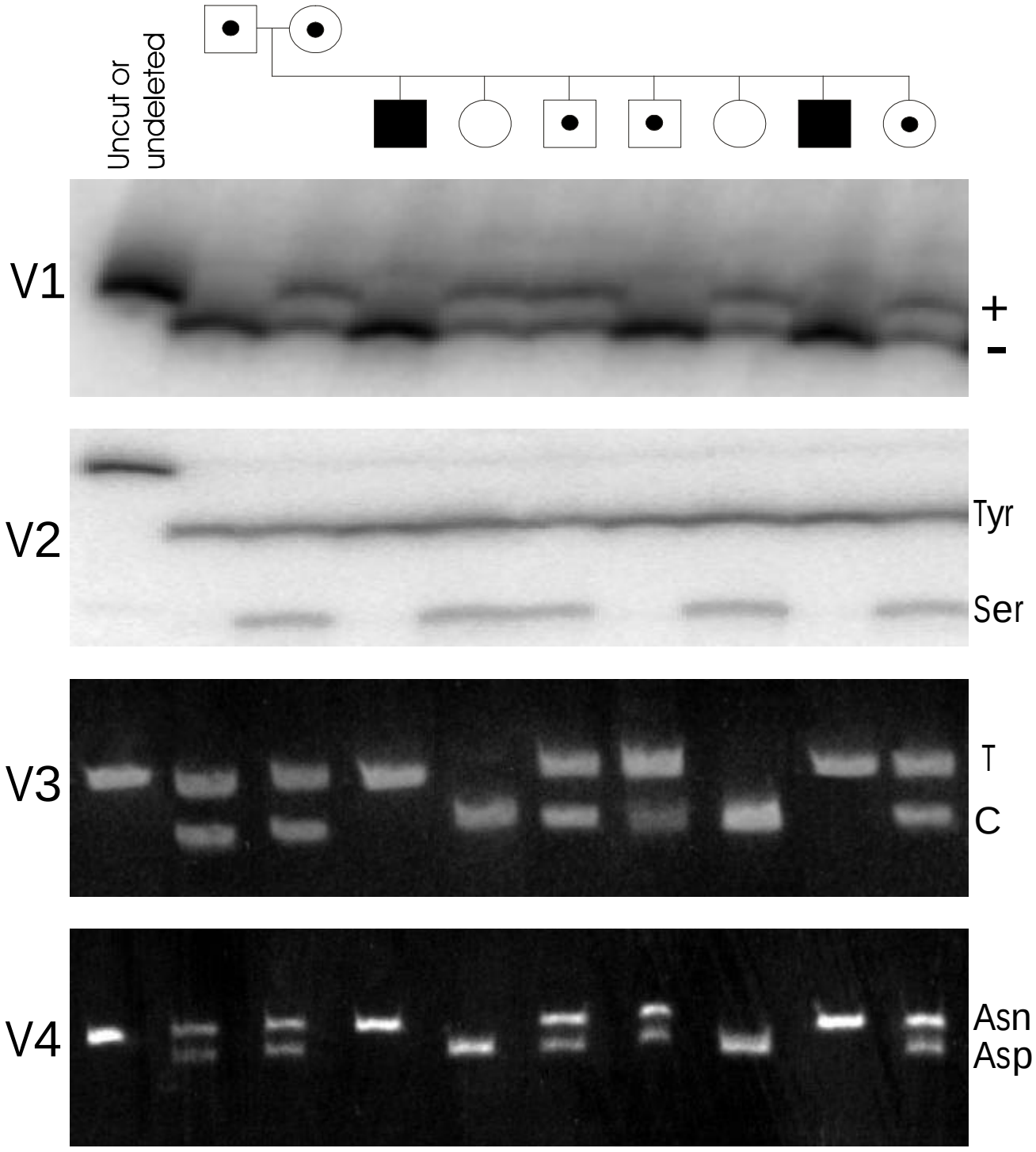


Figure 8: Segregation of the four LGMD2H variants. V1 is a simple size difference whereas the assays for V2-V4 are based on restriction fragment lengths. The nucleotide change in V2-V4 abolishes a restriction site in all cases leading to a larger fragment. Note a secondary control site is present on the V2 fragment leading to a larger uncut control. See text for descriptions of each assay.

region as defined by an inferred ancestral recombination event (D-III:17, Figure 6). Both affected and unaffected family members were homozygous for the deletion and it was common among controls (Figure 6 and Table 7). V2, a missense mutation in codon 1224 of *PAPPA*, can also be ruled out as a disease-causing mutation. All affecteds, and all but one of the obligate carriers (D-II:2, Figure 6) are homozygous for the V2(Tyr) allele. Both alleles of V1 and V2 are common in Hutterite families and controls.

V3 results in a silent substitution in codon 1458 of *PAPPA*. The V3(T) allele was found to have a frequency of 2% in controls. As the V3 mutation does not change the amino acid sequence of PAPPA, it could be disease-causing if it leads to alternate splicing or the codon usage of the V3(T) allele is significantly different. However, codon usage does not appear to be a factor as the two coding triplets are used at similar frequencies in humans; UGC accounts for 55% and UGU accounts for 45% of all cysteine codons (GenBank Version 123.0). Alternative splicing is unlikely because *in silico* analysis of the splice consensus sequences shows no predicted change with the introduction of V3(T) (NIX package, http://www.hgmp.mrc.ac.uk). RT-PCR was used to confirm this prediction by amplifying the region containing V3 (*PAPPA* exons 17-19) from both affected and unaffected RNA isolated from cultured skin fibroblasts. No differences were apparent between the affected and the unaffected samples (data not shown).

V4 results in a nonconservative substitution of an acidic aspartate by a neutral asparagine at amino acid 487 of TRIM32. The V4(Asn) and V3(T) alleles are in complete linkage disequilibrium in the Hutterite families. The V4(Asn) allele was not observed in any of the controls. All affected individuals diagnosed with LGMD2H are homozygous for

V4(Asn) and V3(T). There were six asymptomatic individuals who were also homozygous for these alleles and the disease-associated chromosome. Four of these individuals are under 20 years of age which is consistent with our previous findings that the onset of LGMD2H is age dependent.[279] The other two individuals are in their 30s and do not meet the strict criteria for affected status previously established by our group for the purposes of linkage analysis but do show signs suggestive of an LGMD (mildly elevated SCK of 1.5-2X maximum normal and chronic fatigue).[278]

The V4 amino acid substitution occurs within the third NHL domain of TRIM32. NHL domains are conserved motifs found in many prokaryotic and eukaryotic proteins. The domain was named after the first three proteins in which they were identified; NCL1, HT2A (TRIM32), and LIN-41.[237] This missense mutation occurs at one of the two most highly conserved positions in this domain (Table 8).

(E) Tissue specific expression

LGMD2H is a disease mainly restricted to skeletal muscle. Evidence from ESTs (UniGene Hs.236218) suggests the *TRIM32* transcript is expressed in a wide variety of tissues, including skeletal muscle. In order to confirm the presence of the transcript in skeletal muscle, we probed a multiple-tissue northern blot (Origene) with a 733 bp fragment obtained from the coding sequence of *TRIM32*. The results shown in Figure 9 indicate that the transcript is indeed widely expressed and, in particular, is expressed in skeletal muscle.

In order to confirm that there was protein expression, anti-TRIM32 antibodies were necessary. Antibodies were generated in collaboration with Dr. Jody Berry (Staff

Table 8: Alignment of selected NHL domains from nine eukaryotic species

Source	Amino Acid	
	Position	Sequence
Consensus sequence	1–27	FDYPRGVAVDSDGO-IVVADSE--N
Species and protein[a] (GenBank accession number):		
Homo sapiens:		
TRIM32 (NP_036342.1)	371–398	FNLPVSLYVTSQGE-VLVADRG--N
	428–455	NLTPLSVAMNCQGL-IGVTDSY--D
	469–496	LSKPWGITALPSGQ-FVVTDVE--G
	520–550	TCDAEGTVYFTQGLGLNL-ENRQNE
	575–602	FRCIAGMCVDARGD-LIVADSS--R
	616–643	LTCPVGIALTPKGQ-LLVLDCW--D
PAM (NP_000910.1)	683–711	FTVPHSLALVPLLGQLCVADRE--N
TRIM2 (NP_056086.1)	534–561	FTNLQGVAASTNGK-ILIADSN--N
TRIM3 (NP_006449.1)	533–560	LQRPTGVAVDTNGD-IIVADYD--N
Caenorhabditis elegans:		
NCL1 (AAC14263.1)	586–613	FTEPSGVAVNGQGD-IVVADTN--N
	633–662	LLYPNRVAVNRTTGDFVVTERS--P
	677–704	LQHPRGVCVDSKGR-IIVVECK--V
	720–747	LEFPNGVCTNDKNE-ILISDNR--A
LIN-41B (AAF15530.1)	763–791	TNYPIGVGINSLGE-VVVADNH-NN
	845–872	LCRPWGICVDQRGR-VIVADRS--N
	892–919	FDRPAGITTNSLNN-IVVADKD--N
	939–966	FNYPWGVATNSHNA-IAVSDTR--N
	987–1014	LDSPRGLCYLPDGQ-LLITDFN--N
	1035–1062	FVRPQGVVIDPEGH-ILVCDSR--N
	1120–1147	LDRPTDLAVGPDGR-IYVVDFG--N
YMB4 (CAA79562.1)	712–739	LNWPRGICALSGGL-VATCDSS--N
F26F4.6 (AAA91222.1)	761–788	LHCPSGFCLSDTDD-ILIADTN--N
F21F3.1 (AAB42278.1)	279–307	FVVPHSLSLIEDMNIVCVADRE--N
PAM (AAB37637.1)	424–451	FYLPHGIYVDKDGF-VYTTDVG--S
Mus musculus:		
Pam (NP_038654.1)	581–608	FYLPHGLSIDTDGN-YWVTDVA--L
Trim3 (BAA83343.1)	713–740	LYGPQGLALTSDGH-VVVADAG--N
Rattus norvegicus:		
Pam (NP_037132.1)	581–608	FYLPHGLSIDTDGN-YWVTDVA--L
Trim3 (AAC17997.1)	486–513	FTNLHPLSAASSGR-IVVADSN--N
Bos taurus PAM (AAA30683.1)	779–806	FDMPHDIAASEDGT-VYVGDAH--T
Equus caballus PAM (BAA06104.1)	682–710	FRVPHSLALVPHLGQLCVADRE--N
Xenopus laevis XELCAM (AAA49667.1)	582–610	FRIPHSLTMISDQGQLCVADRE--N
Drosophila melanogaster:		
brat (AAF53771.1)	780–807	FTEPSGVAVNAQND-IIVADTN--N
dpld (AAF59245.1)	537–564	VSRPWGLCVDKMGH-VLVSDRR--N
cg12130 (AAF58870.1)	387–415	FQNPHDVAVTADGNEIYVAELN--P
Lymnaea stagnalis LPAM (AAD42258.1)	1578–1605	YFMPHGIEVDNQGN-LWLTDVA--L

[a] Each protein has up to six NHL domains (InterPro accession number IPR001258); however, only one domain from each protein is shown, except for the three after which the NHL domain was named (i.e., NCL1, HT2A [TRIM32], and LIN-41). There are six domains from TRIM32, and the mutation (i.e., D487N) is in the third NHL domain, at position 20, denoted by the underlined letter in each sequence. This aspartate is one of the most highly conserved amino acids among the NHL domains. There are currently 110 eukaryotic NHL domains in the InterPro database; of these, at position 20, 100 have an aspartate, 9 have a glutamate, and 1 has a lysine. InterPro URL: www.ebi.ac.uk/interpro/

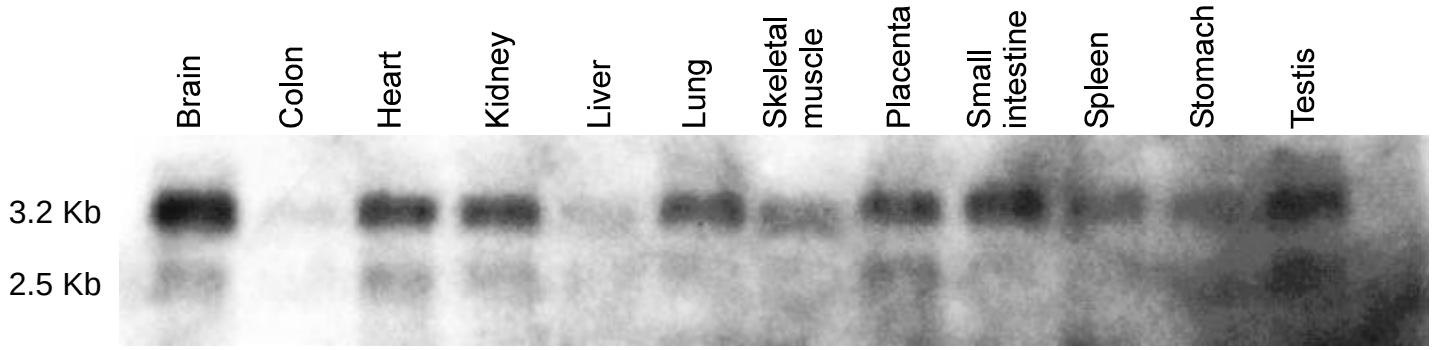


Figure 9: Tissue distribution of the *TRIM32* transcript. Multiple human tissue northern blot analysis using a fragment of the *TRIM32* coding sequence as probe. *TRIM32* shows two transcript sizes, 3.2 Kb and 2.5 Kb. These two mRNAs are produced by alternative polyadenylation as shown by the ESTs R42491(3'end of short transcript) and BX091494 (3'end of long transcript). The larger 3.2 Kb transcript appears to be the major form in all the tissues studied. RNA expression is evident in skeletal muscle, but many other tissues show higher levels.

Immunologist, Canadian Food Inspection Agency) using established protocols.[149,201,284] A 16-mer peptide (C-ASPRASPAKQRGPEA) was synthesized for immunization of BALB/c mice. The TRIM32 protein has, from the N to C terminal end, a RING finger domain, a B1 box, a coiled-coil domain, and six NHL domains (Figure 10). The sequence used for immunization corresponds to residues 334-348 of TRIM32 with an N-terminal cysteine for conjugation to a carrier protein (Figure 10). This portion of the protein is just N-terminal to the first NHL domain and is relatively well conserved in TRIM32 from different species but does not show much conservation with the rest of the TRIM family (Figure 11). A mouse polyclonal antibody in ascites was prepared and affinity purified using the immunization peptide conjugated to a different carrier protein (keyhole limpet hemocyanin for immunization, bovine serum albumin for purification). This purified preparation showed reactivity against a band at approximately 72 kDa in bacterial and mammalian cell extracts expressing recombinant TRIM32 (with or without a 6XHis tag, Figure 12). As an indication of specificity, this band could be blocked by the preadsorbtion of the antiserum with 200 ：M of the 16-mer peptide used for mouse immunization.

A multiple-tissue immunoblot using the above preparation was attempted (Figure 13). No bands corresponding to TRIM32 were detected suggesting that our antibody preparation is not sensitive enough to detect endogenous levels of protein via this method. We were able to obtain small amounts of another anti-TRIM32 antiserum in order to repeat this experiment. The serum was raised against whole recombinant protein and was used in a previously published manuscript.[124] While messy, this blot did show the presence of a band that corresponds to TRIM32 in brain, heart, skeletal muscle, and possibly placenta and testis.

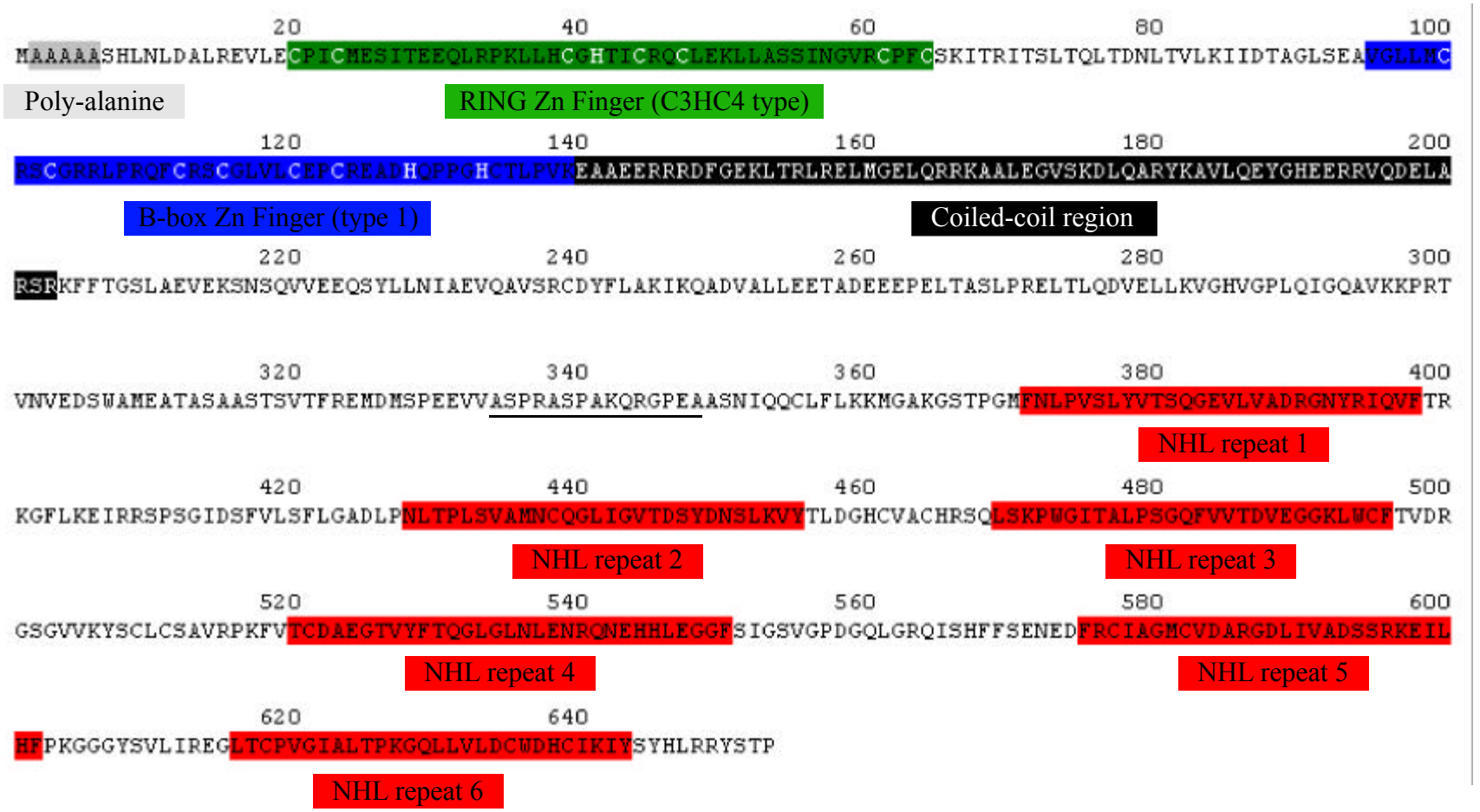


Figure 10: TRIM32 protein sequence. Important domains are highlighted by various colours and labelled beneath. Domains were delineated using the InterPro database, /www.ebi.ac.uk/interpro/. Note that for the two Zn fingers the key Zn chelating residues are in white. The underlined portion of the protein corresponds to the peptide used for producton of our anti-TRIM32 antibody.

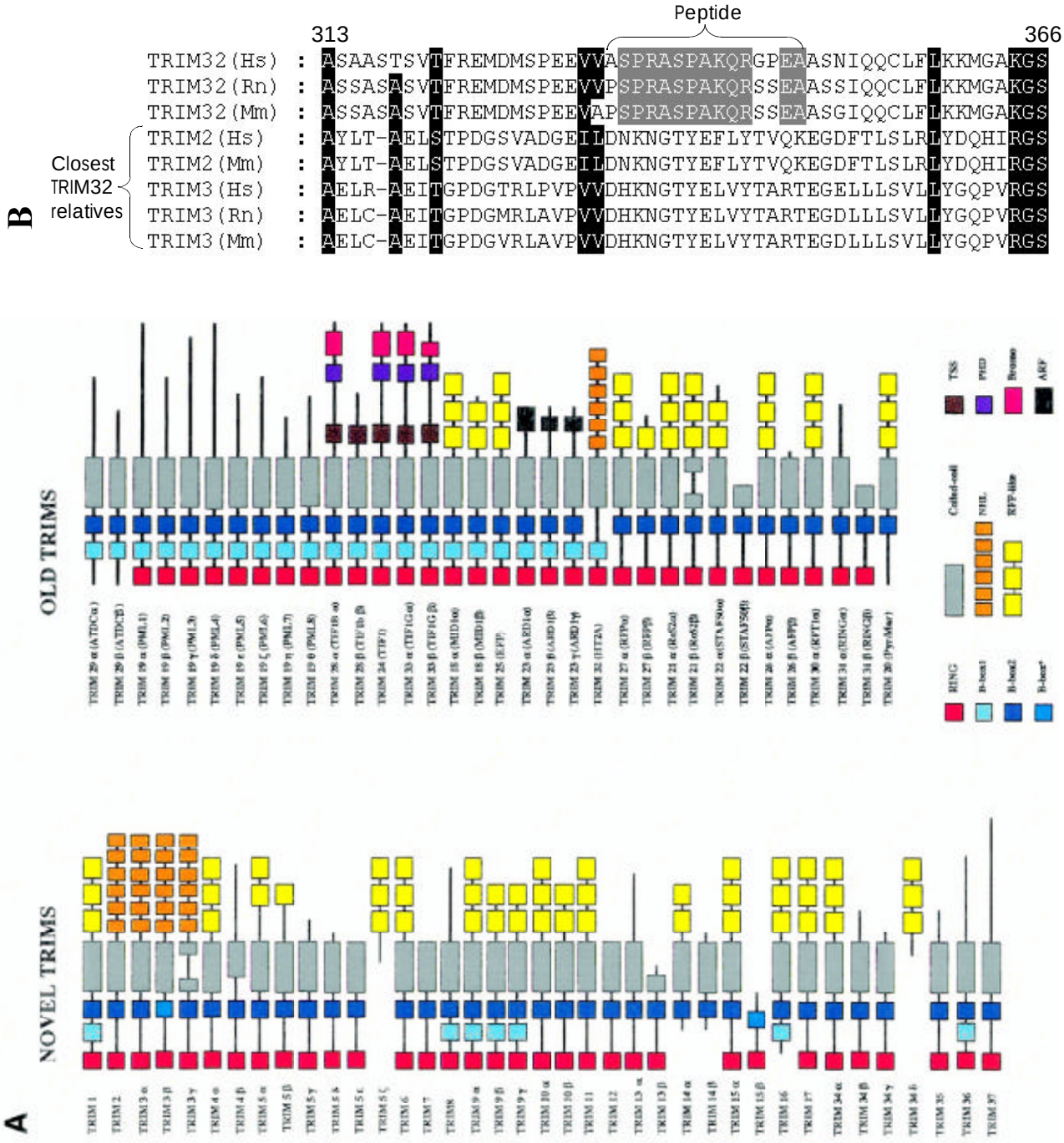


Figure 11: (A) The TRIM family of proteins. Modification of a figure presented in Reymond et al. (2001).[212] Each member contains the tripartite motif (RING finger, B-Box Zn Finger and Coiled-coil domains) and many have additional C-terminal domains such as NHL repeats (TRIM2, TRIM3 and TRIM32). (B) Alignment of a small section of TRIM32 (aa 313-366) with its orthologues in mouse and rat as well as its paralogues, TRIM2 and TRIM3. The immunogenic peptide (C-ASPRASPAKQRGPEA) is well conserved amongst the three TRIM32 sequences but not in the other TRIM familymembers.

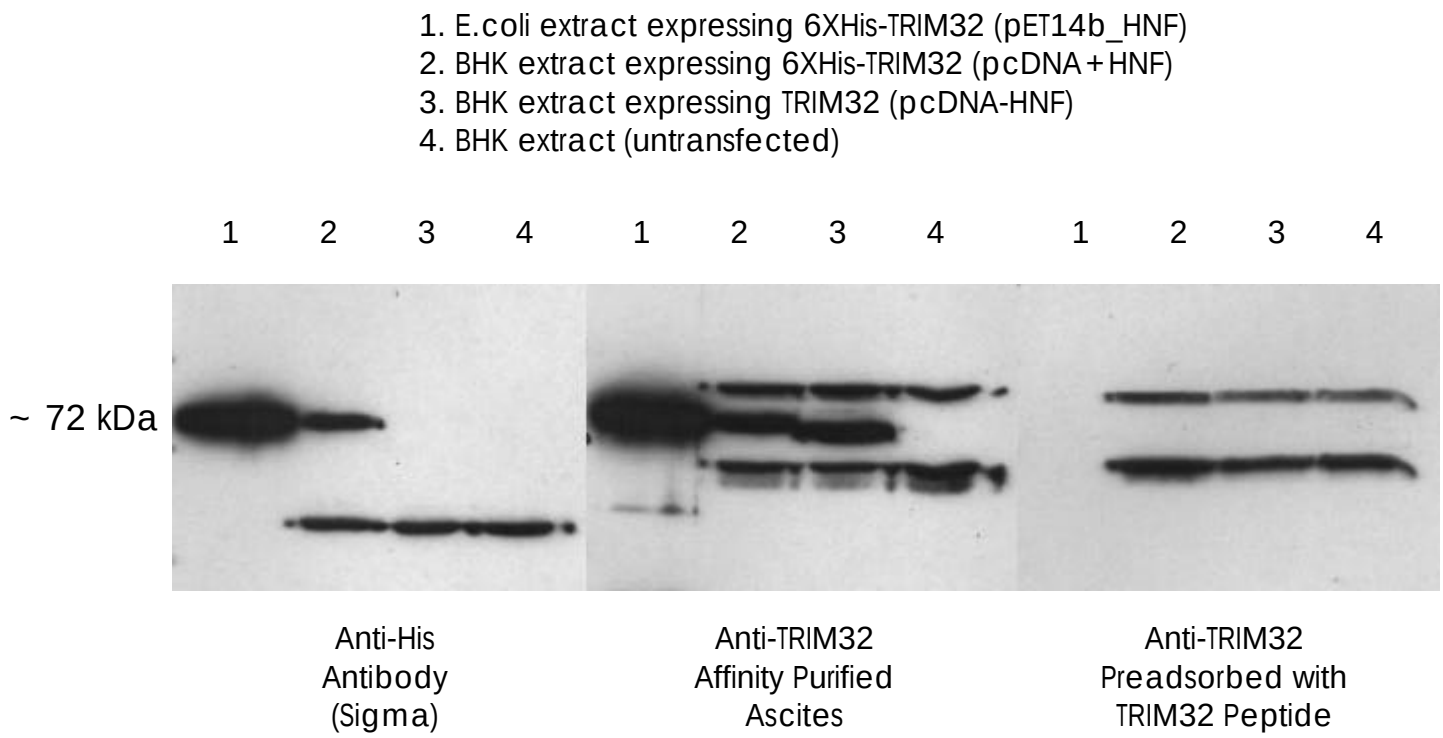


Figure 12: Specificity of anti-TRIM32 antibody by immunoblotting (A) Immunoblots of *E.coli* and mammalian cell protein extracts showing recognition of recombinant TRIM32 by affinity purified ascites (recognition is blocked by synthetic TRIM32 peptide).

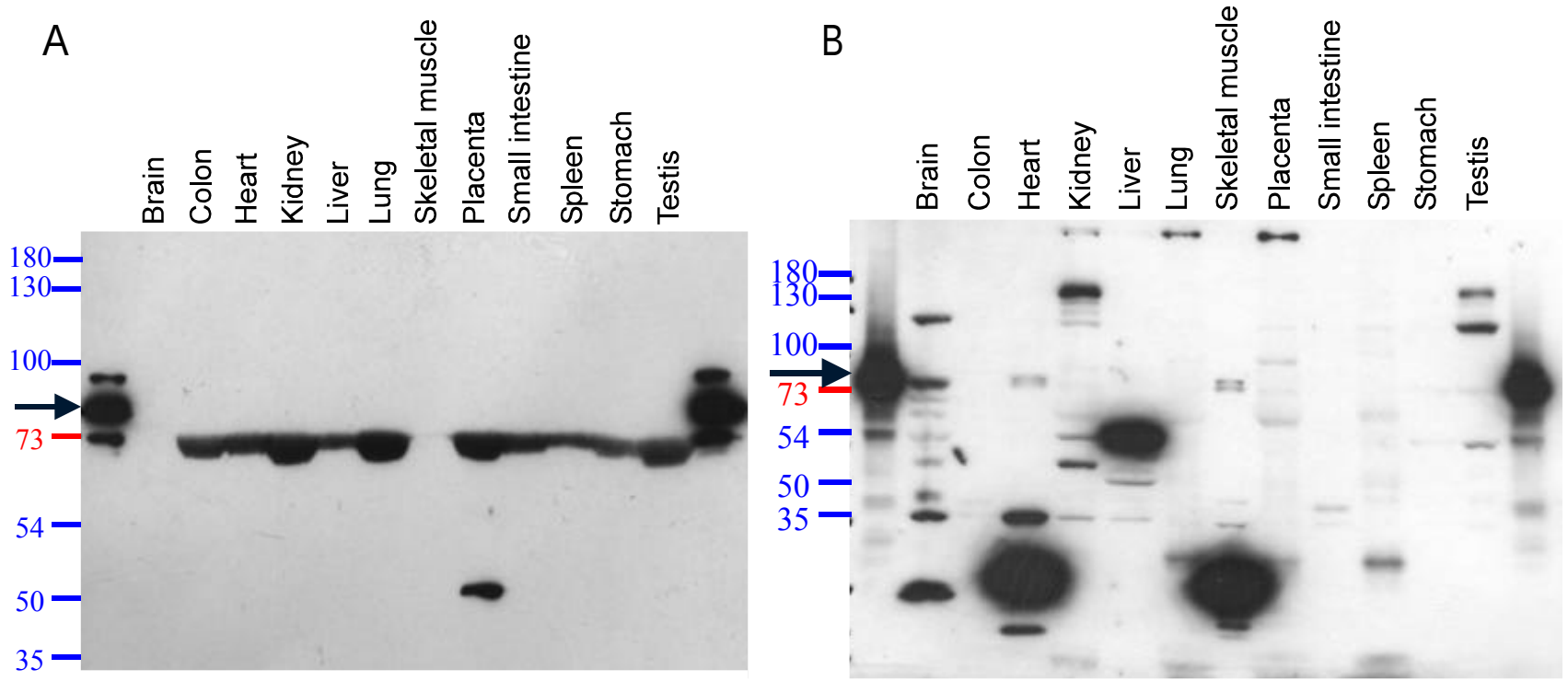


Figure 13: Multiple tissue immunoblot for TRIM32. (A) Immunopurified anti-TRIM32 (anti-peptide) was used at 1/100 (same preparation as shown in figure 12). Tissue samples are arranged identical to the northern blot shown in figure 8 with TRIM32 transfected BHK cell extract loaded on either side as a control (25 µg of each sample is loaded). The appropriate size for TRIM32 is marked with an arrow. The antiserum contains mild reactivity to albumin due to the purification process, hence the strong band migrating at ~70 kDa. No band corresponding to TRIM32 appears to be detected. Note the SDS-PAGE was performed on a 7.5% gel. (B) Similar blot using a whole protein anti-TRIM32 rabbit antiserum at 1:500 kindly provided by collaborators (same as was used in Horn et al 2004). A band with the correct size of monomeric TRIM32 was detected in the tissues with the highest levels of mRNA expression. TRIM32 migrates at ~80 kDa when measured against a prestained protein ladder but at ~72 kDa when using an unstained ladder (data not shown), reflecting the inaccuracy of prestained ladders. Note the SDS-PAGE was performed on a 4 -15% gradient gel.

These were the tissues that showed the highest levels of TRIM32 mRNA by northern blot. Numerous other bands were detected, most of which were probably unrelated cross-reacting proteins. It is not known if any of these other bands are alternate forms of TRIM32.

(F) Subcellular localization

Localization studies of the TRIM32 protein are important for understanding its function and for designing future experiments. The missense mutation found in LGMD2H patients may very well affect the localization as a result of misfolding or failure to interact with normal partners in the cell. The TRIM32 sequence does not contain any localization signals and is predicted to be a cytoplasmic protein.

Immunofluorescence, largely in cell culture, was used to determine the subcellular localization of TRIM32. The mouse anti-TRIM32 preparation discussed above appears to be both sensitive and specific when used to detect endogenous TRIM32 via immunofluorescence (Figure 14). TRIM32 appears to be present mainly in the cytoplasm in a diffuse punctate pattern. This agrees with previous observations on cells transfected with GFP-tagged TRIM32 constructs.[212] TRIM32 staining is not disrupted in patients cells compared to controls suggesting that the D487N mutation does not affect localization or folding (Figure 15). Interestingly, the antibody was able to detect this same pattern of staining in a diverse variety of cells from different tissues and species including: human myoblasts, mouse myoblasts (C2C12D), human fibroblasts, canine tracheal smooth muscle cells, and hamster kidney cells (BHK21) (some data not shown).

Myoblasts were the main subject of study in our experiments and approximate muscle stem cells or satellite cells. When these cells are stressed, usually by serum starvation, they

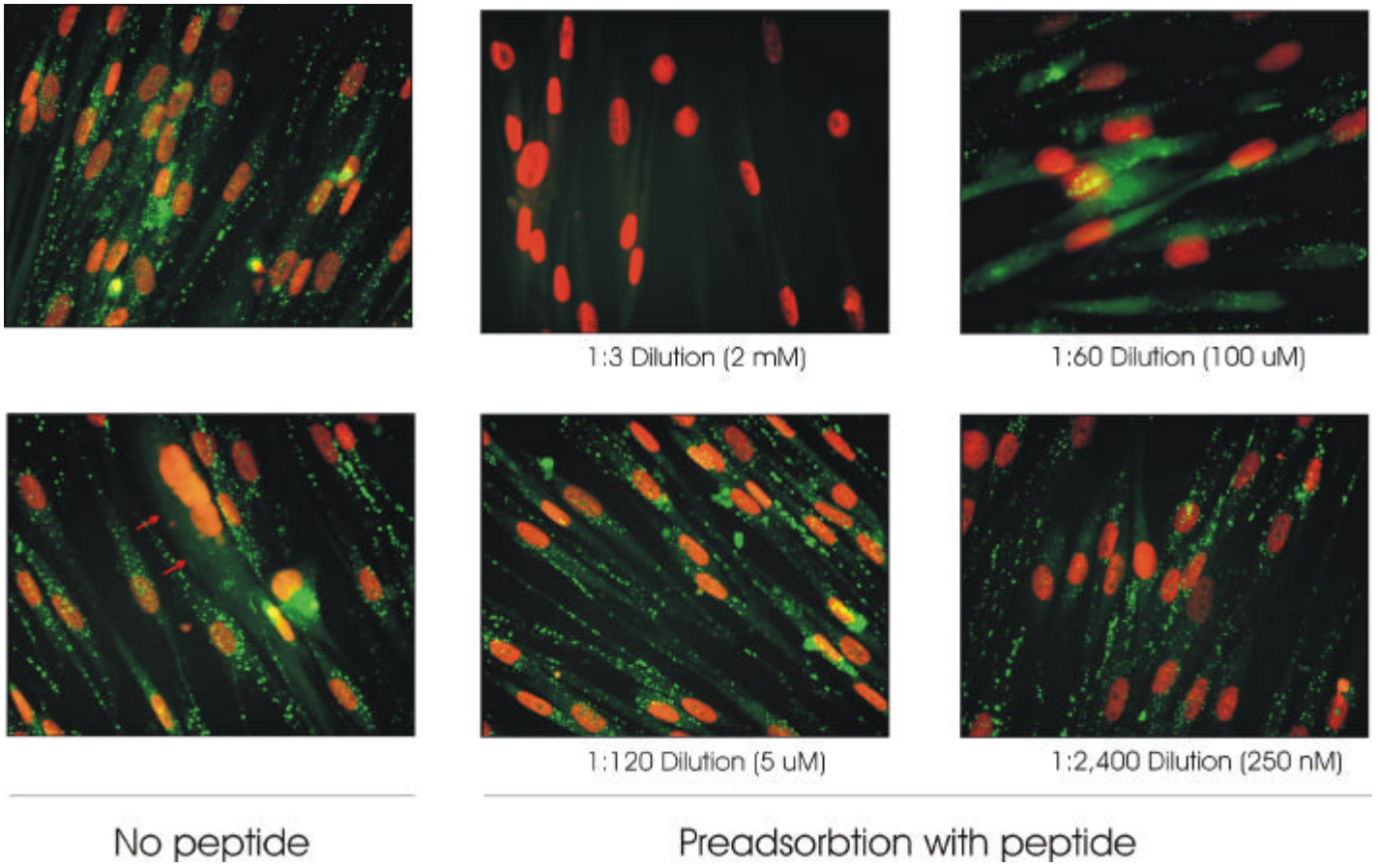


Figure 14: Specificity of anti-TRIM32 antibody by immunofluorescence. Normal human myoblasts were stained using the same anti-TRIM32 antibody as in figure 12. FITC (green) was used to visualize the binding of anti-TRIM32 and propidium iodide (red) was used to stain nuclei. Preadsorbtion with the immunogenic peptide abolishes the TRIM32 signal. Note a large multinucleated myotube is present in the bottom left corner (red arrows), and the TRIM32 signal appears to be absent or no longer punctate.

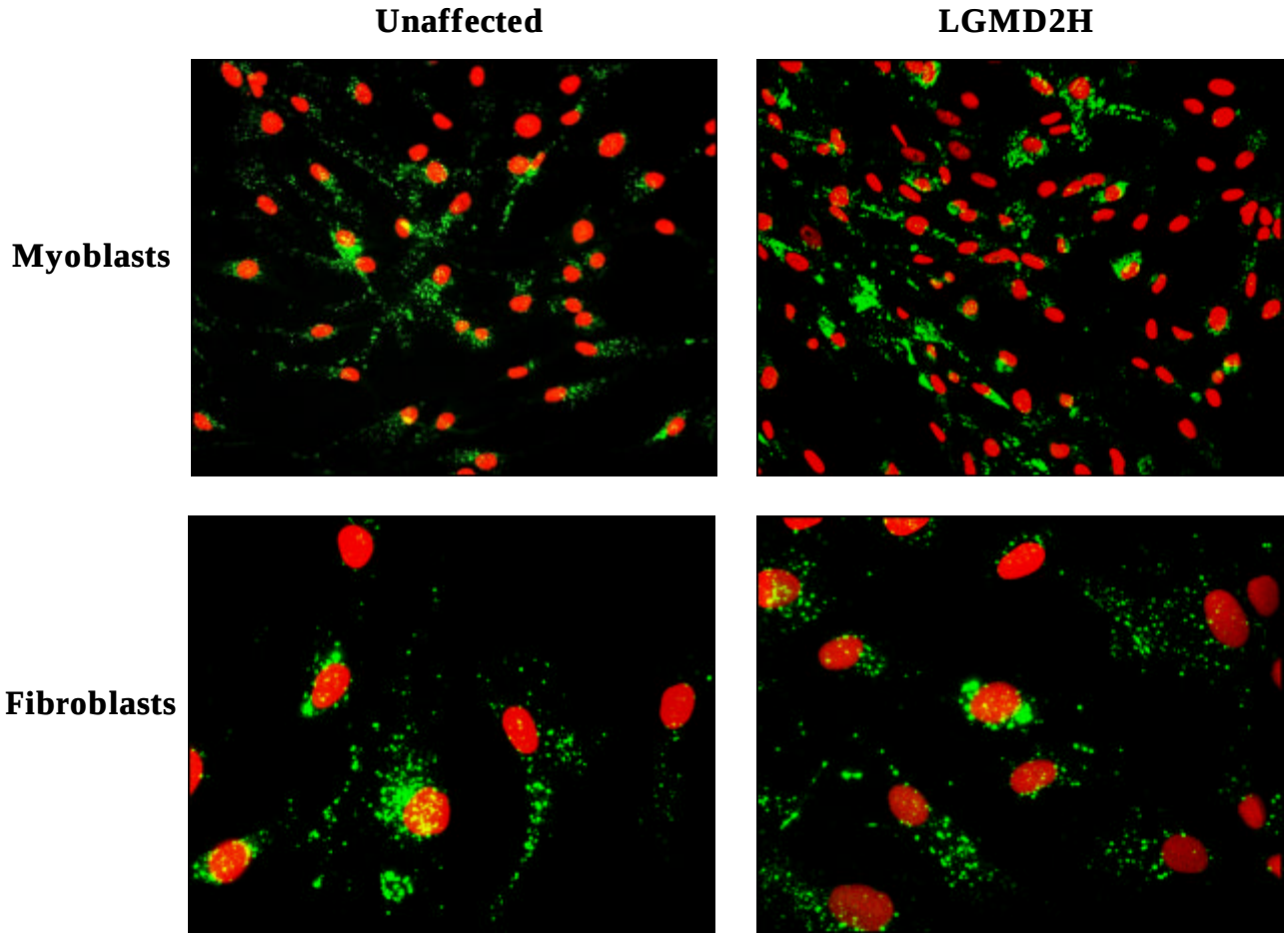


Figure 15: TRIM32 in normal and patient cells. Immunoflourescence microscopy of human cells from patient and control using anti-TRIM32 antibody. FITC (green) was used to visualize the binding of anti-TRIM32 and propidium iodide (red) was used to stain nuclei. No differences are apparent between patient and controls.

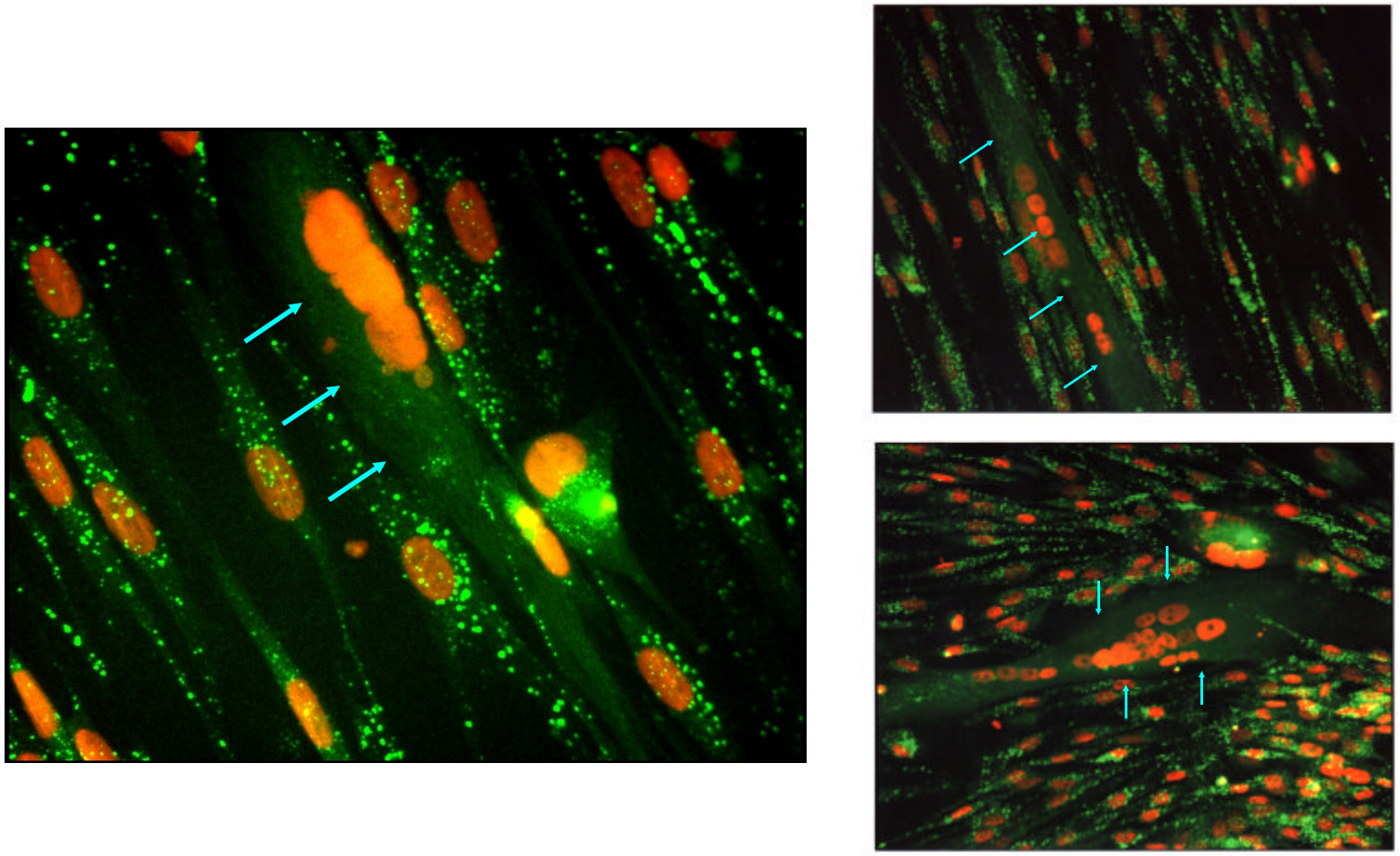


Figure 16: TRIM32 in fused myoblasts. Immunoflourescence microscopy of human cells using anti-TRIM32 antibody. FITC (green) was used to visualize the binding of anti-TRIM32 and propidium iodide (red) was used to stain nuclei. The majority of cells are not fused as expected for semi-transformed myoblasts. Those that did fuse (blue arrows) appear to lack the punctate staining present in those cells that remained unfused.

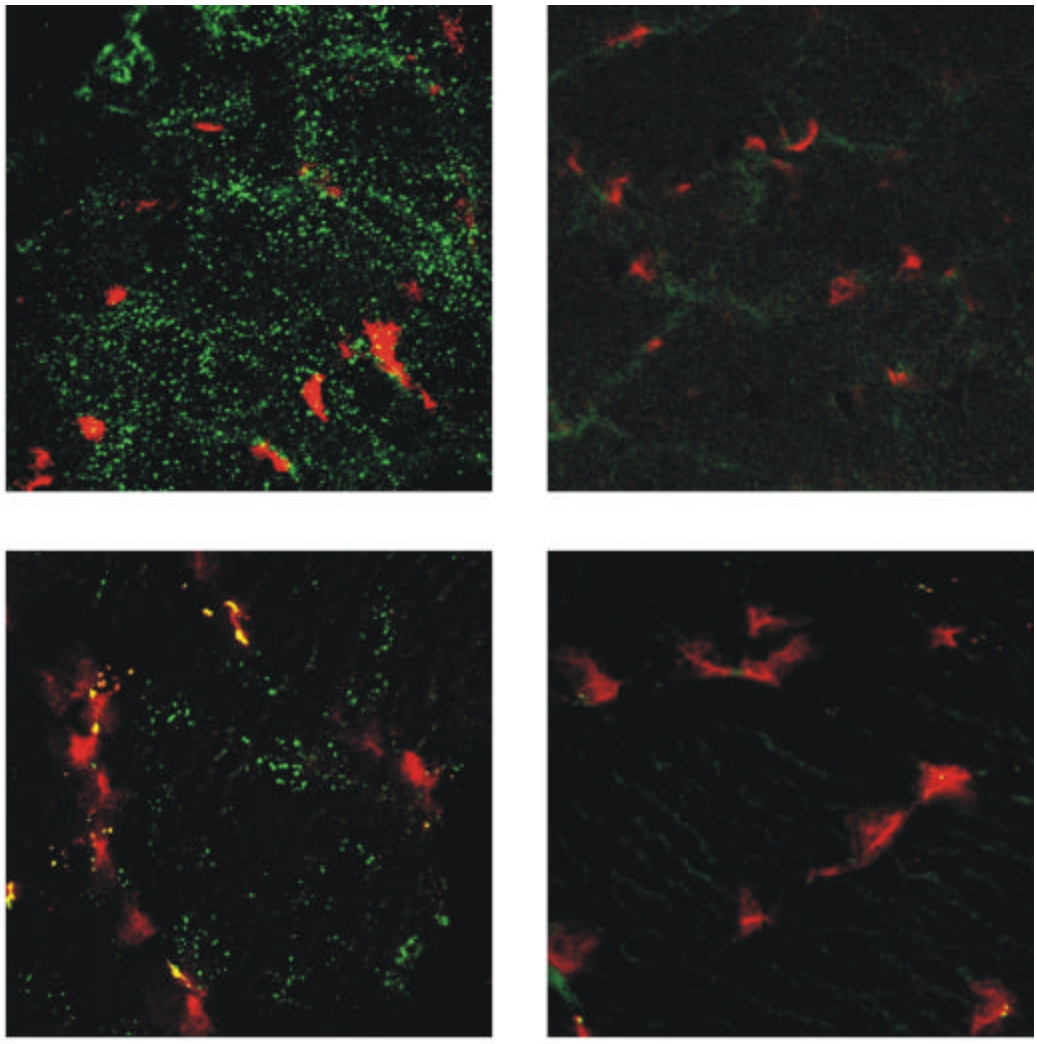


Figure 17: TRIM32 in muscle sections. Mouse (upper panels) and human (lower panels) muscle sections stained with the anti-TRIM32/FITC-secondary (green) and nuclei are stained with propidium iodide (red). Fresh frozen sections were mounted and stained unfixed using the same protocol for cells from tissue culture. Note, these were heavily stained areas in comparison to most of the section.

begin to fuse into myotubes. These myotubes are similar to mature muscle fibres. When TRIM32 staining was done in fused cells it was noticed that in the largest, most evident myotubes, the punctate staining pattern was no longer present (Figure 16). Either the TRIM32 was no longer being expressed or it was no longer punctate and became more diffuse. Similar results were obtained in muscle sections from mouse and human (Figure 17). There appeared to be only focal areas that stained well for TRIM32, although there was no obvious restriction to satellite cells (present along the periphery of the fibres, between the sarcolemma and the basement membrane). These results suggest that TRIM32 may be more important in pre- muscle cells rather than the myofibres themselves.

(G) Interaction screening

The bacterial two hybrid system (Stratagene), a bacterial version of the classical yeast two hybrid system, was used to identify potential interacting proteins of TRIM32. Screening was done according to the protocol outlined in chapter 3 using a rat skeletal muscle library. In short, portions of TRIM32 were fused to the DNA binding domain of 8cI protein and the library mRNAs were fused to the "-subunit of RNA-polymerase (Figure 4). Interaction between bait and target allows recruitment of transcriptionally active complexes to promoters containing 8cI binding sites. A reporter episome is present with both $-galactosidase and $-lactamase under the control of 8cI sites and interaction can be detected by breakdown of X-gal and resistance to carbenicillin. Selection for the presence of the two plasmids and the reporter episome is accomplished by antibiotic resistance: chloramphenicol on the bait plasmid (pBT); tetracycline on the library plasmid (pTRG XR); and kanamycin on the reporter episome. Original screens using the entire coding sequence of human *TRIM32* as

Table 9: Summary of TRIM32 interaction screening

	TRIM32 Bait	
	RING/B-Box - N-Terminal	NHL Repeats - C-Terminal
Cfu's Screened	3.12 X 10^6	1.08 X 10^7 Low Stringency: 2 X 10^5 High Stringency: 1.06 X 10^7
Library Coverage	1.2X	4X
Positives Picked	58	137 Low Stringency: 84 High Stringency: 53
Identified	28	48
Confirmed	None	None

bait were plagued by very high levels of background. Therefore, separate screens were done using only the N-terminal portion or the C-terminal portion of TRIM32 (Table 9).

The N-terminal screen was performed using a low stringency (250 μg/ml carbenicillin) dual reporter setup (β-galactosidase and β-lactamase). This screen yielded 58 positive clones, 28 of which we were able to identify (Table 10). There were seven genes associated with energy metabolism - *ALDOA*, *CKM*, *GAPD*, *LDHA*, *ENO1*, *mt-CO1*, and *PKM2*; three genes associated with the contractile apparatus - *ACTA1*, *TPM1*, and *ACTN3*; two genes associated with nucleotide metabolism - *AMPD1* and *AK3*; two chaperone proteins - *HSPA8* and *HSPB6*; and two sarcoplasmic reticulum proteins - *HERPUD1* and *CASQ1*. In addition, there are three genes with no clear function - *NDRG2*, *ISLR*, and *H19*. Four of these genes were found in multiple clones including - *ALDOA*, *CKM*, *GAPD*, and *ACTA1*, but it is not clear whether this is an important finding or whether they are false positives since all of them are known to be highly expressed in muscle.

The C-terminal screen was originally performed in the same way as the N-terminal screen. However, the percentage of positives compared to the number of plasmids screened was extremely high. A second screen at a high stringency (500 μg/ml carbenicillin) was performed that yielded 84 positives, 48 of which we were able to identify (Table 11). There were again seven genes associated with energy metabolism - *GAPD*, *CKM*, *ALDOA*, *mt-CO1*, *IDH3A*, *PKM2*, and *LOC361713*; five genes associated with the contractile apparatus - *TNNT3*, *LOC311029*, *DES*, *ACTA1*, and *TPM1*; two chaperone proteins - *CABC1* and *HSPB6*; and two sarcoplasmic reticulum genes - *LOC360310* and *LOC305234*. Interestingly, in addition to alpha-actin itself, there were five genes identified that were involved in actin

Table 10: TRIM32 N-terminal interacting proteins (TRIPs)

TRIP #	Gene Symbol	Full Name / Alternates	Function
2,7,13	*ALDOA* [a]	Aldolase A, Fructose Bisphosphate Aldolase	Glycolysis (myopathy w/ hemolytic anemia)[b]
3	*AMPD1*	Adenosine monophosphate deaminase 1 (isoform M)	Purine nucleotide cycle (exercise-induced myopathy)
4,10,11,15,34,47	*CKM*	Creatine Kinase, muscle	Energy metabolism
5A	*HSPA8*	Hsp70 #8	Constitutive chaperone, uncoating vesicles
5B,9	*GAPD*	Glyceraldehyde-3-phosphate dehydrogenase	Glycolysis
12	*NDRG2*	N-myc Downstream regulated gene	Wide expression, neurite outgrowth?
14	*CASQ1*	Calsequestrin	SR lumen, stores Ca^{2+} (dec. in DMD)
16,46	*ACTA1*	Alpha actin 1	Contraction, thin filaments (nemaline myopathy)
18	*TPM1*	Tropomyosin 1 alpha	Contraction, regulatory (hyper. cardiomyopathy 3)
25	*LDHA*	Lactate dehydrogenase	Glycolysis (exertional myoglobinuria)
29	*ENO1*	Enolase alpha	Glycolysis
31	*ISLR*	Ig Domain LR-repeat	Membrane protein, function unknown
36	*HERPUD1*	Homocysteine, ER stress-inducible Ub-like domain member 1	SR membrane, recruits proteasome during ER-stress
39	*AK3*	Adenylate kinase 3	Mitochondrial matrix, regulates phospho-nt pools
43	*mt-CO1*	Cytochrome c oxidase subunit 1	Respiration
44	*HSPB6*	Heat shock protein, alpha-crystallin-related, B6	Contraction? Partner of αB-crystallin (MFM)
45	*ACTN3*	Alpha actinin 3	Component of the Z-line
51	*H19*	Expressed Non-translated RNA	Maternally imprinted locus, Near IGF2 gene
54	*PKM2*	Pyruvate kinase (muscle)	Glycolysis

[a]Yellow highlighting signifies genes that were identified in both N-terminal and C-terminal screens
[b]Human muscle disorders associated with the gene in question

Table 11: TRIM32 C-terminal interacting proteins (TNIPs)

TNIP #	Gene Symbol	Full Name / Alternates	Function
1	*TNNT3*	Troponin T3	Contraction, regulatory (DA1 congenital contractures)
2	*MPST*	Mercaptopyruvate sulfurtransferase	Transfers sulfer to thiol compounds like cyanide
3	*LOC364451*	Similar to enlongation factor 1-gamma	Translation
4	*RT1-Da*	RT1 class II, locus Da	MHC class II antigen
5, 8, 43, 44	*GAPD*	Glyceraldehyde-3-phosphate DeH	Glycolysis
6	*OAZ1*	Ornithine Decarboxylase antizyme 1	Regulation of polyamine synthesis
7, 11, 13, 21, 26, 38, 47	*CKM*	Creatine Kinase, muscle	Energy metabolism
9, 10	*LOC360310*	related to Serpinh1 (serine protease inhibitor)	SR lumen protein, may be chaperone for collagen
12	*CABC1*	Chaperone, ABC1 activity of BC1 complex-like	Chaperone, may suppress cell growth
14	*AP1G1*	Adaptor protein complex AP-1, gamma 1 subunit	Vesicular formation and trafficking
17, 23, 32	*ALDOA*	Aldolase A, Fructose Bisphosphate Aldolase	Glycolysis
18	*GSN*	Gelsolin	Actin depolymerization
19, 22	*SYNPO2*	Synaptopodin 2, Myopodin, Genethonin 2	Actin bundling, myoblast fusion (decreased in DMD)
20	*LOC311029*	Similar to Nebulin	Thin filment length, assembly (nemaline myopathy)
25	*mt-CO1*	Cytochrome c oxidase subunit 1	Respiration
27, 40	*DES*	Desmin	Intermediate filament at Z-disc (MFM)
28, 33	*IDH3A*	Isocitrate DeH 3 alpha	TCA cycle
29	*PKM2*	Pyruvate kinase (muscle)	Glycolysis
30	*NMT1*	N-myristoyltransferase 1	Myristoylation of N-term glycines
31	*LOC366742*	Similar to Ig heavy chain V region precursor	??
34	*LOC361713*	Similar to muscle glycogen phosphorylase	Glycogen breakdown (McArdle myopathy)
35, 39, 45, 48, 50, 52	*ACTA1*	Alpha actin 1	Contraction, thin filaments (nemaline myopathy)
36	*DUSP13*	Dual specificity phosphatase	Active on phospho Tyr and Ser/Thr
37	*TPM1*	Tropomyosin 1 alpha	Contraction, regulatory
41	*Unknown*	Unknown Gene	??
46	*HSPB6*	Heat shock protein, alpha-crystallin-related, B6	Contraction? Partner of α B-crystallin (MFM)
49	*ARPC1A*	Supressor of profilin/p41 of actin related complex	Controls actin polymerization
51	*LOC305234*	Similar to Genethonin 1	Found in T-tubule and SR membrane

[a]Yellow highlighting signifies genes that were identified in both N-terminal and C-terminal screens
[b]Human muscle disorders associated with the gene in question

dynamics - *GSN*, *SYNPO2*, *LOC311029*, *TPM1*, and *ARPC1A*. As with the other screen, many of these genes were found in multiple clones. One of these, *SYNPO2*, is not known to have a particularly high expression in muscle compared to some of the components of the sarcomere or enzymes of energy metabolism, suggesting it may be a true interacting protein.

Due to limitations in the bacterial two hybrid system it has not been possible to reliably confirm the positives that we have obtained by either of these screens. As such, it is very possible that many of these positives are spurious and do not reflect a true interaction with TRIM32. All of the potential positives will have to be recloned and tested by more traditional methods such as coimmunoprecipitation before it will be clear whether they are able to bind to TRIM32. An alternative to this has recently become available from Stratagene due to the many problems researchers have had with this system. They have created a new reporter strain that apparently reduces background by 100 fold (use of streptomycin resistance and an auxotrophic marker). Between these two methods, confirmatory results will undoubtedly be obtained in the near future.

4.3 Discussion

On the basis of observed recombinants, the candidate gene for LGMD2H was previously defined to be in a 3.9-6.2 cM interval between *D9S302* and *D9S1850*.[279] This interval is now estimated to be 6.4 Mb. Through the study of additional families with LGMD2H and additional microsatellite markers, we reduced the candidate region to a 560 kb interval flanked by *D9S1126* and *D9S737* on the basis of haplotype analysis. The region contains three annotated genes, *PAPPA*, *ASTN2*, and *TRIM32*, and one unannotated gene.

TRIM32 is located within intron 12 of *ASTN2* in the opposite orientation, and part of the unannotated gene is within introns 9 and 10 of *PAPPA* in the opposite orientation (Figure 7).

All exons within the candidate region were screened for mutations. Of the four sequence variations detected, V1 and V2 could readily be excluded as disease causing because they do not consistently segregate with the disease. V3(T) appears to be an unlikely candidate because it does not lead to an amino acid change, does not lead to a codon with significantly altered usage, and does not appear to lead to alternative splicing. The latter was confirmed by RT-PCR of mRNA isolated from skin fibroblasts obtained from one patient and a control. However, at this time we cannot exclude the possibility that the V3(T) allele disrupts an exonic splicing enhancer which functions only in muscle[160,161] or alters the secondary structure of the mRNA.[227]

The best candidate mutation for LGMD2H is the missense mutation V4 in TRIM32 that changes codon 487 of the TRIM32 protein from aspartate to asparagine (D487N). There is strong evidence that this D487N causes LGMD2H, because (i) all affected individuals in our study are homozygous for V4(Asn); (ii) it was not observed in 100 controls; (iii) it occurs in a protein domain referred to as an NHL domain[237] in an aspartate residue that is one of the two most conserved amino acids in NHL domains (Table 8); and (iv) the change from aspartate to asparagine is a nonconservative change that could result in a significant alteration in the structure and function of the TRIM32 protein. Thus, we consider D487N to be the disease-causing mutation for LGMD2H in our patients. The information we currently possess does not exclude the possibility that V3 may contribute additional effects to the phenotype.

The TRIM32 protein has, from the N to C terminal end, a RING finger domain (InterPro accession IPR001841), a B1 box (IPR002991), a coiled-coil domain (IPR003649), and six NHL domains (IPR001258)(www.ebi.ac.uk/interpro/).[7] It is a member of a growing family of TRIM or RING-B-box-coiled-coil proteins (Figure 10).[212,257] Current knowledge concerning the RING finger domain indicates that it may have intrinsic ubiquitin (Ub) ligase activity[15] and it is thought that all RING finger proteins are E3 Ub ligases.[88] This information suggests that TRIM32 is an E3 Ub ligase that may function in the ubiquitin-proteasome pathway.

The ubiquitin-proteasome pathway is a specialized mechanism for the posttranslational regulation of protein levels. The initial step in the Ub-proteasome pathway is the activation of Ub by an E1 Ub activating enzyme. The activated Ub is transferred to the E2 Ub conjugating enzyme. The E3 ligase interacts with E2-Ub complex and recruits a protein to be targeted to the 26S proteasome. The E3 ligase catalyzes the transfer of Ub to the target protein. The ubiquitinated protein is released from the complex and is recognized by factors associated with the 26S proteasome. The 26S proteasome digests the ubiquitinated protein into small peptides. The E3 enzymes are responsible for the selection of proteins for ubiquitination by this pathway and it is thought that a large number of these enzymes exist.[88] It has been shown that the RING finger domain of an E3 ligase mediates interaction with the E2 conjugating enzyme and is the source of the ligase activity.[136] This suggests that recognition of target proteins by E3 ligases occurs through protein-protein interactions with areas of the protein other than the RING finger domain. In the case of TRIM32 this function may be fulfilled by a series of NHL domains.

NHL domains have been shown to be important for the function of a number of proteins including LIN-41 of *C. elegans* and Brat of *D. melanogaster* both of which act as post-transcriptional repressors. Of the seven apparent loss-of-function mutations in lin-41 discussed by Slack et al. (2000), five led to amino acid substitutions found in the NHL domains.[236] Loss-of-function mutants of Brat include both deletions of the NHL domains as well as missense mutations.[8,239] All of these mutations abolish an interaction with another protein, thereby leading to functional consequences.

This information suggests interesting possibilities as to the role of TRIM32 in LGMD. If TRIM32 is indeed an E3 ubiquitin ligase, the NHL domains could mediate interaction with either other members of an E3 Ub ligase complex or the proteins that the complex targets to the proteasome. Considering the limited distribution of symptoms in LGMD2H patients and the wide tissue distribution of TRIM32, it is tantalizing to postulate that the third NHL repeat is involved in interacting with and therefore targeting of one or a group of proteins to the proteasome to be degraded. A mutation in the NHL domain would prevent this process and these proteins would accumulate to higher concentrations (Figure 18). The restriction of symptoms to muscle tissue may have two explanations: (i) the third NHL domain may be interacting with muscle specific proteins; (ii) although the increased level of proteins is not restricted to muscle, it may only have a toxic effect in muscle. Increased levels of some proteins have been reported to cause myopathic symptoms, as shown by work involving transgenic mouse lines that over-express caveolin-3 or 8-sarcoglycan.[96,289] Furthermore, the gene mutated in rare forms of Parkinson disease, *PARK2*, is thought to work via a similar pathogenic mechanism. Parkin, the product of

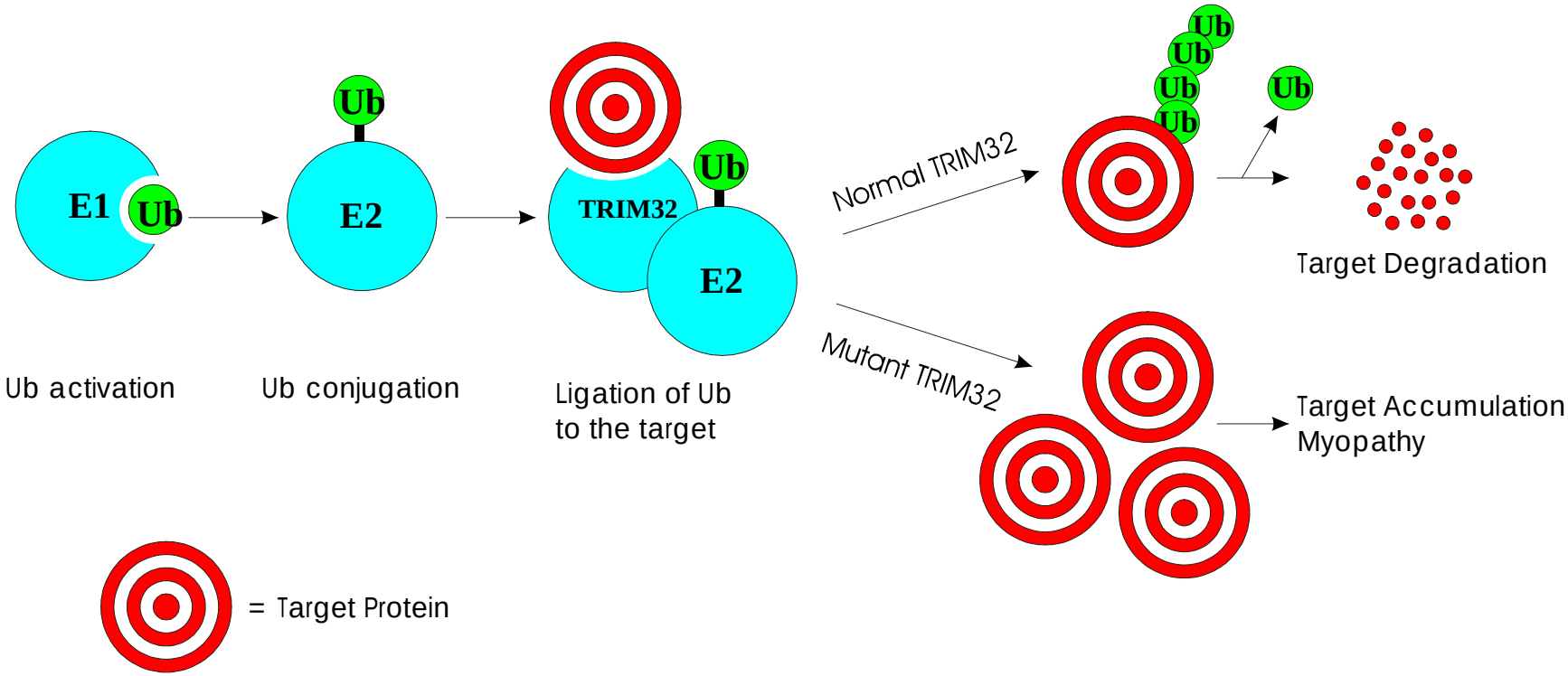


Figure 18: Schematic of the proposed pathogenic mechanism of LGMD2H. The figure shows TRIM32 as an E3 ubiquitin ligase responsible for the recognition of a target protein (bullseye). When TRIM32 is functional, the target gets ubiquitinated and degraded whereas when TRIM32 is non-functional (LGMD2H) it accumulates and potentially causes a myopathy.

PARK2, is known to be a RING finger containing E3 Ub ligase.[229] The neuronal death seen in Parkinson disease is thought to be a direct result of the accumulation of proteins which are no longer ubiquitinated by mutant forms of parkin and therefore are not degraded by the proteasome.[133,230] We favour such a model in the pathogenesis of LGMD2H.

Recently a paper was published, linking TRIM32 to skin cancer, that provided some evidence that this hypothesis may be correct.[124] TRIM32 was shown to be upregulated in certain models of skin cancer as well as in human skin tumours. They found that TRIM32 was associated with ubiquitinated proteins and was ubiquitinated itself. These characteristics are suggestive of an E3 Ub ligase. The authors concluded that TRIM32 exerted its effects during carcinogenesis by inhibiting apoptosis. This suggests that LGMD2H may be a result of increased apoptosis due to inactivation of TRIM32 by mutation.

We have been able to perform limited characterization of the *TRIM32* gene product ourselves and while we have not been able to show a direct link between this protein and the Ub proteasome system we have uncovered some very interesting information. TRIM32 appears to exist in large 'bodies' in the cytoplasm of the cell. This leads to a diffuse punctate staining when the cells are subjected to immunofluorescence microscopy. These bodies no longer appear to be present in fused myotubes and there is only small focal areas of staining in mature muscle tissue. This suggests that TRIM32 may be more important in the regeneration of muscle via myoblast fusion rather than in the maintenance of individual myofibres.

The potential interacting proteins identified by bacterial two hybrid screen, while confusing, hold promise to shed some light on the native function of TRIM32. Our original

intention was to identify an E2 enzyme using the N-terminal portion of TRIM32 (RING and B-Box) and a substrate with the C-terminal portion of TRIM32 (NHL repeats). None of the genes found are known to have E2 conjugating ability and it remains to be seen if any of the potential interacting proteins are substrates for TRIM32. There is the indication that TRIM32 may have some role in regulating actin dynamics or in maintaining a functional sarcoplasmic reticulum. Six genes that are known to bind actin were identified as well as four SR resident proteins and three chaperones. The identification of myopodin (*SYNPO2*) as a potential interacting protein is particularly intriguing as it is thought to be involved in actin polymerization during myoblast fusion. This further strengthens the hypothesis that TRIM32 is involved in regenerating muscle.

It remains to be determined how TRIM32 causes LGMD when mutated. It is unclear how these observations regarding apoptosis, muscle regeneration, actin dynamics, and SR function relate to one another. There are many potential avenues of research that have to be explored before there are any definitive answers regarding the pathogenic mechanism underlying LGMD2H.

5. Identification of the LGMD2I gene

The bulk of the data presented in this chapter have been published in the following three articles:

- *Poppe M, Bourke J, Eagle M, **Frosk P**, Wrogemann K, Greenberg C, Muntoni F, Voit T, Straub V, Hilton-Jones D, Shirodaria C, Bushby K. Cardiac and respiratory failure in limb-girdle muscular dystrophy 2I. Ann Neurol 2004; 56(5):738-741.*
- ***P Frosk**, CR Greenberg, AA Tennese, R Lamont, E Nylen, C Hirst, D Frappier, NM Roslin, M Zaik, K Bushby, V Straub, M Zatz, F de Paula, K Morgan, TM Fujiwara, K Wrogemann. The most common mutation in FKRP causing limb girdle muscular dystrophy type 2I (LGMD2I) may have occurred only once and is present in Hutterites and other populations. Hum Mutat 2005; 25(1):38-44.*
- ***P Frosk**, MR Del Bigio, K Wrogemann, CR Greenberg. Hutterite brothers both affected with two forms of limb girdle muscular dystrophy: LGMD2H and LGMD2I. Eur J Hum Genet 2005 (In Press).*

I would like to acknowledge the contributions of the following individuals: (1) Ryan Lamont for performing the genome scan described in this chapter, (2) Ted Nylen, Cheryl Hirst, Alyssa Tennese, and Amanda Fortier for the determination of some genotypes, (3) Dr. Ken Morgan and Mary Fujiwara for help with the genome scan, calculating LOD scores, and preparing the Human Mutation manuscript, and (4) Drs. Kate Bushby, Mayana Zatz, and Volker Straub for providing non-Hutterite LGMD2I patient samples.

5.1 Introduction

Prior to the identification of the gene causing LGMD2H (chapter 4), it became apparent that a selection of Hutterite LGMD families did not show linkage to chromosome nine. As expected, none of the affected individuals in this group tested positive for the TRIM32 mutation once it had been discovered. We hypothesized that at least one other locus was responsible for LGMD in this subset. A genome-wide scan was undertaken in five of these families and significant LOD scores were obtained for markers at chromosome region 19q13.3, the site of a previously described LGMD locus, *LGMD2I*. *LGMD2I* was first reported in a large consanguineous Tunisian family and mapped to a 3.2 Mb region delineated by the markers *D19S412* and *D19S879*.[66] Haplotyping was performed in our Hutterite families and we were able to reduce this region to 1 Mb, flanked by the markers *D19S412* and *D19S606*. This region contained five genes, none of which were good candidates for a muscular dystrophy gene.

Concurrent with our study, another group (Brockington et al.) searching for congenital muscular dystrophy (MDC) genes by a bioinfomatic / homology method identified a novel gene on chromosome 19.[36] This approach was used because MDC is very heterogeneous and families are generally quite small because the disease is severe and manifests at birth. This makes linkage analysis very difficult when looking for novel loci. The gene that was identified, *FKRP* (fukutin-related protein), was highly homologous to the fukutin gene, in which mutations cause a common MDC in Japan (Fukuyama congenital muscular dystrophy, OMIM #607440).[145,256] Mutations in *FKRP* were found to be responsible

for a subset of MDC patients and the disorder was given the name MDC1C (OMIM #606612).[36]

FKRP was an ideal candidate for the *LGMD2I* gene and we were able to localize it within the region delineated in our study. We screened *FKRP* and found c.826C>A, a mild leucine to isoleucine mutation in the Hutterite patients (L276I). While attempting to determine if this was the true causative mutation and not a common polymorphism, the group that identified *FKRP* published a manuscript describing a later-onset LGMD caused by mutation in *FKRP*.[37] Subsequently, Driss et al. identified *FKRP* mutations in the original Tunisian family, confirming that *FKRP* is the *LGMD2I* gene.[67] Interestingly, the L276I mutation identified in the Hutterites was also found by Brockington et al. in a majority of their LGMD2I patients, which they attributed to a mutational hotspot.[37] Recent work has shown that LGMD2I is one of the most common forms of LGMD worldwide, largely because of the prevalence of the L276I mutation.[43]

FKRP is thought to be a glycosyltransferase that may function in the O-linked glycosylation (mucin-type) of proteins such as "-dystroglycan, potentially disturbing the DGC link to the extracellular matrix.[74,122] Since the discovery of this gene there has been an enormous amount of work done on this and other similar proteins.[113] O-linked glycosylation defects have been found to underlie many congenital forms of muscular dystrophy including Fukuyama Congenital Muscular Dystrophy (FCMD, OMIM #607440), Walker Warburg Syndrome (WWS, OMIM #236670), Muscle-Eye-Brain Disease (MEB, OMIM #253280) and Muscular Dystrophy Congenital type 1D (MDC1D, OMIM #608840).[165,186] These disorders usually show neurological and occasionally optic defects in association with the

muscular dystrophy, largely due to the importance of "-dystroglycan in the brain and eye.[176,180] FKRP is peculiar in this respect because not only do severe mutations usually not cause extra-muscular manifestations but mild mutations lead to a more benign later-onset disorder.

Due to the unique population that we were working on, we have been able to perform certain analyses that could not have been done otherwise. A comparison of the clinical characteristics of the two LGMD patient groups in the Hutterites has allowed us to make some genotype-phenotype correlations. LGMD2I patients generally have an earlier age at diagnosis, a more severe course, and higher SCK levels. A subset of individuals affected with LGMD2I show calf hypertrophy, cardiac symptoms, and severe reactions to general anaesthesia. None of these features are present amongst LGMD2H patients.

In addition, we have been able to make some conclusions regarding the natural history of the L276I mutation in *FKRP*. A single common haplotype surrounding the *FKRP* gene was identified in the Hutterite LGMD2I patients. An identical core haplotype was also identified in 19 other non-Hutterite LGMD2I patients from Europe, Canada, and Brazil. The occurrence of this mutation on a common core haplotype suggests that L276I is a founder mutation that is widely dispersed among Caucasian populations.

5.2 Results

(A) Subjects

DNA samples from 39 Hutterites, including 12 affected with LGMD, from 4 families were included in a genome-wide scan (pedigrees L-O, Figure 19). In addition, to these larger

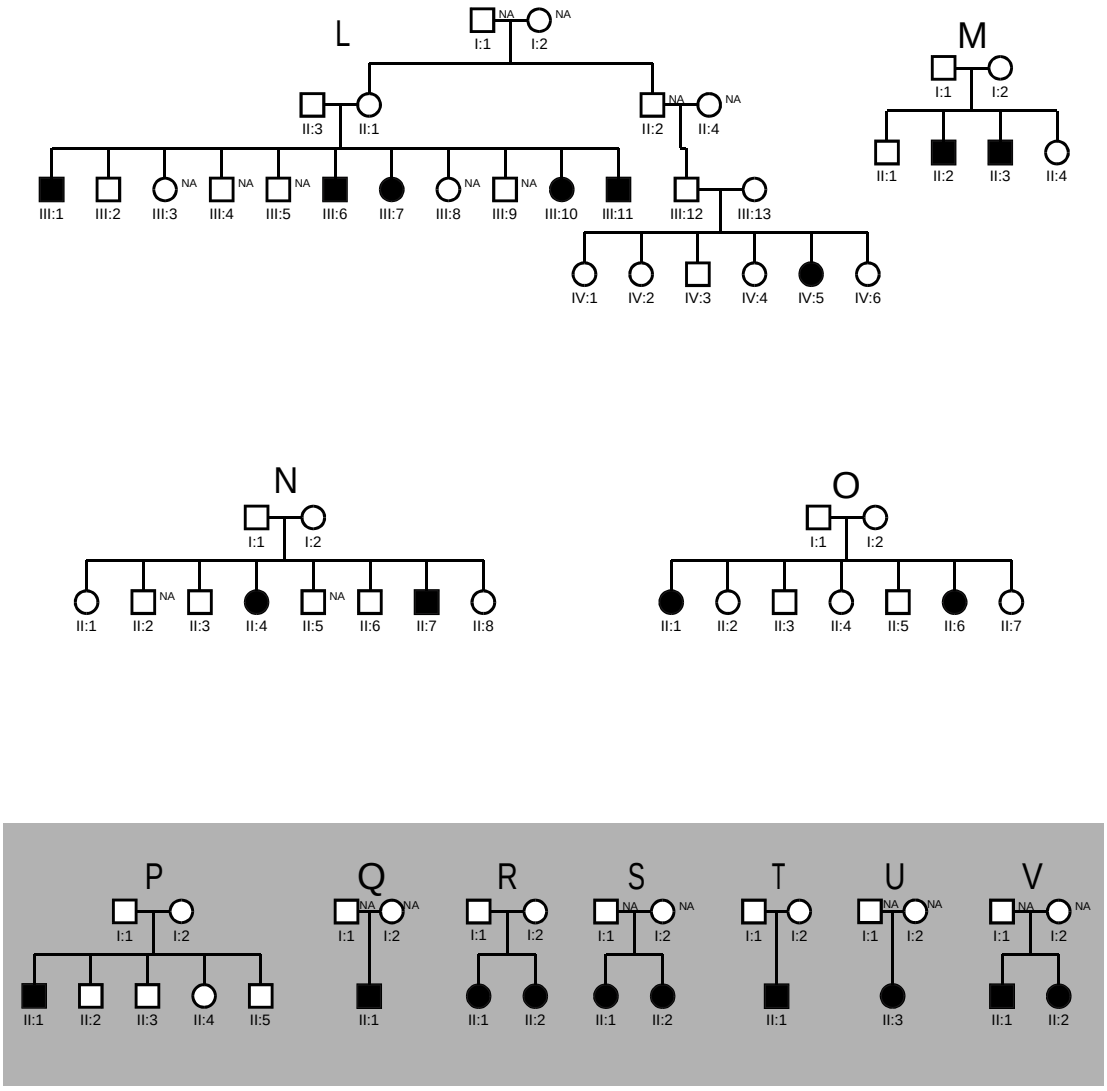


Figure 19: LGMD2I pedigrees. Each pedigree is assigned an identifier (L-V) and individuals are numbered according to generation. Birth order has been randomized to protect anonymity. Individuals denoted with "NA" were not available for study. Individuals highlighted by a grey background were not used in the original genome scan.

families, we collected 7 other small Hutterite families with 10 individuals affected with LGMD (pedigrees P-V, Figure 19). There are families from all 3 subdivisions of the Hutterites: 4 are Schmiedeleut (M, O, Q, and U), 6 are Dariusleut (L, N, P, R, S, and T), and 1 is Lehrerleut (V). All families, with the exception of pedigree V, can be linked into one large, complex pedigree on the basis of our two available genealogical records (Schmiedeleut and Dariusleut).[105,281] The LGMD2H families referred to in this study are those in chapter 4 (pedigrees A-K, Figure 5). Note that individual Q II:1 is a carrier of one LGMD2H allele (Figure 19). A slightly larger control group of 111 healthy individuals was used, compared to the original 100 used in the LGMD2H study.

(B) Genome scan and haplotype analysis

A full genome-wide scan was performed in collaboration with the Montreal Genome Centre. The five LGMD families were included alongside samples for another Hutterite disorder, Bowen-Conradi syndrome (BCS), and the scan was performed by a colleague. The fine details of the scan have been recently published in a manuscript describing the discovery of the BCS locus.[150] In short, the genome-wide scan consisted of 389 microsatellite markers with an average spacing of 9.1 cM. Singlepoint parametric linkage analysis with a fully penetrant autosomal recessive disease model was performed using GENEHUNTER 2.1.[147,168] LOD scores were calculated for each of the 4 families individually because inclusion of all the interrelations made the calculation intractable. A maximum LOD score of 1.50 at *D19S587* was obtained. The second highest score was 0.87 at the adjacent marker, *D19S178*, and the third highest score was 0.67 at D2S407. These LOD scores were all at zero recombination. Using multipoint linkage analysis, the maximum multipoint LOD score was

3.18 at *D19S178*. The region with the next highest multipoint LOD score was 0.67 at *D2S407*.

To define a candidate gene interval, families were genotyped for 14 additional microsatellite markers in a 13 cM region between two genome-scan markers, *D19S178* and *D19S246* (Figure 20). Excluding markers used in the genome scan ~2500 genotypes were generated for markers on chromosome 19. One of these markers, *D19S902*, had a singlepoint LOD score of 5.33 at zero recombination, clearly identifying this region of chromosome 19 as the causative locus in these families. This was the site of the *LGMD2I* locus, a 3.2 Mb region delineated by the markers *D19S412* and *D19S879*.[66] Recombinations in our families were observed between markers *D19S902* and *D19S596* (L III:1) and somewhere between *DM* and *D19S540* (L III:6)(Figure 20). We were also able to infer two ancestral recombinations in pedigree N that further reduced the region of homozygosity to between the markers *DM* and *D19S606* (Figure 20). Using the data presented in the original *LGMD2I* mapping paper we can conclude that the *LGMD2I* gene must lie in the 1 Mb region between the markers *D19S412* and *D19S606* (Figure 21).

(C) Mapping and mutation identification

When we had identified the *LGMD2I* locus, the human genome project had just recently published the draft sequence.[151,273] The chromosome 19q13.3 area had some major gaps and rearrangements but in the most recent build the sequence is high quality and contiguous.[139,141] Surprisingly, very little has changed regarding gene content or the relative positions of the genes. Within the region delineated by haplotype analysis were five known genes; *PRKD2*, *STRN4*, *SLC1A5*, *AP2S1*, and *GRLF1* (Table 12). All of these genes were

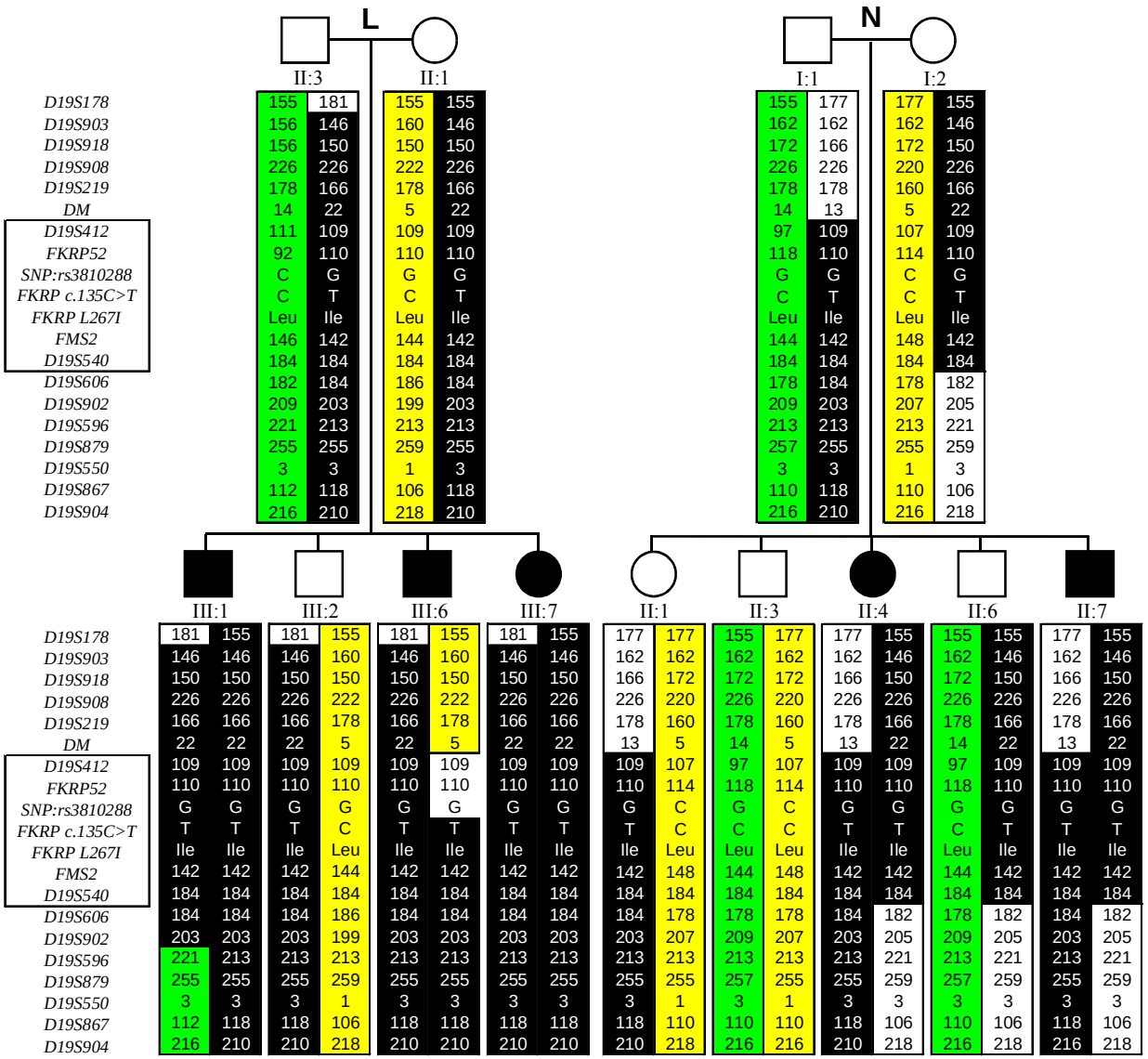


Figure 20: Chromosome region 19q13 haplotypes. Recombinations are evident (L III:1 and L III:6) that define a candidate region for *LGMD2I* between the markers *DM* and *D19S596* (an area now known to be 3.0 Mb). Inferred ancestral recombinations do not change the upper boundary (N I:1) but do raise the lower boundary (N I:2). This reduces the region to 1.7 Mb, flanked by *DM* and *D19S606* (region enclosed by the open rectangle). Microsatellite alleles are given in bp, 0 indicates no data. SNP alleles are presented in nucleotides unless there is a coding change and then it is in three letter amino acid code. Black background on haplotypes indicates consensus disease-associated alleles, white indicates non-consensus disease associated alleles, and yellow/green indicates a normal parental chromosome. Alleles without an outer vertical border indicate that the allele is uninformative for a crossover. Note the five central markers from *FKRP52* to *FMS2*, one of which is the causative mutation, were not available at the time of fine mapping. They were included to allow for comparison with haplotypes presented in figure 24.

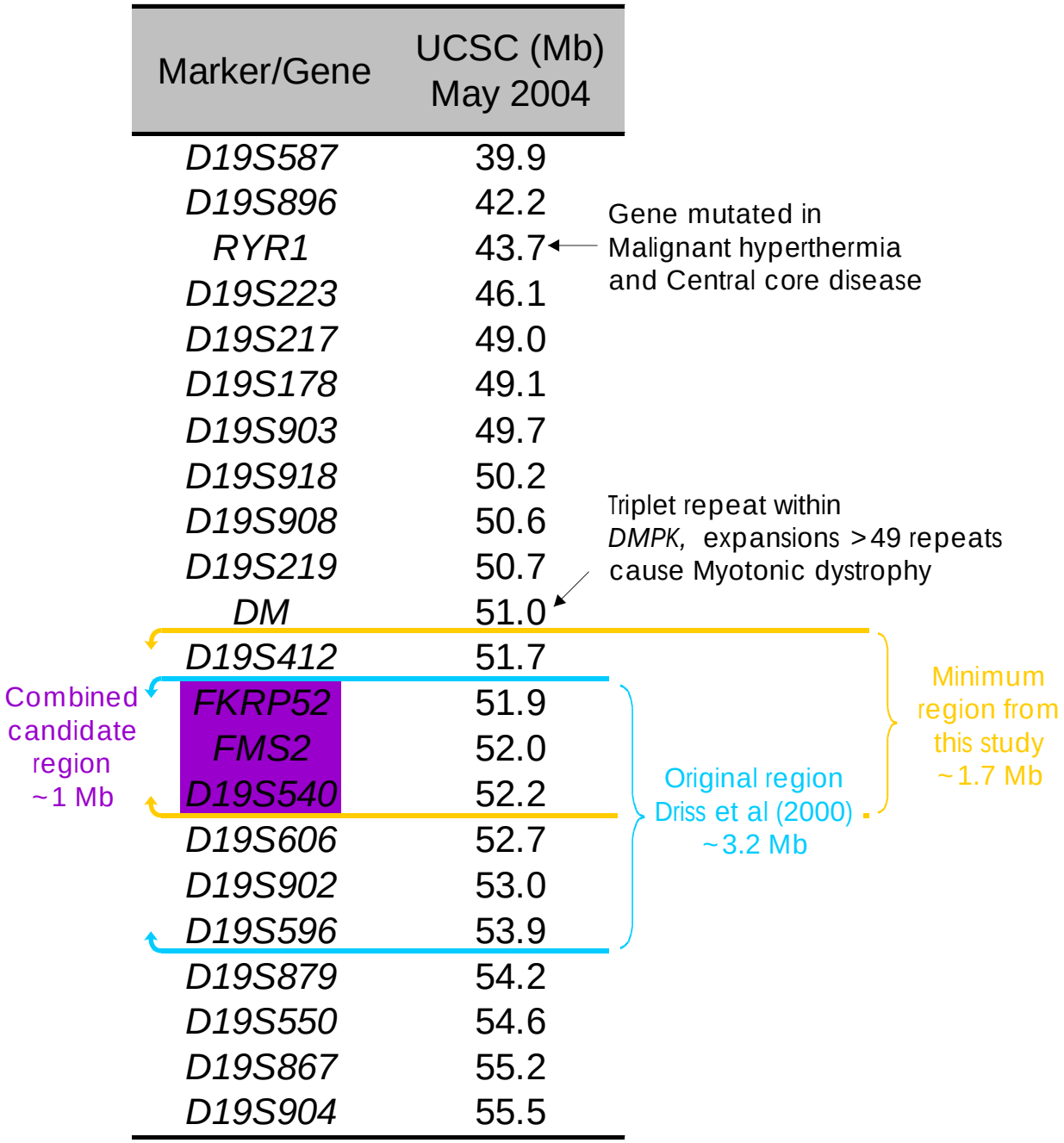
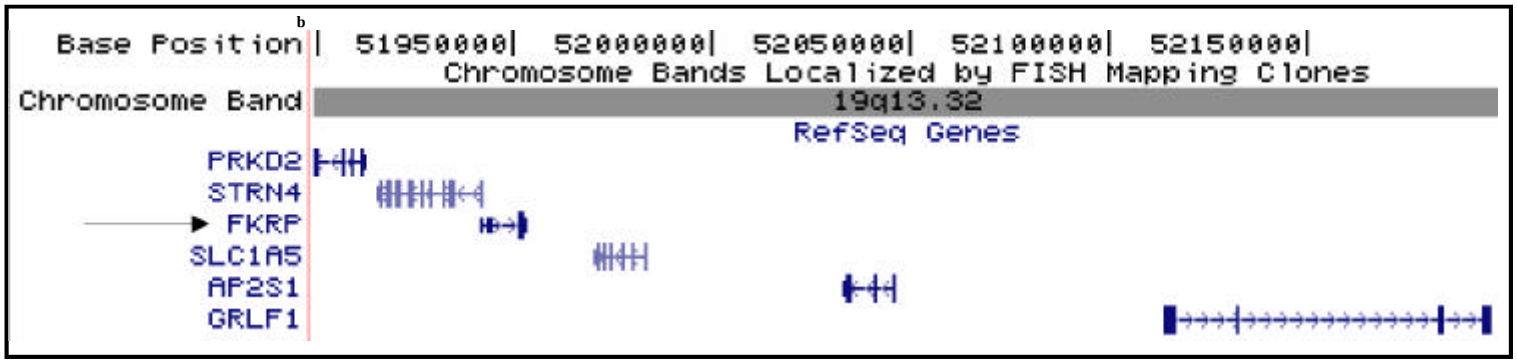


Figure 21: LGMD2I candidate region. A selection of markers and genes in chromosome region 19q13. The minimal region identified in this study is shown in yellow. The original published *LGMD2I* locus is shown in blue. Combined, these data suggest a smaller 1 Mb region in which to search for potential causative genes.

Table 12: Genes within the *LGMD2I* region and positions in relation to *FKRP*

Locus	Locus ID[a]	Full Name	Description
PRKD2	25865	Protein Kinase D2	Serine / Threonine kinase, may control exit from *trans*-golgi
STRN4	29888	Striatin, calmodulin binding protein 4	Scaffolding / signalling protein, expressed in brain
SLC1A5	6510	Solute carrier family 1, member 5	Neutral amino acid transporter
AP2S1	1175	Adaptor-related protein complex 2, sigma 1 subunit	Links clathrin to receptors on coated vesicles
GRLF1	2909	Glucocorticoid receptor DNA binding factor 1	Represses glucocorticoid receptor mediated transcription



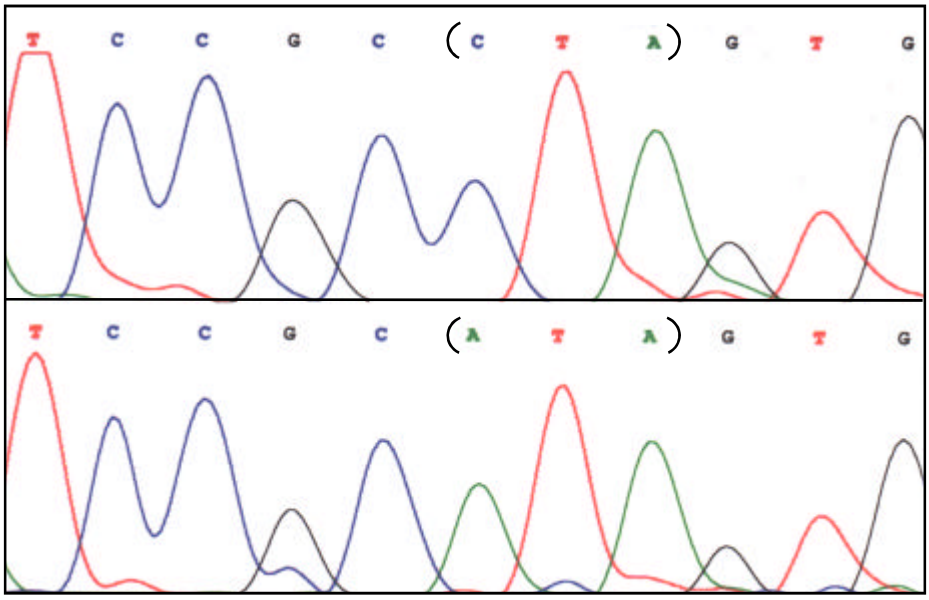
[a] NCBI locuslink; www.ncbi.nlm.nih.gov/LocusLink
[b] Map showing gene positions - UCSC Genome Browser, May 2004 Freeze; /genome.ucsc.edu/index.html

relatively well characterized and none of them showed any similarity to previously characterized muscular dystrophy genes. In addition, there were no obvious indications for novel uncharacterized genes such as spliced ESTs. There were two known muscle disease genes in the relative vicinity, *DMPK* causing myotonic dystrophy (DM1, OMIM #160900) and *RYR1* causing a sensitivity to malignant hyperthermia episodes (MHS1, OMIM #145600) and central core disease (CCD, OMIM #117000), but they were outside of the region (Figure 21). In fact, they were outside of even the large region determined by Driss et al.[66]

The same week that we completed our fine mapping studies, Brockington et al. reported that a form of congenital muscular dystrophy was caused by mutations in *FKRP*. This novel gene was a promising candidate gene for LGMD2I and we found that it was indeed localized within the 1 Mb region defined by our studies. The reason that it was not apparent originally was that the 5' ends of *FKRP* and *STRN4* overlap, with *FKRP* facing centromeric>telomeric and *STRN4* facing telomeric>centromeric, confounding many of the gene prediction and alignment programs.

The entire coding region of *FKRP* was sequenced in one Hutterite LGMD2I patient who was found to be homozygous for a missense mutation, c.826C>A (L276I), that results in the substitution of isoleucine for leucine. A testing method was devised to detect the L276I mutation based on a natural restriction site that is abolished by the C>A change (Figure 22). Upon digestion of a 686 bp fragment by *Bfa*I (NEB) the C allele yields 438 and 248 bp fragments whereas the A allele remains uncut. This test only works consistently if very small amounts of fragment are digested (0.5 ：1 of PCR amplicon digested in a 20 ：1 volume), if

A



B

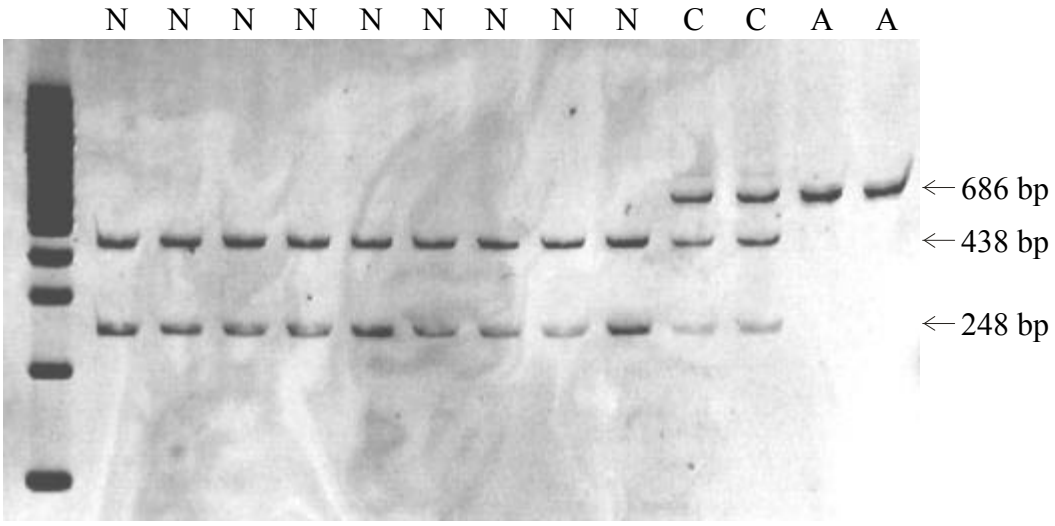


Figure 22: Detection of L276I in FKRP. (A) Electropherogram of sequence from exon 4 of *FKRP* showing the L276I mutation. The upper sequence was obtained from an unaffected individual and the lower from an affected individual. The mutation changes a Leu codon (CTA) to an Ile codon (ATA). (B) Assay for L276I based on a natural *Bfa*I site (CTAG) abolished by the change. The full length fragment is 686 bp and when the site is present, *Bfa*I digestion will produce 438 bp and 248 bp fragments. N = homozygous L276, C = heterozygous, A = homozygous L276I. Individuals shown were randomly selected, in all cases L276I segregates with the disease phenotype.

larger amounts are used much of the fragment remains uncut regardless of the genotype. We found that every Hutterite LGMD patient, who was not homozygous for the *TRIM32* mutation, was homozygous for *FKRP* L276I. In addition, L276I was not found in our control group of 111 individuals.[90]

As we were completing this work, Brockington et al. reported a later-onset LGMD being caused by mild mutations in *FKRP*.[37] The suggestion was that depending on the nature of the mutation you could get phenotypes ranging from congenital muscular dystrophy to LGMD. The same mutation found in the Hutterite patients was also found by their group, with the patients from 15 of their 17 *LGMD2I* families, being homozygous (five families) or heterozygous (10 families). The group that originally identified the *LGMD2I* locus in a large Tunisian family was able to identify mutations in *FKRP* although they were novel and did not include L276I.[67] Since then many studies have been done on patients with LGMD2I and it is found that the bulk of Caucasian patients carry at least one copy of L276I.[174,208,275]

(D) Genotype-phenotype correlations

To date we have identified 74 Hutterite individuals from 22 families who are homozygous for either *TRIM32* D487N or *FKRP* L276I. Table 13 shows clinical details on individuals who are homozygous or heterozygous for the mutations. There is considerable clinical variability even among siblings. SCK levels tended to be higher in the LGMD2I than in the LGMD2H patients. Severe dilated cardiomyopathy was the presenting symptom in one LGMD2I patient and has subsequently been found in another two patients. Calf hypertrophy with proximal muscle wasting reminiscent of that seen in patients with Becker muscular

Table 13: Clinical differences between LGMD2H and LGMD2I in Hutterites

Mutation	Status	Number of Individuals	Age of Onset (years)	Calf Hypertrophy	Cardiac Status	Highest Resting CK (U/litre)	Mean Multiples of Max Normal	Current status (age range in years)
LGMD2H / *TRIM32* D487N	Homozygote	46[a]	9-42 Mean = 24	Absent (46)	Normal (14), Not studied (32)	81 - 5,556	10 X	13 Asymptomatic (7-42), 26 Ambulatory (9-53), 4 Assisted ambulation (42-47), 3 Wheelchair bound (60-66)
	Heterozygote	54	na	Absent (54)	Not studied (51)	44 - 1,460	1 X	54 Asymptomatic (6-77)
LGMD2I / *FKRP* L276I	Homozygote	22[b]	2-25 Mean = 12	Absent (11), Borderline (2), Present (9)	Dilated cardiomyopathy (3), Normal (11), Not Studied(8)	322 - 26,087	33 X	20 Ambulatory (5-45), 1 Assisted ambulation (40), 1 Deceased / congestive heart failure (40)
	Heterozygote	39	na	Absent (39)	Not studied (39)	52 - 411	1 X	39 Asymptomatic (6-70)
Both	Double Heterozygote	8	na	Absent (7), Not studied (1)	Not studied (8)	35 - 294	1 X	7 Asymptomatic (12-47), Not studied / Deceased (1)

[a] SIx of these are also heterozygous for LGMD2I with no obviously different phenotype

[b] One of these is also heterozygous for LGMD2H with no obviously different phenotype

dystrophy has been observed in the LGMD2I patients in our study but is not a constant feature. No other muscular hypertrophy, including macroglossia, was noted.

Two children presented with reactions to inhalation anaesthetics (succinylcholine / halothane) during dental surgery, one with a masseter spasm and the other with severe rhabdomyolysis. Both of these patients recovered post-operatively. They showed persistently elevated resting SCK levels and muscle biopsies with dystrophic features. Both were subsequently found to be L276I homozygotes. Recently a German LGMD2I patient (compound heterozygote for L276I and V121E) was reported to also have had a malignant hyperthermia-like episode subsequent to inhalation anaesthetic [275]. This suggests that LGMD2I patients, in general, are at risk for reactions to anaesthetic, possibly due to linkage disequilibrium with the *RYR1* locus, in which mutations are known to cause malignant hyperthermia susceptibility (Figure 21).[24]

Unlike LGMD2I, to our knowledge LGMD2H patients have shown none of the following characteristics: muscle hypertrophy, reaction to general anaesthetics, or development of cardiomyopathy. In addition, none of the LGMD patients of either type that we have studied have shown signs of facial weakness or any respiratory symptoms; however, subtle respiratory difficulties cannot be ruled out [62].

Among the Hutterite LGMD families that we studied, there was one individual who was homozygous for *FKRP* L276I and heterozygous for *TRIM32* D487N. This individual (Q II:1, Figure 19) has proximal muscle weakness and a highly elevated SCK level of 9,190 U/L. There were also six individuals who were homozygous for *TRIM32* D487N and heterozygous for *FKRP* L276I, representing two families (a father and daughter from one

family [D III:1 and IV:2] and a set of four siblings from another family [F III:4, III:5, III:6, and III:10])(Figure 5).Individual D II:1 is ambulatory but has proximal muscle weakness, a dystrophic muscle biopsy, and a SCK level of 669 U/L. His daughter is asymptomatic at this time and her SCK level is 267 U/L. In the remaining family, all four siblings have SCK levels ≥10× maximum normal (range 1,700 - 2,960 U/L), one has had a clearly dystrophic muscle biopsy, and two show proximal weakness but they are all ambulatory. Within both of these families there are eight individuals who are heterozygous for both mutations. Seven of these individuals were available for study and were clinically normal. SCK values for these individuals ranged from 35 - 294 U/L.

Recently, a new nuclear family that is part of one of the LGMD2H pedigrees (F, Figure 5) came to our attention. The father, F III:4, had participated in our original LGMD2H study and was identified to be homozygous for the *TRIM32* mutation.[91,278] Due to parental concern of decreased stamina in F IV:10 and IV:11 (10 and 7 yrs), a detailed clinical examination of the entire family was performed and findings are shown in Table 14 (none of these individuals are included in Table 13 with the exception of the father, FIII:4). Both of the boys in question had slender builds with no muscle hypertrophy or atrophy and no joint contractures. There were no objective signs of muscle weakness, normal cranial nerves, a negative Gower sign, and normal running in the clinical setting. The children had normal cardiac and respiratory exams, and echocardiograms showed no evidence of cardiac disease. SCK levels were elevated at 1555 and 1055 U/L respectively (normal <165 U/L). The other 3 sons F IV:9, IV:12, and IV:13 (11, 6 and 4 yrs), had similar builds to their brothers and normal physical examinations. Their SCK levels were 160, 151 and 213 U/L respectively

Table 14: Clinical data on Hutterite family with both LGMD mutations

ID	Current age (yrs)	Age at onset (yrs)	Presenting complaint	Muscle Testing	Cardiac Assessment	Highest Resting CK (U/L)	Other Comments	Current status
F III:4	40	Late twenties	Chronically fatigued legs	Moderate proximal muscle weakness	Normal	2030	Marked trendelenberg gait	Slowly progressive weakness
F III:22	33	NA	None	No sign of atrophy or weakness	Normal	117		Asymptomatic
F IV:9	11	NA	None	No sign of atrophy or weakness	Normal	213		Asymptomatic
F IV:10	10	8	Decreased stamina	No sign of atrophy or weakness	Normal	1055	Actively dystrophic biopsy	Decreased stamina
F IV:11	7	5	Decreased stamina	No sign of atrophy or weakness	Normal	1555		Decreased stamina
F IV:12	6	NA	None	No sign of atrophy or weakness	Normal	151		Asymptomatic
F IV:13	4	NA	None	No sign of atrophy or weakness	Normal	160		Asymptomatic

(normal <165 U/L). Their 33 year old mother, III:22, was also asymptomatic (CK level 117 U/L; normal 28-110 U/L) and their 40 year old father, III:22, had moderate slowly progressive proximal muscle weakness and an SCK level of 2030 U/L (normal 52-175 U/L). A muscle biopsy was performed on individual F IV:10 that showed moderately severe dystrophic changes (see chapter 6, Figure 29).

Genotyping for the two LGMD-causing mutations and surrounding markers was performed. Both parents (F III:4 and III:22) and all five sons (F IV:9–13) were found to be homozygous for the LGMD2H mutation (Figure 23). Genotyping of the LGMD2I mutation revealed that both F III:4 and III:22 were carriers as were their three asymptomatic sons. The two sons with parental concerns of decreased exercise tolerance (IV:10 & IV:11) were also homozygous for the LGMD2I mutation (Figure 23).

There is clearly an enormous amount of variation within our LGMD2H cohort (Table 13). We have identified a number of individuals that are asymptomatic even in their third decade. Our estimate of the average age at onset is about 24 yrs and there is a tendency for males to manifest signs and symptoms earlier and have higher SCK values.[90] It is not surprising to us that the three boys and their mother, unlike the father, are not showing signs of muscular dystrophy, although we expect disease to manifest eventually. There is also a large amount of variation within our LGMD2I cohort, however we estimate the average age at onset to be about 12 yrs (range is 2 - 25 yrs). This appears to explain why only the two boys homozygous for the LGMD2I mutation are symptomatic at a young age.

These observations seem to indicate that while similar, LGMD2H and LGMD2I are different in many respects and can often be differentiated. Also on the basis of patients with

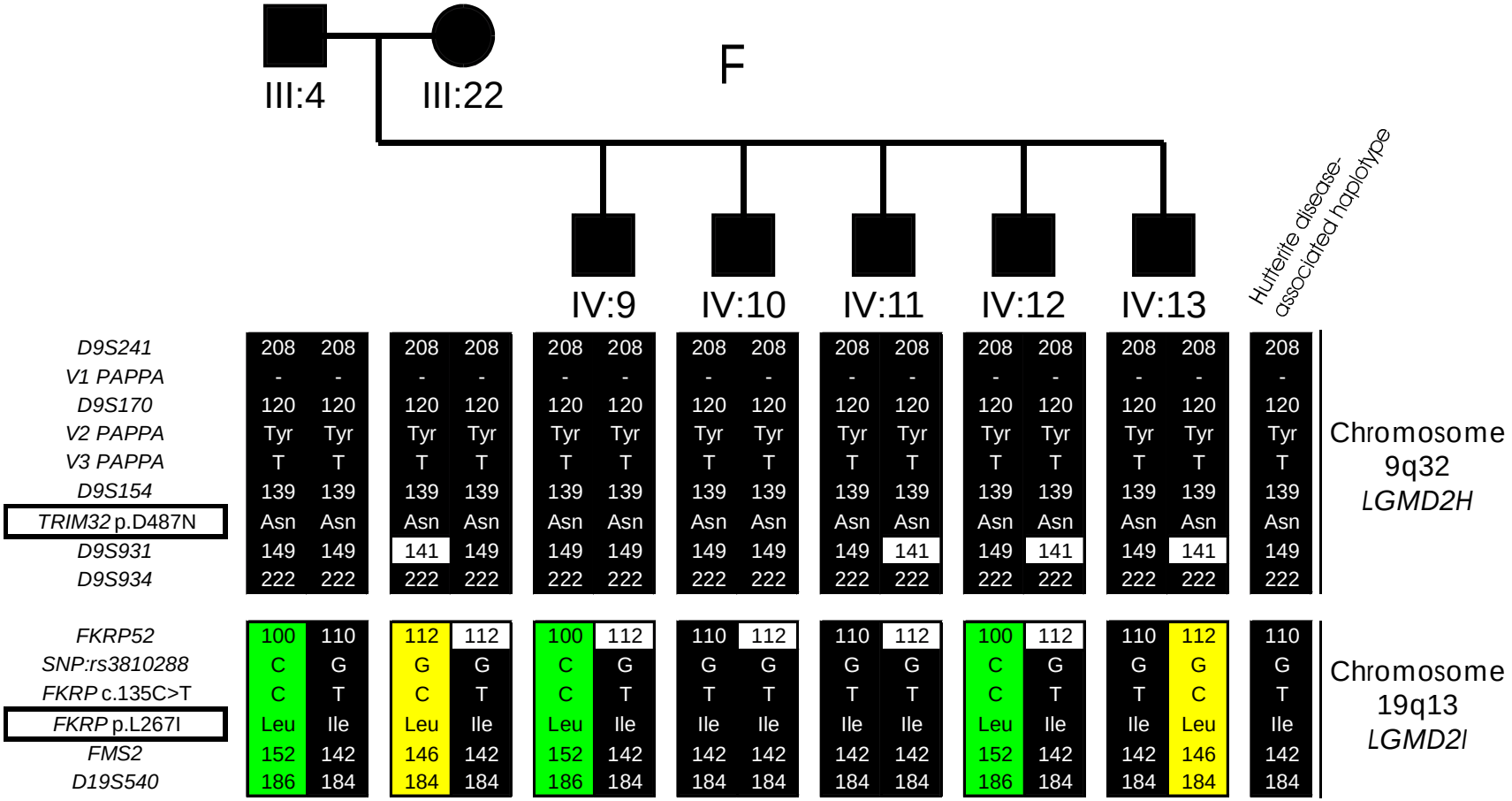


Figure 23: Haplotypes of Hutterite family with both LGMD mutations. Shown are the chromosome regions surrounding the causative mutations of LGMD2H and LGMD2I respectively (in boxes). Alleles corresponding to the consensus Hutterite disease-associated haplotypes are outlined in black, alleles not corresponding are white. Green and yellow haplotypes are the paternal and maternal non-disease-associated haplotypes respectively. Alleles for microsatellite markers are designated by length in bp, single nucleotide polymorphism (SNP) alleles are designated by nucleotide or amino acid (for coding SNPs), and deletions are designated by +/- (not deleted/deleted).

various combinations of genotypes at the two loci it appears that there is very little interaction between them. Even a double homozygote does not differ objectively on physical examination from a homozygote for either LGMD2H or 2I, at least at an early age.

(E) Natural history of the common LGMD2I mutation

In addition to our work involving genotype-phenotype correlations, we wished to determine why the L276I mutation in *FKRP* was so common. The original publication by Brockington et al. contained a figure with numerous haplotypes and they implied that there was a hotspot for mutation. However, closer inspections of the haplotypes revealed that the markers used in the study were widely spaced and we postulated that markers located closer around the L276I mutation might reveal a different picture. We therefore obtained DNA from 19 non-Hutterite LGMD2I patients. Twelve are from the UK, five from Brazil (4 Caucasian, 1 African-Brazilian), one is from Germany, and one is German-Canadian. Fourteen of these patients are homozygous for the *FKRP* L276I mutation and the remaining five are heterozygous for this mutation. The second mutation in four of the five compound heterozygotes is c.928G>T (E310X), c.836G>A (W279X), c.898G>A (V300M), or c.919T>A (Y307N) while no other mutation was identified in the coding sequence of *FKRP* for the remaining heterozygote.

We also discovered four novel markers nearer to the L276I mutation to supplement those used for our gene identification studies. These novel markers were identified by sequence comparison between the two available drafts of the human genome sequence.[151,273] The markers thus identified were *FKRP52*, *rs3810288*, *FKRP* c.135C>T, and *FMS2*. *FKRP52*, a CA/CAA repeat found within an intron of the *PRKD2* gene, was amplified by

polymerase chain reaction (PCR) with FKRP52_F and FKRP52_R.[166] Rs3810288 in the *FKRP* promoter was amplified in a 118 bp fragment using rs3810288_F and rs3810288_R. A mismatch in rs3810288_R creates an *Hpy*188I site (NEB), the C allele yields two fragments when digested (96 bp and 22 bp) and the G allele remains uncut. *FKRP* c.135C>T is a silent variant within codon 45 of *FKRP*. It was amplified in a 702 bp fragment using FKRP_ex.4(1)F and FKRP_ex.4(1)R. The fragment was then digested with *Ngo*MIV (NEB), the C allele yields two fragments (499 bp and 203 bp) and the T allele remains uncut. *FMS2*, a CA repeat found within the 3' UTR of *SLC1A5*, was amplified with FMS2_F and FMS2_R.[137]

Genotypes were obtained and compared to those found in the Hutterite patients. The majority of Hutterites who are homozygous for the L276I mutation are also homozygous for *D19S412* (109 bp), *FKRP52* (110 bp), rs3810288 (G allele), c.135C>T (T allele), *FMS2* (142 bp) and *D19S540* (184 bp) (for example see individuals in Figure 20). This indicates that a genomic segment of about 0.5 Mb is shared among Hutterites carrying L276I and is likely identical by descent from a common ancestor. In comparison to the allele frequencies of our 111 controls (Table 15) there is a strong association of L276I in the non-Hutterite patients with the 110 bp allele of *FKRP52* (52 kb centromeric of L276I), the G allele of rs3810288 (10 kb centromeric of L287I), the T allele of c.135C>T (0.7 kb centromeric of L276I), and the 142 bp allele of *FMS2* (19 kb telomeric of L276I). The association of L276I with the 109 bp allele of *D19S412* (250 kb centromeric from L276I) and the 184 bp allele of *D19S540* (250 kb telomeric of L276I) does not appear as strong (Table 15). The markers flanking this

Table 15: Frequency of L276I-associated alleles

Marker	Allele	Hutterite 276I chromosomes (%)	Non-Hutterite 276I chromosomes (%)	Frequency in control chromosomes (%)	Physical distance from the L276I mutation (bp)[a]
DM	22 repeats	92[b]	0	nd	-986,085
D19S412	109 bp	100	70	36	-248,199
FKRP52	110 bp	97[b]	82	2	-52,237
rs3810288	G	100	97	34	-10,554
FKRP c.135C>T	T	100	100	14	-691
FKRP L276I	Ile	100	100	0	0
FMS2	142 bp	100	94	18	+18,767
D19S540	184 bp	100	79	31	+247,548
D19S606	184 bp	87[b]	10	nd	+714,207

[a]UCSC Human Genome Assembly website (http://genome.ucsc.edu/index.html; April 2003 assembly)
[b]The other observed alleles were *DM* / 5 and 13 repeats; *FKRP52* / 112 bp; *D19S606* / 180 and 182 bp

	Hutterite	NCL-01		NCL-02		NCL-03		NCL-04		NCL-05		NCL-06		NCL-07		NCL-09		NCL-11		G-01		C6.625		C8.749		C10.882		C11.975	
DM	22	6	13	7	7	7	7	12	12	6	6	6	6	12	12	14	15	5	15	13	13	11	11	14	14	0	0	13	13
D19S412	109	99	101	109	109	109	109	109	109	97	97	97	109	109	109	99	109	109	109	109	109	99	109	109	109	109	109	99	111
FKRP52	110	110	110	110	110	110	110	110	110	110	110	110	110	110	110	110	110	110	110	110	110	110	110	108	108	108	108	110	110
SNP:rs3810288	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
FKRP c.135C>T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
FKRP L276I	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile
FMS2	142	142	142	142	142	142	142	142	142	142	142	142	142	142	142	142	142	142	142	142	142	144	142	142	142	142	142	142	148
D19S540	184	188	188	188	188	184	184	184	184	184	184	184	184	184	184	184	184	188	188	184	184	184	184	184	184	184	184	184	186
D19S606	184	180	186	180	180	180	180	180	182	182	184	180	184	180	182	0	0	180	184	0	0	184	184	180	180	180	180	178	178

Compound Heterozygotes	E310X/L276I		W279X/L276I		?	/L276I	V300M/L276I		Y307N/L276I	
	NCL-08		NCL-10		NCL-12		C17.553		G-04	
DM	12	15	10	5	5	5	0	0	11	11
D19S412	109	109	89	99	97	109	111	109	99	109
FKRP52	114	110	100	103	94	110	116	108	103	110
SNP:rs3810288	C	G	C	C	C	G	C	G	C	G
FKRP c.135C>T	C	T	C	T	T	T	C	T	C	T
FKRP L276I	Leu	Ile	Leu	Ile	Leu	Ile	Leu	Ile	Leu	Ile
FMS2	148	142	146	142	142	142	152	142	144	142
D19S540	190	184	186	184	184	184	184	184	184	184
D19S606	0	0	178	180	180	180	186	180	0	0

Figure 24: Genotypes of non-Hutterite LGMD2I patients. Markers are spread across a 1.7 Mb region flanking *FKRP*. The Hutterite haplotype corresponds to the most frequent haplotype in the Hutterites. NCL-01 through 12 are Caucasian patients collected in the UK. C6.625, C8.749, C11.975 and C17.553 are Caucasian Brazilian patients and C10.882 isAfrican-Brazilian . G-01 is German and G-04 is German-Canadian. Black regions indicate alleles that are consistent with the consensus Hutterite haplotype; grey shading indicates alleles that are one repeat unit different and may have arisen by slipped mispairing during DNA replication; boxed areas indicate alleles that were phased by PCR cloning. Alleles for microsatellite markers are designated by length in bp except for *DM* which is reported in the number of CAG repeats. For the c.135C>T and rs3810288 alleles are designated by nucleotide and for *FKRP* L276I alleles are designated by amino acid.

region (*DM*, 1 Mb centromeric and *D19S606*, 0.7 Mb telomeric) show no association with L276I in the patients that we studied (Table 15).

Using these genotypes we were able to construct probable haplotypes in these non-Hutterite LGMD2I patients (Figure 24). Recombination appears to have occurred between L276I and *FMS2* on one chromosome (C11.975) and between rs3810288 and c.135C>T on another (NCL-10). This results in a very small common core haplotype consisting of the mutation itself and c.135C>T. The likelihood that this set of associations has occurred by chance is low and reflects strong linkage disequilibrium. Thus, L276I appears to have arisen only once and is identical by descent in most, and possibly all patients. The presence of a homozygous African-Brazilian individual showing these same associations (C10.882) raises the possibility that the mutation is not specific to Caucasians. However, with our limited data this cannot be conclusively determined, particularly in light of the extreme amount of admixture amongst Brazilians [202].

Of note are the discrepancies present at the *FKRP52* locus. A possible mutation in *FKRP52* due to slipped strand mispairing during DNA replication is present on five of the nine Brazilian chromosomes (110 bp > 108 bp). This suggests a recent Brazilian mutational event in a common ancestor of three of these five Brazilian patients [58]. In addition, within the Hutterite population there are also individuals with a 112 bp allele instead of a 110 bp allele on the same haplotype as the L276I mutation. Through cloning and sequencing we have found a large amount of variation in *FKRP52* (Figure 25). There is variation in both a dinucleotide (CA) and trinucleotide (CAA) stretch between the *FKRP52* primers, however, trinucleotide variation is much less common. We have found that the range of variation in

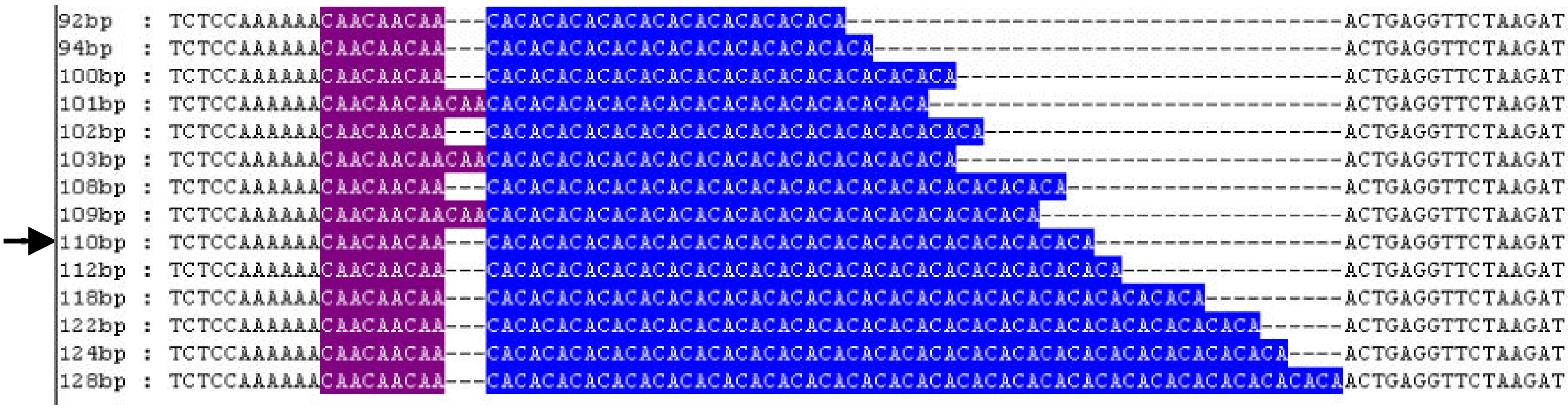


Figure 25: Sequences of selected *FKRP52* alleles. Genomic DNA from patients and controls (both homozygous and heterozygous) was used to amplify the *FKRP52* locus and fragments were PCR cloned. Cloneswereanalysed on a polyacrylamide gel to identify both alleles in heterozygotes (individuals were chosen on the basis of large size differences between the two alleles to allow for differentiation) and one of each was sequenced. Only a portion of the entire fragment is shown, the full size of each is shown on the left side. The arrow points to the allele that is in linkage disequilibrium with the L276I mutation in *FKRP*. Note the presence of two polymorphic stretches, one is a CAA repeat (purple) and the other a CArepeat (blue).

the CAA stretch is limited to two possibilities (three or four CAA repeats, four being rare), whereas the range of variation in the CA stretch is much greater (13 to 31 CA repeats). The net result is allele sizes in the range of 92 - 128 bp, with variation in the CAA stretch showing up as odd-sized alleles within this range. Seventeen different alleles were found to be present in our 111 control samples at frequencies ranging from 0.5% to 26.1%. This hypervariability readily explains the discrepancies that we have detected at this locus in our patient samples.

5.3 Discussion

We have demonstrated unexpected locus heterogeneity for LGMD in the Hutterite population, and have identified patients who are homozygous for a missense mutation in *TRIM32* or homozygous for a missense mutation in *FKRP*. This provides another example of genetic heterogeneity of an autosomal recessive disease in a genetically isolated population. Both locus and allelic heterogeneity were found for LGMD in the Amish, another Anabaptist isolate.[68] At this time, there is no evidence for a third locus causing LGMD in this population.

LGMD2H appears to be more frequent in the Schmiedeleut subdivision of the Hutterites, whereas LGMD2I appears to be more frequent in the Dariusleut. Currently, we do not have an accurate estimate of the relative frequencies of the two mutations in each of the subdivisions. Overall, we have identified 74 Hutterite LGMD patients.

There is considerable clinical heterogeneity for both LGMD2H and LGMD2I in spite of a uniform communal lifestyle and only one mutation for each of the LGMDs. A wide spectrum in clinical severity has been previously reported for LGMD2I and ascribed to the

various mutations found in compound heterozygotes.[174] We have similarly large clinical variation although all our patients are homozygous for the *FKRP* L276I mutation. This was also seen in a recent study by Walter et al. (2004) in which 13/20 patients from nonconsanguinous matings were homozygous for this mutation and showed similar clinical variability.[275] Our impression is that patients with LGMD2I present earlier, follow a more severe course with possible cardiomyopathy,[207] and have higher SCK levels than LGMD2H patients. In addition there is no indication of any interaction between the two loci, at least at a young age, as individuals with mutations at both loci are indistinguishable from those with mutations at only one locus (Table 13 and 14). This is not surprising due to the apparently different mechanisms by which these genes appear to cause muscular dystrophy.[74,91]

It appears that all Hutterite LGMDs are of either type 2H or type 2I. This will make it possible to provide accurate non-invasive diagnostic and carrier testing for LGMD in Hutterites. Such a DNA-based approach is not yet practical for the non-Hutterite LGMD population because of the marked locus and allelic heterogeneity. Given the high incidence of LGMD2I carriers in the Hutterite population, and the risk of cardiomyopathy and anaesthetic reactions in this group, we would suggest that genetic testing of at-risk individuals even below the normal age of consent for such testing should be considered and discussed with the families.

The *FKRP* L276I mutation appears to be common worldwide, with respect to LGMD-causing mutations. Carrier frequency is estimated to be 1/306 on the basis of controls typed for L276I.[37,58,90,275] In addition, patients homozygous for L276I from 28 non-consanguineous families have been reported.[37,208,275] Our analysis indicates that L276I

may be a founder mutation, as all Hutterite and non-Hutterite disease chromosomes tested to date carry the low frequency T allele (14%) at an intragenic SNP (c.135C>T) and the G allele at rs3810288 in the putative promoter of FKRP (with one exception). The L276I mutation with the C allele at rs3810288 (NCL-10, Figure 24) is consistent with being a recombinant chromosome but due to the lack of phase information for most of the genotypes this cannot be firmly established. The T allele of c.135C>T and the G allele of rs3810288 have been shown to be associated with a further 26 L276I chromosomes from German patients,[275] strengthening the evidence for a founder mutation. Markers as far away from the L276I mutation as 0.25 Mb in each direction show readily detectable linkage disequilibrium; however, markers 0.75 - 1.0 Mb away show very little linkage disequilibrium in the samples used in this study. This is strong evidence that L276I has arisen only once. The small common core haplotype is an indication that the mutation may have occurred long ago. Further analysis of SNPs in and around *FKRP* and additional patients will be needed to confirm that most, if not all, copies of L276I in the contemporary population are identical by descent and to accurately estimate the age of the mutation.

The relative frequency of L276I in Caucasians, compared to other LGMD-causing mutations, and the high likelihood that it is a founder mutation are readily explained by genetic drift. However, given the postulated function of *FKRP* it is tempting to speculate that a selective advantage may also be contributing to the maintenance of this allele. FKRP is thought to be a glycosyltransferase and mutations affect the glycosylation of " -dystroglycan, an essential component of muscle cell membranes.[70,74,122] An immunoblot of muscle from L276I homozygotes showed a decreased level of fully glycosylated " -dystroglycan.[39,90,275] " -

Dystroglycan is known to be expressed in numerous tissues and has been shown to be the receptor for the entry of two different types of pathogens.[45,71,210] It is possible that an L276I heterozygote with a mild defect in glycosylation may have a partial resistance to these or other pathogens. Over long periods this advantage would then increase the prevalence of the L276I allele in areas where the pathogen is endemic. On the basis of the data presented here it appears that the frequent occurrence of L276I is not the result of multiple 'de novo' mutations as was previously thought.[43] Instead, our findings suggest this mutation has occurred once and became prevalent through either genetic drift, selective advantage, or some combination of both.

6. Identification of the STM gene

Most of the data presented in this chapter were published in the following article:

- *B.G.H. Schoser,* ***P. Frosk*** *, A.G. Engel, U Klutzny, H. Lochmüller, K. Wrogemann. Commonality of TRIM32 mutation in causing sarcotubular myopathy and LGMD2H. (2005) Ann. Neurol. 57:591-595.*

I would like to acknowledge the contributions of the following individuals: (1) Isha rivera and Jossette Douville for determination of some genotypes, (2) Drs. Marc Del Bigio and Andrew Engel for the preparation of patient muscle samples and pathological examination, and (3) Dr. Benedikt Schoser for noticing the possible connection between Hutterite LGMD and STM and kindly initiating an ongoing collaboration with our group. Note, all of the detailed genetic aspects of this study were performed by our laboratory and the submitted version of the manuscript was largely written by me.

6.1 Introduction

Sarcotubular myopathy (STM) was originally described in 1973 by Jerusalem and coworkers as a congenital disorder in two Hutterite brothers aged 11 and 15 years.[135] Both boys had mild to moderate muscle weakness and wasting (predominantly proximal and symmetrical) and experienced minor difficulties on strenuous activity. SCK levels were normal in one brother (X II:4) and moderately elevated to 360 U/L in the other (X II:2, normal < 71 U/L) (Figure 26). The disorder was thought to be autosomal recessive in nature because the parents of the two boys were asymptomatic as well as consanguineous. STM was distinguished from other myopathies by its unique structural features.[135,185]

The second report of STM was of two brothers aged 33 and 35 yrs from a small village in Southern Germany.[185] The younger brother (W II:3, Figure 26) had moderate proximal muscle weakness by age 8 yrs. By age 31, he could walk no further than 10-20 m, experienced increasingly severe exercise-induced myalgias, and had winged scapulae, hypertrophied calf muscles, and a positive Gower sign. The older brother (W II:2, Figure 26) had a more benign course with only slight weakness, mild exercise-induced myalgias, and moderate calf hypertrophy. Both had elevated SCK levels, W II:3 to 430 U/L and W II:2 to 1546 U/L (normal <80 U/L). Muscle biopsy studies demonstrated the same vacuolar changes observed by Jerusalem and coworkers in the two Hutterite brothers as well as other myopathic features.

As mentioned previously, in 1976, the original description of autosomal recessive LGMD in Manitoba Hutterites was reported.[232] The eleven affected individuals had a slowly progressive proximal weakness and wasting with mild to moderately elevated SCK levels

(57 - 562 U/L, normal <40-50). The facial muscles were also affected and it was concluded that the disorder combined phenotypic features of limb girdle (LGMD) and facioscapulohumeral (FSHD) dystrophies. The muscle fibres varied in size and contained an increased number of internal nuclei, and some fibres harbored small vacuoles, although no connection to sarcotubular myopathy was made. A similar paper was published in 1985 by the same physician regarding LGMD in Saskatchewan Hutterites and it was presumed to be the same disorder.[233] Our group followed-up on these publications as described in chapter 4 and 5. We discovered that there were in fact two disorders, LGMD2H and LGMD2I, and they were caused by mutations in *TRIM32* and *FKRP* respectively.[90,91]

A physician treating the two German brothers hypothesized that STM may be caused by one of the above two LGMD-causing mutations found in the Hutterites. This was mainly because the original STM family was of Hutterite origin and the clinical presentation of STM was like that of an LGMD. Our group was contacted and we began a collaboration to determine if this hypothesis was indeed true.

6.2 Results

(A) Subjects

We were able to obtain genomic DNA from the four reported STM patients as well as their families through collaboration with Dr. Andrew Engel, one of the authors of the original Hutterite STM paper (family X), and Dr. Benedikt Schoser, the physician treating the two German STM patients (family W, Figure 26). Informed consent in writing was obtained from patients and their families with the exception of one of the Hutterite brothers who is deceased (X II:4). His death appears to be independent from the myopathy (Dr. C.R.

Greenberg, personal information). Dr. Engel was able to extract DNA (Qiagen) from muscle tissue of this individual that was stored at the Mayo Clinic and sent it to our laboratory for genotyping.

(B) DNA analysis

We genotyped the Hutterite (pedigree X) and German (pedigree W) families for the D487N mutation in *TRIM32* and L276I in *FKRP* by the methods described in chapters 4 and 5 respectively. All four patients (W II:2, W II:3, X II:2, and X II:4) proved to be homozygous for the D487N mutation and none harbored the L276I mutation. Genotypes were also obtained for ten markers surrounding *TRIM32*, a subset of the closest markers used in the LGMD2H study. Haplotypes were constructed and they revealed not only that the two families have the same mutation but also that it was on a chromosome that shared identity by descent with the original LGMD2H disease chromosomes (Figure 27).

(C) Muscle Pathology

In all STM patients, segments of many muscle fibers harbored a myriad of small abnormal spaces (Figure 28A & B). Electron microscopy studies revealed that the smallest abnormal spaces arose from focal dilations of the sarcoplasmic reticulum (Figure 28D). Coalescence of the smaller vacuoles gave rise to larger ones whose limiting membranes often degenerated (Figure 28C). The membranes limiting the vacuoles showed sarcoplasmic reticulum (SR)-associated ATPase reactivity (Figure 28D),[255] confirming that the vacuoles arose from the SR. Regenerating or necrotic fibers were not observed and inflammatory changes were absent in the original Hutterite STM patients. However, the two German STM

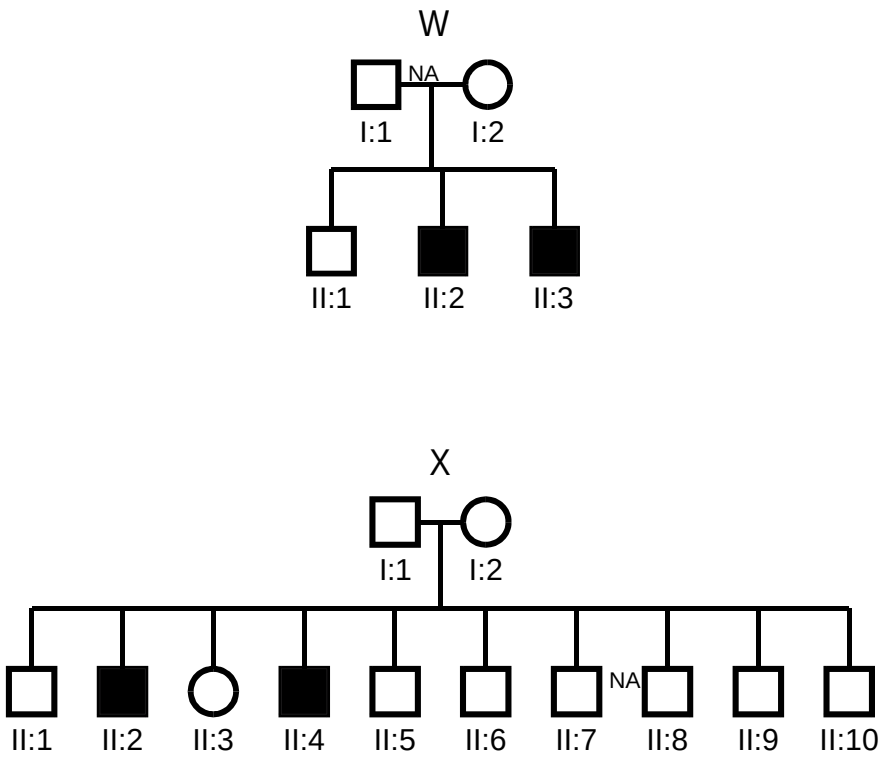


Figure 26: STM pedigrees. Each pedigree is assigned an identifier (W & X) and individuals are numbered according to generation. Birth order has been randomized to protect anonymity. Individuals denoted with an NA were not available for study. Family W is non-Hutterite from a small Southern German village and family X is a Hutterite family from South Dakota (Schmiedeleut). Individual X II:4 is deceased but DNA was available for study.

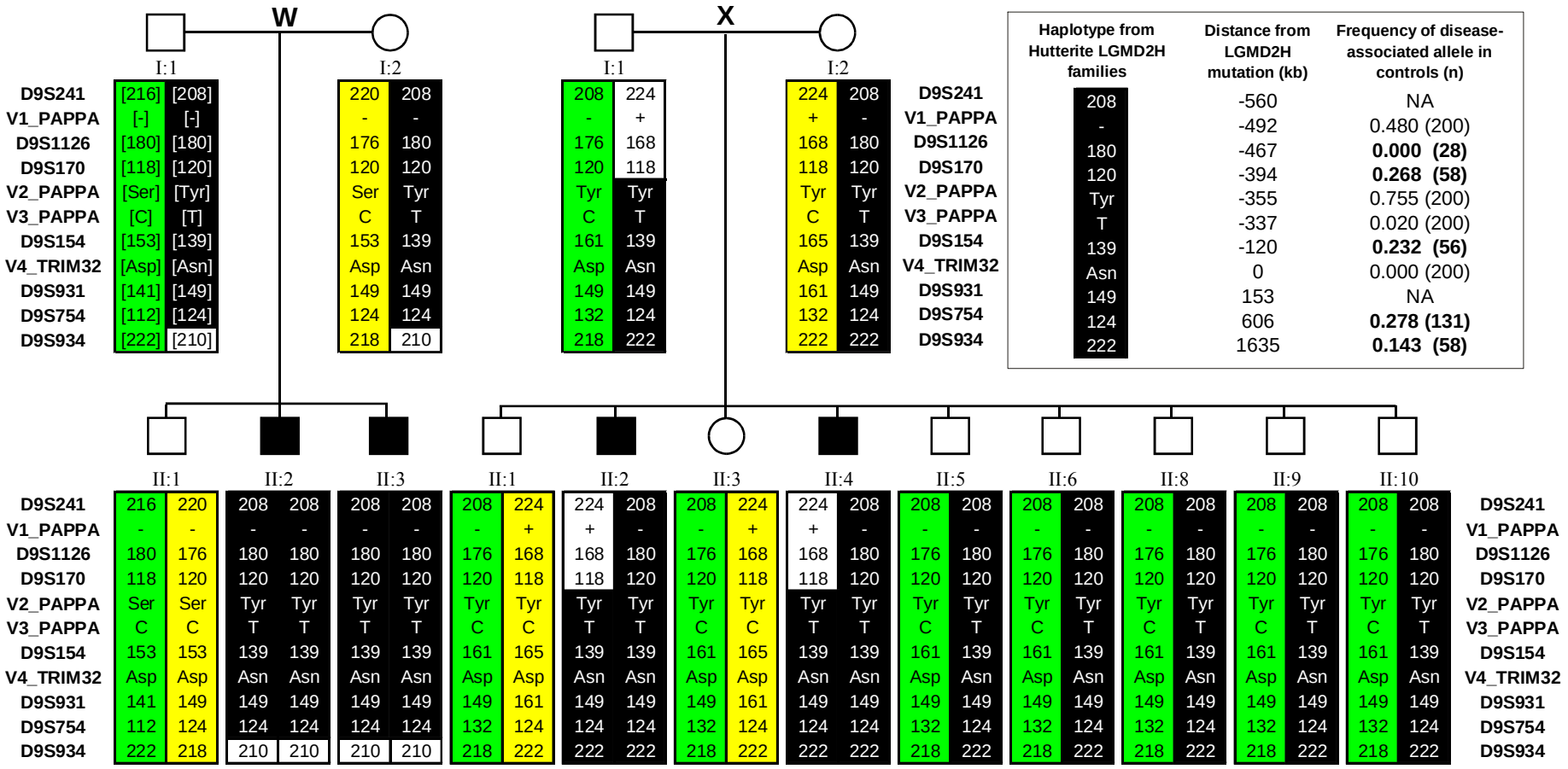


Figure 27: Chromosome region 9q31-33 haplotypes in STM families. STM is caused by the same mutation as LGMD2H. This mutation is on the same chromosomal background despite the fact that family W is not Hutterite. Alleles are given in bp, 0 indicates no data. Black background on haplotypes indicates consensus disease-associated alleles, white indicates non-consensus disease associated alleles, and yellow / green indicate a normal parental chromosome. Alleles enclosed in square brackets are inferred. V4 is the causative mutation of LGMD2H, the remaining variants are described in chapter 4.

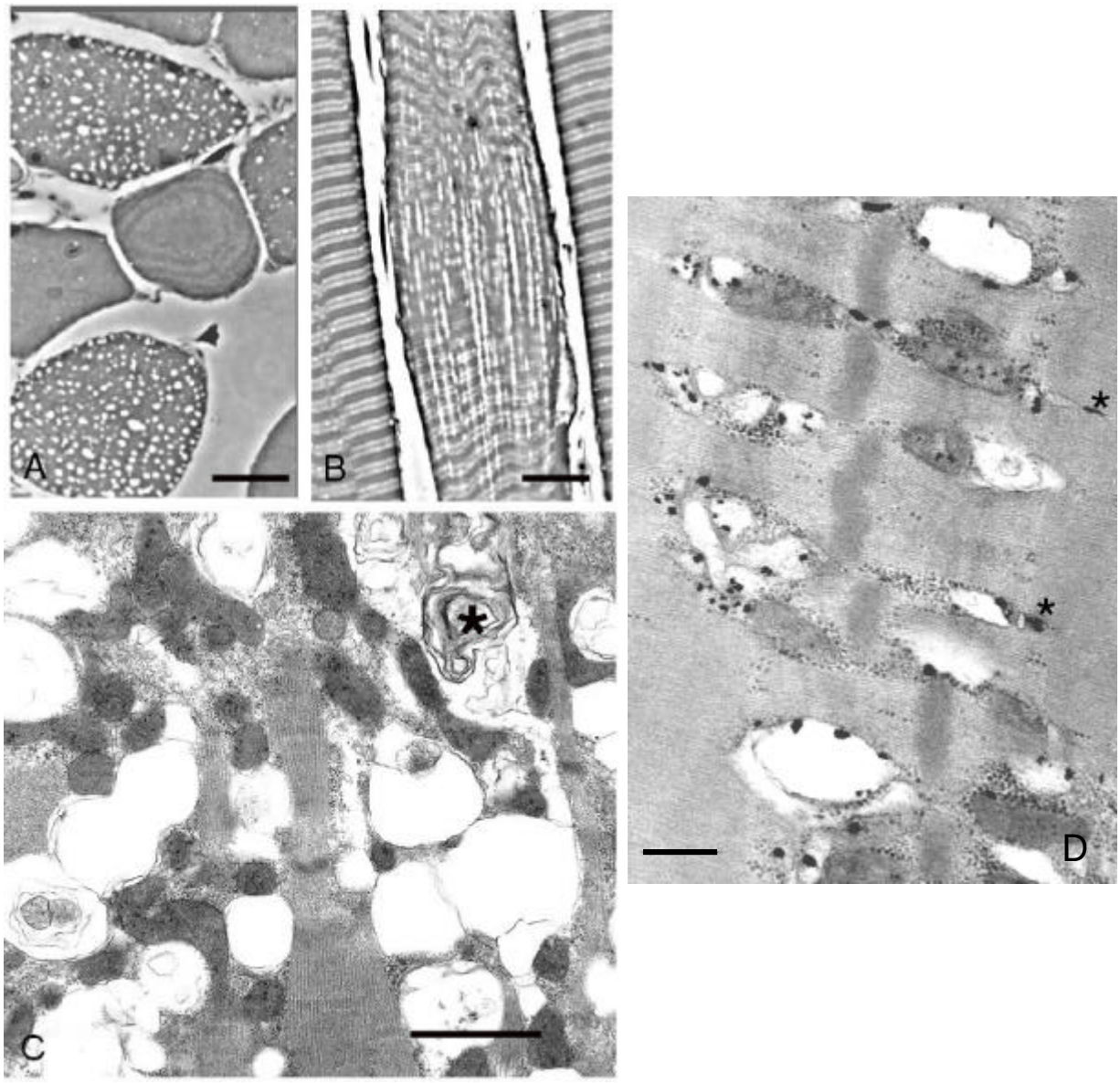


Figure 28: Muscle pathology of the original Hutterite STM family. (A) and (B) 1 μm-thick resin sections reveal myriad small abnormal spaces in a proportion of the muscle fibers. Note segmental distribution of the abnormal spaces. (C) Electron microscopy shows coalescing membrane bound spaces. Some of the limiting membranes are degenerating (asterisk). Bars = 10 μm in (A) and (B), and 1 μm in (C). (D) Electron cytochemical localization of sarcoplasmic reticulum (SR)-associated ATPase by the method of Tice and Engel (1966).[255] The black reaction product is associated with normal (asterisk) as well as dilated components of the SR. Bar = 0.5 μm. Note, all samples were prepared and analysed in the laboratory of Dr. Andrew Engel.

patients had other myopathic features that included abnormal variation of muscle fiber diameters, an increased number of internally located nuclei, and small foci of Z disk streaming.[185]

In light of the genotyping results, our most recent Hutterite LGMD biopsy was re-examined to see if we could identify similar pathology to the STM patients. Even though it was from individual F IV:10 (Figure 5), a homozygote for both the LGMD2I and LGMD2H mutations (see chapter 5), it was used because there was an abundance of material available for study. The muscle showed moderately severe dystrophic changes with increased fiber size variation, scattered necrotic fibers, regenerating fibers, negligible fibrosis, and segmental vacuolation in at least 5% of fibers. Vacuoles were not associated with acid phosphatase activity and did not stain with periodic acid-Schiff. Electron microscopic examination demonstrated clusters of empty vacuoles associated with membranes. In addition, a few otherwise intact fibers had degenerating nuclei with condensed heterochromatin (apoptosis-like) immediately next to normal nuclei, a very unusual finding (Figure 30). Preliminary results from other biopsies suggest that vacuolation is present in many other LGMD2H patients but there is a wide range from very few vacuoles to amounts rivalling the STM patients. This vacuolation is clearly not present in the muscle of the one LGMD2I individual that was studied (Figure 31).

6.3 Discussion

The four STM patients were found to be homozygous for the D487N mutation in *TRIM32*. Haplotyping revealed that the chromosomal region surrounding *TRIM32* is identical

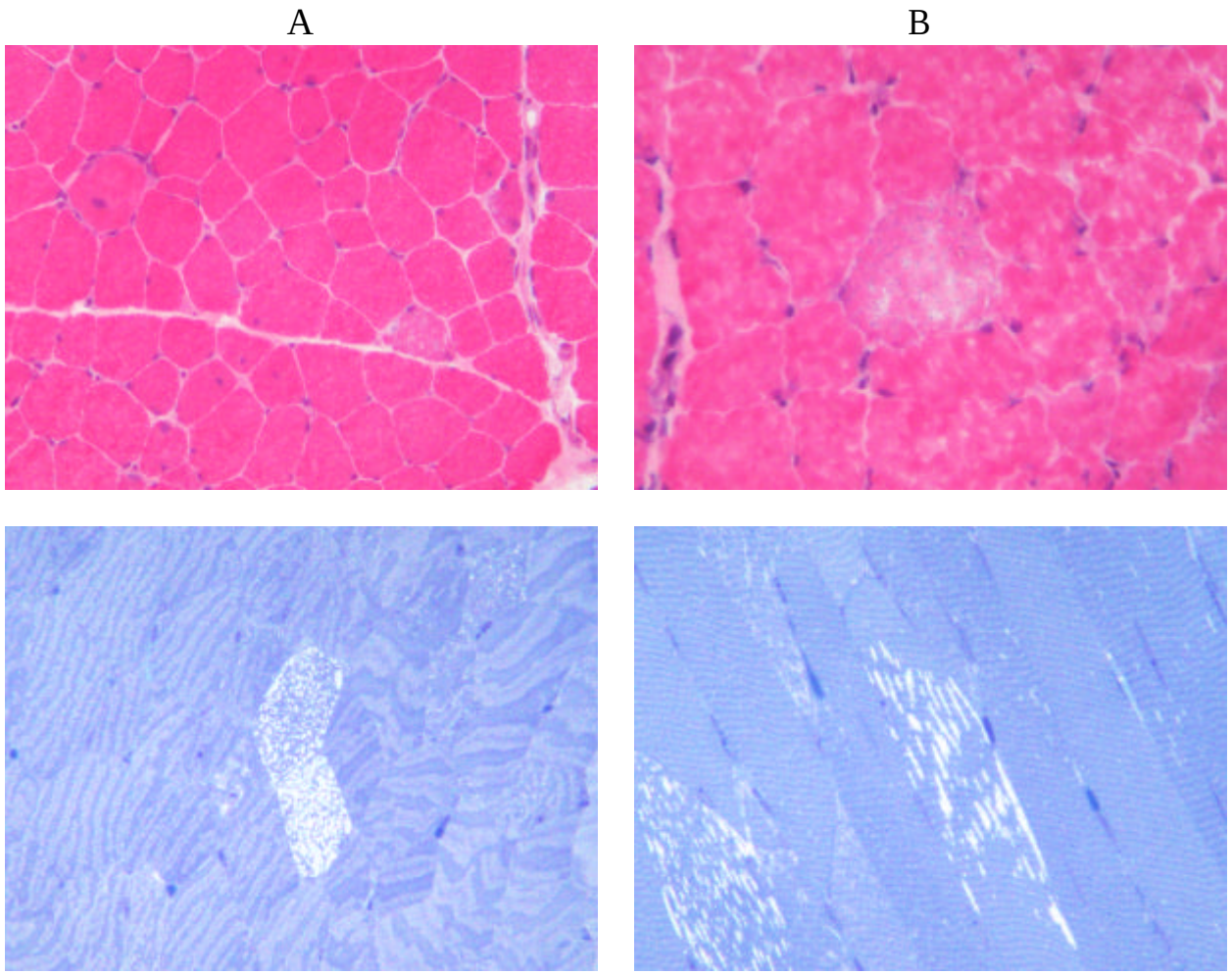


Figure 29: Muscle biopsy from individual F IV:10 (figure 23), a homozygote for both the LGMD2H and LGMD2I mutations. Panels A and B show photomicrographs of cross-sections of frozen muscle stained with hematoxylin & eosin at 20X and 40X magnification respectively. There is increased fiber size variation and rare fibers with internal nuclei. Panels C and D show photomicrographs of plastic sections in cross (C) and longitudinal (D) orientation taken at 40X magnification. Small empty appearing vacuoles are present in a subset of fibers.Note, samples were prepared and analysed in the laboratory of Dr. Marc Del Bigio.

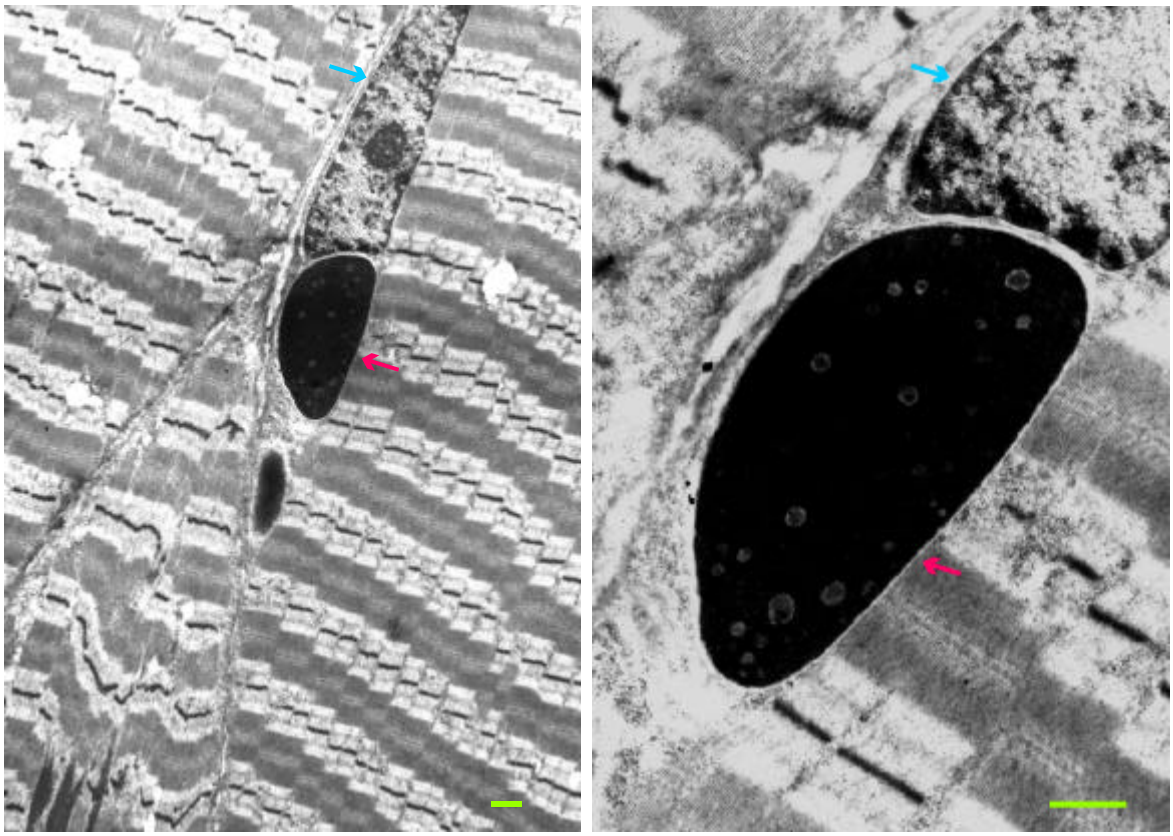


Figure 30: Electron micrograph of a nucleus undergoing apoptosis. Prepared from the same biopsy specimen as figure 29. A dark, degenerating nucleus with morphologic features of apoptosis is shown (red arrow). The nucleus has defined foci of chromatin condensation, an unusual finding in muscle. Adjacent is an apparently normal nucleus in the very same fiber (blue arrow). The green bars in the lower right corners correspond to 0.4 μm. Note, samples were prepared and analysed in the laboratory of Dr. Marc Del Bigio.

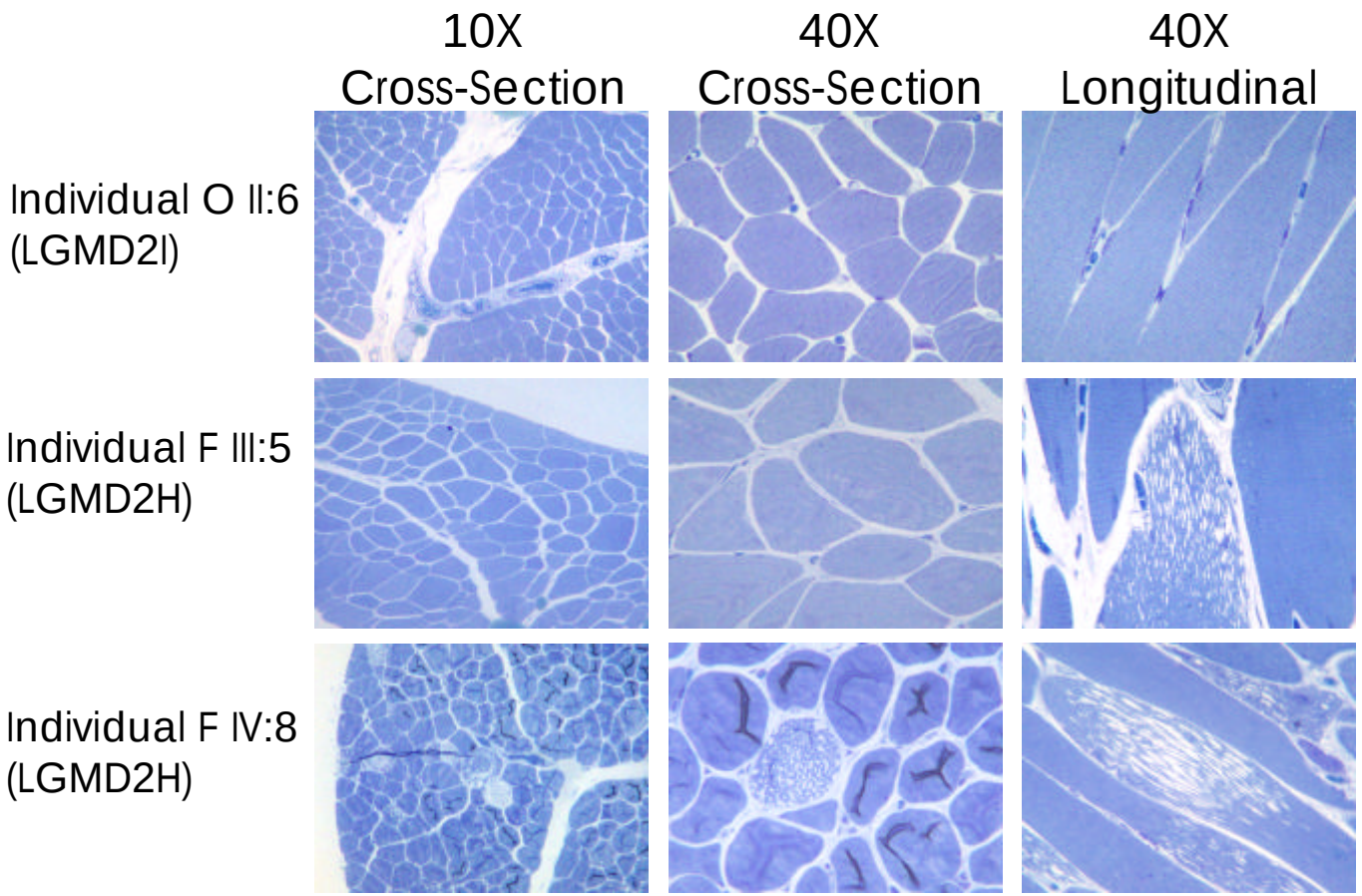


Figure 31: Muscle biopsies from a selection of LGMD2H and LGMD2I patients. All panels show photomicrographs of plastic sections, cross-sectional or longitudinal, and at the indicatedmagnification. Empty appearing vacoules are present in themuscle of the two individuals with LGMD2H but not in the muscle of the patient with LGMD2I. Note the dark fold appearing in the tissue of individual F IV:8 are preparatory artifacts. Note, all sampleswereprepared and analysed in the laboratory of Dr.MarcDelBigio.

by descent in all STM and LGMD2H patients. This is surprising because the German family has no apparent connection with the Hutterite population other than the fact the Hutterites are largely Germanic in origin. This raises the possibility that the D487N mutation arose prior to the emergence of the Hutterite religion in central Europe in the 16th century.

We hypothesize that STM and LGMD2H are the same disorder. The phenotypic variability is large amongst the LGMD2H cohort, from virtually asymptomatic to some older individuals that require a wheelchair for ambulation. As such, there is little to differentiate them from the STM patients clinically. Our initial impressions from examining muscle specimens of LGMD2H patients is that the vacuolar pathology is not usually present at the frequency or severity that it is in the two families classified as having STM. However, ascertainment of the vacuolar change requires artifact free sections and the minute size of the vacuoles (most smaller than 4 ：m) may limit their detection in 10-：m thick sections. To address this hypothesis, we are conducting a detailed retrospective study of available LGMD2H muscle specimens and are planning to obtain follow-up biopsies on some patients.

A given mutation may lead to different phenotypes in different families and even within the same family. For instance, all four different caveolinopathy phenotypes can arise from the same amino acid substitution (chapter 2).[282] If our hypothesis is correct, then the D487N mutation is associated with a spectrum of vacuolar pathology and STM may prove to be the more histologically severe variant. At this time it is not known whether the presence or absence of vacuoles has an effect on the progression of the disease or on patient prognosis.

As discussed in chapter 4 initial evidence suggests that TRIM32 is an E3-ubiquitin ligase and may have anti-apoptotic activity, which may relate to the presence of a potential

apoptotic nucleus in the muscle of an LGMD2H patient.[124] Perhaps even more interesting is that the vacuoles present in LGMD2H / STM muscle may originate from the sarcoplasmic reticulum. This finding suggests that TRIM32 is likely to be involved in the generation of the SR system or in maintaining its structural integrity. This ties in to the data that we collected using the bacterial two hybrid system where we found a number of SR membrane and lumen proteins that potentially interact with TRIM32. The presence of these vacuoles reinforces the idea that TRIM32 may be intimately involved with the SR and SR-associated proteins.

7. Conclusions and future directions

Since the advent of positional cloning, muscular dystrophies as a group have received an enormous amount of attention. Historically, the dystrophin gene which causes Duchenne muscular dystrophy, was the first major human disease gene to be identified solely on the basis of its chromosomal position. Since that time the genetics of muscle disorders have been very fruitful because of the large amount of heterogeneity. With each new gene identified we not only begin to understand more about muscle function in health and disease, but it becomes even clearer how much we do not know regarding this complex tissue.

Another thing that has become clear is that our classification systems for these disorders are sorely lacking. As I have shown in chapter 2, even in a small subset like the LGMDs there is considerable phenotypic overlap between disorders caused by mutations at different loci, considerable phenotypic differences between individuals with mutation at the same locus, and even different modes of inheritance for a given locus depending on the mutation. Of the thirteen LGMDs with an identified disease gene, seven are known to cause at least one other distinct disorder depending on the mutation (eg/laminopathies) or on the genetic context in which the mutation is expressed (eg/dytrophinopathies). The barriers between simple differentiation schemes like, myopathy versus muscular dystrophy and proximal versus distal, are quickly breaking down. There is no easy solution for this but perhaps a combined clinical and pathological approach to classification would yield the most useful results.

The study of large kindreds segregating for muscle disorders has been crucial in the development of our current state of knowledge and remains important, as this study shows.

We have been able to define at the molecular level the cause of all myopathies of increased prevalence in the Hutterite population. It is currently possible for us to diagnose any potential Hutterite patient without resorting to invasive muscle biopsy by simply testing them for the mutations in *TRIM32* and *FKRP*. We have provided over 200 Hutterite individuals with an accurate genotype and diagnosis in the process of discovering these two genes. DNA-based diagnosis like this is currently not feasible in the general population due to the sheer number of mutations that would have to be assayed for and the high probability of novel mutations. The next step is to develop a way in which to easily identify other patients (non-Hutterite) with mutations at the same loci without having to screen for novel mutations in every potential patient. This is normally done through the development of an antibody that can be used to screen muscle biopsies, not only as a way to identify novel mutations but also for use in diagnosis.

For LGMD2H / STM this has not yet been realized. The antibody against TRIM32 we have produced in this study is limited in amount and preliminary results indicate that this antibody detects no differences in the expression level or distribution of TRIM32 between affected and control cells. While there is a need for better antibodies toward TRIM32, particularly with more utility for immunoblotting, it is not yet clear how they could be used in a clinical setting. The assay for D487N in *TRIM32* has some use outside of the Hutterites with the discovery of the German STM patients but this mutation is not likely to be particularly common amongst the general population. The characteristic vacuolar pathology seen in LGMD2H / STM muscle is currently the most promising way to identify patients for *TRIM32* screening. Finding patients in this manner is not perfect however since we do not

know that every mutation in *TRIM32* will cause such pathology and it is not always obvious that these vacuoles are present due to their small size. We have already begun screening such patients although we have not yet identified any novel mutations in *TRIM32* this way.

In the case of LGMD2I, such an antibody already exists in the form of an anti-glycosylated "-dystroglycan antibody.[176] This antibody also has the advantage that it will detect any of the disorders that result in "-dystroglycan hypoglycosylation and is clearly a useful clinical tool. In addition, due to the prevalence of the L276I mutation in *FKRP*, a specific assay for this one mutation would also have utility outside of the Hutterite population.

The research potential for both of these genes is quite large. *FKRP* is part of a group genes causing disorders that result from a defect in O-glycosylation and as such there are many tools available to researchers in this area. At the same time there are also many groups working on *FKRP* and there is already quite a bit known about this gene. Very little is known about *TRIM32,* both its function and the mechanism by which it causes disease when mutated are obscure. Since our group is in a very good position to study *TRIM32* it is important that we concentrate our efforts in this area.

Many avenues are open in the study of TRIM32 function and the pathogenic mechanism underlying LGMD2H / STM. All the current evidence points to TRIM32 being an E3 ubiquitin ligase. A recent study of all 469 RING finger proteins in the *Arabidopsis thaliana* genome showed that the vast majority have detectable ubiquitin ligase activity.[249] In addition, the study by Horn et al. linking TRIM32 to skin cancer provided some evidence that TRIM32 is an E3 ubiquitin ligase.[124] An enzyme assay will be necessary before TRIM32

can be definitively classified as an E3 but a compatible E2 enzyme will have to be identified first. Nevertheless it is extremely likely that TRIM32 is an E3. If so, the hypothesis proposed in chapter four is probably valid, in LGMD2H / STM a protein(s) that undergoes TRIM32-mediated ubiquitination is no longer being degraded by the proteasome and as such accumulates to toxic levels. The most pressing question is what this substrate is and how does it cause its toxic effect.

One possibility is that the key substrate that is being controlled by TRIM32 is pro-apoptotic. This would explain the observations regarding skin cancer whereby increased levels of TRIM32 in skin cells prolong cell survival and promote transformation.[124] With defective TRIM32 one would expect this pro-apoptotic factor to accumulate and induce apoptosis. This may explain the apoptotic nucleus found in the biopsy of an LGMD2H patient (Figure 30). This process may occur in mature fibers as suggested by this electron microscopy or in satellite cells as suggested by our immunofluorescence work (Figure 16).

In addition, it could be that the key substrate of TRIM32 travels through the sarcoplasmic reticulum and is therefore either a secreted protein, a transmembrane protein, or is within a membrane-bound organelle (excluding the nucleus and mitochondria). This is based on the observation that the vacuoles found in LGMD2H / STM muscle are of SR origin and four SR resident proteins were identified via interaction screening (*CASQ1*, *HERPUD1*, *LOC360310*, and *LOC305234*). Particularly interesting in this respect was the finding of *HERPUD1* (Table 10) as a binding partner of TRIM32. This SR transmembrane protein lies largely in the cytoplasm and is thought to recruit the 26S proteasome to the surface of the SR during periods of stress (accumulations of misfolded proteins within the

lumen of the SR).[271] Since the process of degrading these misfolded proteins requires ubiquitination prior to proteasomal action it is not unreasonable to hypothesize that E3 enzymes such as TRIM32 would be required at these same sites. If one takes these observations into account the most likely candidates for the key substrate are muscle membrane proteins already known to be involved in muscular dystrophies. Caveolin-3 is a particularly attractive candidate as it is already known to be a folded in the SR,[123] degraded by the ubiquitin system in pathological states,[94,95] has the tendency to aggregate,[123] causes a myopathy when there are increased levels present at the membrane,[96] and also leads to impaired fusion when at increased levels in myoblasts.[274] Studies are ongoing to determine if there are increased levels of caveolin-3 in LGMD2H / STM patient samples.

Regardless of the ultimate mechanism underlying the pathogenesis of LGMD2H / STM there are a number of things that must be done. The first is to obtain an unlimited source of anti-TRIM32 antibody that is more active via immunoblot. We are currently working to obtain such antibodies through the production of monoclonals. Next we need to fully confirm the potential interacting proteins that were identified by bacterial two hybrid screen. We will not be able to provide convincing evidence that any of the identified proteins are binding partners or possible substrates unless we can show that they truly interact and to define the regions that are interacting. Two of the more promising interaction candidates, *HERPUD1* and *SYNPO2* (see chapter four discussion), are currently being tested through biochemical means and the remaining clones will be confirmed via the new bacterial two hybrid reporter strain (Stratagene). We will eventually need an animal model of LGMD2H / STM to fully realize the potential of work in this area and are working with collaborators

to produce mouse lines that recapitulate the LGMD2H phenotype. Finally we would like to directly address our hypothesis through proteomic means by identifying proteins of increased prevalence in patient cells.

The work outlined in this thesis is evidence that through the use of large families and / or inbred populations we can learn much about genetic disorders and the normal functioning of the human body. However we will only be able to go so far using this approach, particularly in the field of muscle disorders where there is so much heterogeneity. It is estimated that just among LGMDs there are 25% of familial cases and 40% of isolated cases that do not show mutations at any of the known loci.[191] We are entering an era where more global approaches such as large-scale expression profiling based on classical transcript microarrays, the new protein-based antibody microarrays, or mass spectrometry will be necessary as a starting point to target mutation detection to a specific gene or pathway. These techniques may even work on easy to obtain tissues such as lymphocytes or skin, eliminating the need for muscle biopsy. In the last five years there have been an explosion of such approaches in research and it is only a matter of time before they are translated into the clinical realm. It is clearly an exciting time to be working on muscular dystrophies and in the area of human genetics in general.

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Appendix 1

Limb Girdle Muscular Dystrophies (LGMDs) in Manitoba Populations

Participant Information and Consent Form

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You are being asked to participate in a research study. Please take your time to review this consent form and discuss any questions you may have with the study staff. You may take your time to make your decision about participating in this study and you may discuss it with your friends, family or (if applicable) your doctor before you make your decision. This consent form may contain words that you do not understand. Please ask the study staff to explain any words or information that you do not clearly understand.

Purpose of Study

This research study is being conducted to identify the genetic factor or factors which lead to the form of muscular dystrophy in your family. There are many forms of muscular dystrophy. Muscular dystrophy is a group of disorders with different causes but all leading to a variable but slowly progressive loss of muscle power. We are studying the muscular dystrophy seen in individuals from your family in the attempt to identify the exact type of limb girdle muscular dystrophy and to try to learn more about why some individuals and not others develop this condition. The approach we are taking is a genetic one, involving nucleic acid (DNA) analysis and the analysis of gene function using RNA and protein studies. DNA is a chemical compound found at the center (nucleus) of almost every cell in the human body. DNA carries the complete genetic blueprint for all inherited traits. Differences in the DNA make-up of different individuals can be used to track the DNA changes that result in traits such as muscular dystrophy. It is necessary that we examine the DNA of family members who do not have muscular dystrophy as well as those who do. RNA is another chemical compound that is found in almost every cell in the body. RNA is used to study gene function. Gene function can also be studied by looking at the expression of specific proteins in muscle.

Study Procedures

If you take part in this research study you will have the following procedures :

i) We will review your past medical history and test the muscle strength manually in your arms and legs.

ii) For DNA analysis, a blood sample is requested. We will prepare the DNA from the blood sample and in some instances we will take a fraction of blood cells and grow them in the laboratory. Growing the cells in the laboratory means that we have an essentially permanent source of DNA and RNA. A very small portion of the blood will be used to measure the muscle protein known as creatine kinase (CK). In individuals in your family affected with limb girdle muscular dystrophy, the level of CK is usually greater than 10 times the normal level. It is unlikely, if you have no symptoms or signs of muscle weakness, that your CK level will indicate that you are in the "affected" range. In the unlikely event that we find that you have a grossly elevated CK level, this may indicate that you will develop signs and symptoms of this disorder in the future. The level of CK in your blood will, however, at this time not help us differentiate between a carrier and a non-carrier for limb girdle muscular dystrophy, remembering that silent gene carriers do not develop signs of muscle weakness.

iii) Analysis of gene function by RNA analysis will proceed from the RNA isolated from your blood sample. In addition, if there is a sample of muscle left over from the muscle biopsy you underwent for diagnostic purposes, protein expression studies will be done in this tissue. These protein studies will only be performed if there is stored muscle tissue. No biopsy of muscle will be requested solely for research purposes.

Drs. Greenberg and Wrogemann will be performing the DNA and RNA analysis and will coordinate the overall project. We will not use the blood samples for any unrelated studies. This study may involve long-term followup.

Risks and Discomforts

Your blood sample will be obtained using the routine technique known as venipuncture with withdrawl of blood from a vein in the arm. Blood sampling is a very routine and safe procedure but may be associated with a small amount of discomfort and/or bruising at the site of the venipuncture. Otherwise there are no physical or non- physical (such as inability to work) risks by participating in this study.

Benefits

There may or may not be direct benefit to you from participating in this study. We hope the information learned from this study will benefit other people with limb girdle muscular dystrophy in the future.

Costs

All the procedures, which will be performed as part of this study, are provided at no cost to you.

Confidentiality
Information gathered in this research study may be published or presented in public forums, however your name and other identifying information will not be used or revealed. Despite efforts to keep your personal information confidential, absolute confidentiality cannot be guaranteed. Your personal information may be disclosed if required by law.
Medical records that contain your identity will be treated as confidential in accordance with the Personal Health Information Act of Manitoba.

The University of Manitoba Health Research Ethics Board may review records related to the study for quality assurance purposes.

Voluntary Participation/Withdrawal from the Study
Your decision to take part in this study is voluntary. You may refuse to participate or you may withdraw from the study at any time. Your decision not to participate or to withdraw from the study will not affect your care at this center. If the study staff feel that it is in your best interest to withdraw you from the study, they will remove you without your consent.
We will tell you about any new information that may affect your health, welfare, or willingness to stay in this study.

Questions
You are free to ask any questions that you may have about your treatment and your rights as a research participant. If any questions come up during or after the study, contact Dr. Cheryl R. Greenberg at 204-787- 2711 or Dr. Klaus Wrogemann at 204-789-3701.

For questions about your rights as a research participant, you may contact The University of Manitoba, Bannatyne Campus Research Ethics Board Office at (204) 789-3389

Do not sign this consent form unless you have had a chance to ask questions and have received satisfactory answers to all of your questions.

Statement of Consent
I have read this consent form. I have had the opportunity to discuss this research study with Dr. Cheryl Greenberg and/or her study colleagues and staff. I confirm that the DNA and gene expression studies proposed to identify the genes causing limb girdle muscular dystrophy have been explained to me. I have had my questions answered by them in language I understand. The risks and benefits have been explained to me. The results of these tests will be conveyed to me and this information may be shared, if requested, with professionals involved with my medical care including my family physician. The results will also be kept on file in the research laboratory of Dr. Klaus Wrogemann at the University of Manitoba. I have been assured that my record will be kept confidential and that no information will be released or printed that will reveal my identity without my permission. I understand that I will be given a copy of this consent form after signing it. I understand that my participation in this study is voluntary and that I may choose to withdraw at any time. I freely agree to participate in this research study.

I understand that information regarding my personal identity will be kept confidential, but that confidentiality is not guaranteed. I authorize the inspection of any of my records that relate to this study by The University of Manitoba Research Ethics Board for quality assurance purposes.

As DNA may be stored indefinitely, for the following statements I am indicating my choices as to the long-term handling and storage of my DNA, specifically:

1. I give my consent for the DNA sample extracted from my blood to be used in the search for genes causing LGMD, but my DNA will be discarded once the initial results of the investigation are available.	YES	NO
2. If my DNA is destroyed, I understand that if I want any further genetic testing to be done in the future I will need to have another blood sample taken.	YES	NO
3. The DNA obtained from me may be stored for 20 years in a DNA bank so that further testing of genes related to limb girdle muscular dystrophy may be performed in the future.	YES	NO
4. I wish to be re-contacted regarding the results of any new tests for LGMD that are performed on my DNA in the future.	YES	NO
5. Samples may be used in this laboratory or sent to other laboratories for research on other genetic diseases after all the identifying information has been removed so that my identity cannot be known or traced either by members of the research team or by the requesting research laboratory.	YES	NO
6. Prior to my death, members of my family are allowed access to my stored DNA only if I give my written permission.	YES	NO
7. My first-degree relatives will be allowed access to my stored DNA after my death.	YES	NO

I may request that my DNA be removed from the DNA bank at any time.

By signing this consent form, I have not waived any of the legal rights that I have as a participant in a research study.

Participant signature: _____ Date:

Participant printed name: _____

Parent/legal guardian's signature: _____ Date:

Parent/legal guardian's printed name: _____

Child's signature: _____ Date: _____

Child's printed name:

Legal guardian's signature: _____ Date:

Legal guardian's printed name: _____

I, the undersigned, attest that the information in the Participant Information and Consent Form was accurately explained to and apparently understood by the participant or the participant's legally acceptable representative and that consent to participate in this study was freely given by the participant or the participant's legally acceptable representative.

Witness signature: _____ Date: _____

Witness printed name: _____

I, the undersigned, have fully explained the relevant details of this research study to the participant named above and believe that the participant has understood and has knowingly given their consent

Printed Name: _____ Date: _____

Signature:

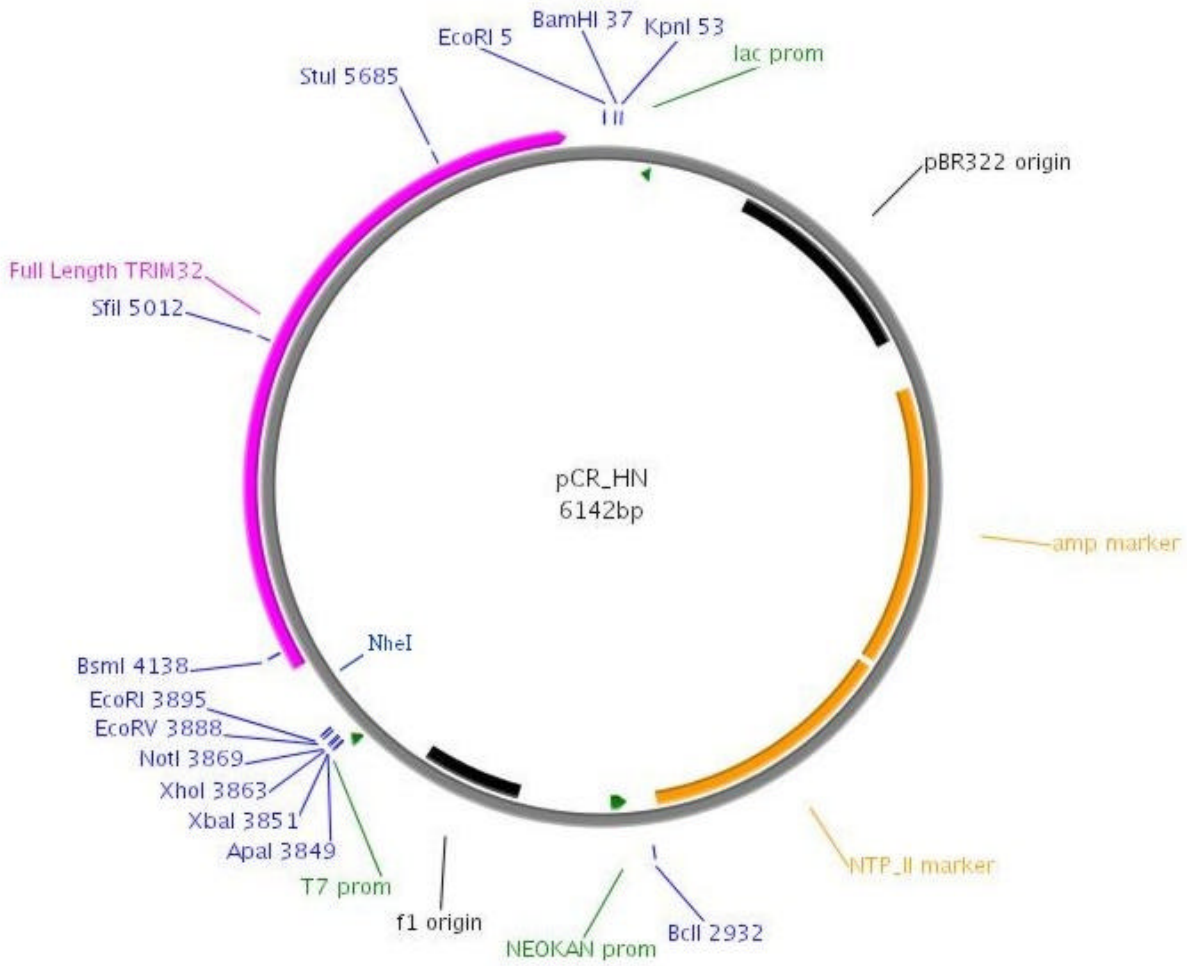
Role in the study:

Appendix 2

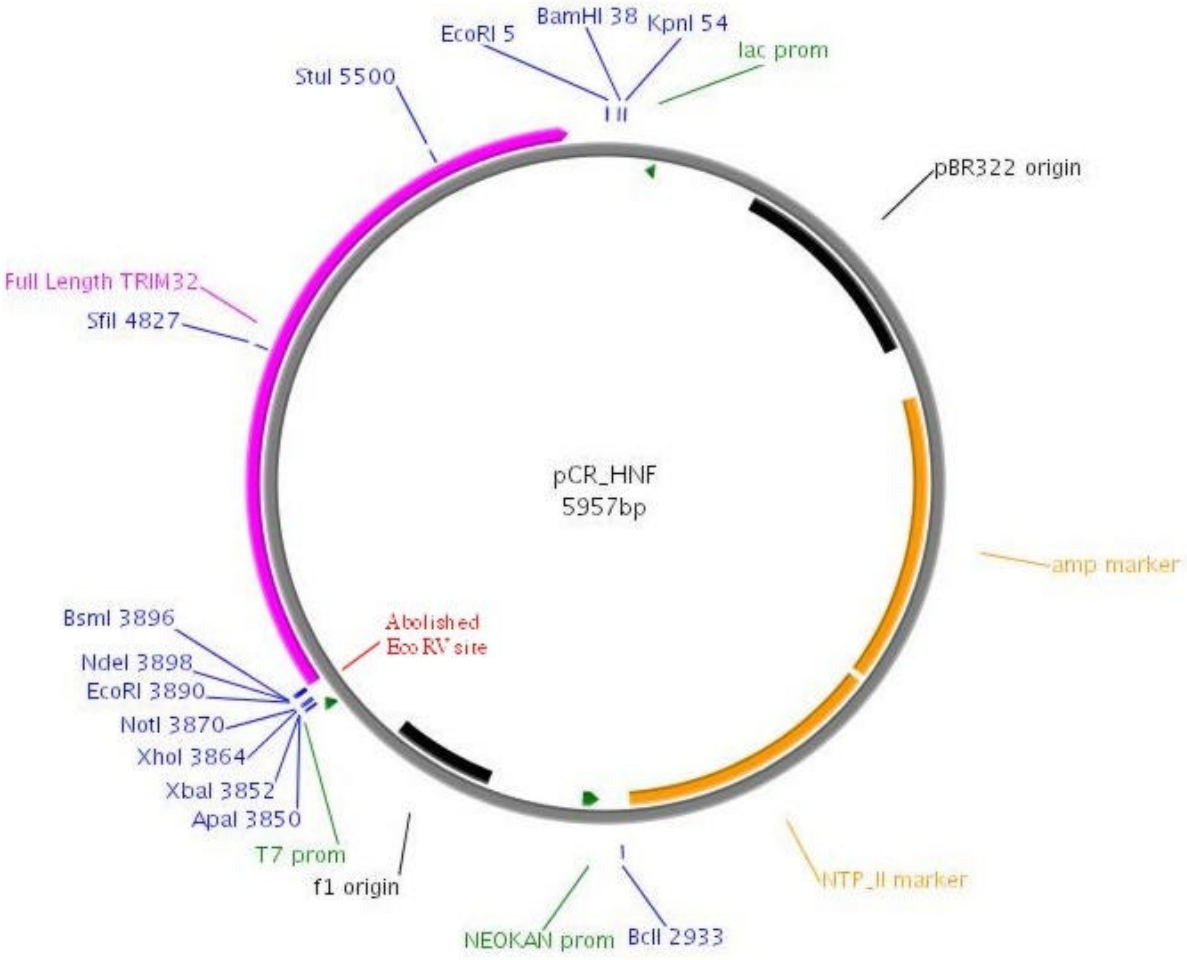
Appendix 3

Name	Forward (5'-3')	Name	Reverse (5'-3')	Amplifies	Size (bp)
PAPPA_ex.1(1)F	GGGGAGCAGATTAGCATACG	PAPPA_ex.1(1)R	CAAATCCAGAGCCGCATGTC	*PAPPA* exon 1 (1)	557
PAPPA_ex.1(2)F	AAGAAGGGTGAAGAAGCGAAG	PAPPA_ex.1(2)R	GAGCAAAGTGAGGCTCTCAG	*PAPPA* exon 1 (2)	692
PAPPA_ex.2F	TTAACCCCCCCTCCTTTTC	PAPPA_ex.2(1)R	TCACAGTTGGCCAGGATGAG	*PAPPA* exon 2 (1)	889
PAPPA_ex.2(2)F	GCAGTGCCCTGAATCACAAC	PAPPA_ex.2R	CAAGTTCAACTTTCAACTGGG	*PAPPA* exon 2 (2)	800
PAPPA_ex.3F	GCTCTAAATTATTTGGAGAGG	PAPPA_ex.3R	GCACTAAGTGTATTACTGTCC	*PAPPA* exon 3 / **V1**	291
PAPPA_ex.4F	GCACCAAGAAGCAGAGTACC	PAPPA_ex.4R	ACATCTGACATCCAGACCTG	*PAPPA* exon 4	395
PAPPA_ex.5F	GTCATTACTCTCTCATATGCC	PAPPA_ex.5R	GCTTCCCTTCCAAGTTTCC	*PAPPA* exon 5	335
PAPPA_ex.6F	CATTCAACTGTTCCTAAGTCG	PAPPA_ex.6R	AGCTGTTCTTTGCACACTCC	*PAPPA* exon 6	227
PAPPA_ex.7(1)F	GAATAAAGCTCTTTCCCCAAG	PAPPA_ex.7(1)R	CCAGGGAGATGTTCTTCCCAC	*PAPPA* exon 7 (1)	301
PAPPA_ex.7(2)F	GTTGGCTGTCAGTGGGAAG	PAPPA_ex.7(2)R2	CCACCCACTTAATAAACTCC	*PAPPA* exon 7 (1)	320
PAPPA_ex.8F	GTCTGCCCAGTATTGTAATTG	PAPPA_ex.8R	CTCTTGACCTAACCAGCATCC	*PAPPA* exon 8	232
PAPPA_ex.9F	CATGGTTTTAAGACTAAATTGG	PAPPA_ex.9R	ACCGTTCATTTCTTCATGAAGG	*PAPPA* exon 9	189
PAPPA_ex.10F	GTCTGCAGGAAATGCCACAC	PAPPA_ex.10R	CTCTCCCTTTCTATGTCAATC	*PAPPA* exon 10	259
PAPPA_ex.11F	CTTCTGACACTCTCTAAAACATG	PAPPA_ex.11R	GATTCCAAAGCTCTCAGAGC	*PAPPA* exon 11	193
PAPPA_ex.12F	GAGTGCACATGTGACCCTCC	PAPPA_ex.12R	GAAAGCAAGCCAAGACCAAG	*PAPPA* exon 12	206
PAPPA_ex.13F	GGCCTAGGGCGAGTCTGC	PAPPA_ex.13R	GGCCTTGAGTAATGAGCC	*PAPPA* exon 13	296
PAPPA_ex.14F	ATATTGCAGGTGGCATGTGAG	PAPPA_ex.14R3	CTGTCCTGTGAGGAGACTCC	*PAPPA* exon 14	258
PAPPA_ex.15F	CTGCCACTCCTCACTATGC	PAPPA_ex.15R	CAAAGGAAGGCTGAGCTGG	*PAPPA* exon 15	257
PAPPA_ex.16F	GACTCCTCCCTCCTCTAACC	PAPPA_ex.16R	TCCCAGAGACCATAAGTTTGC	*PAPPA* exon 16	291
PAPPA_ex.17F	GAGATGTCTCCTGTTTGATCC	PAPPA_ex.17R	CATGGTATCAATCTCAAGTTCC	*PAPPA* exon 17	237
PAPPA_ex.18F	CTCTCTTGGTCCTAACTCTG	PAPPA_ex.18R	GAATTGCCCCTCCTACTGC	*PAPPA* exon 18 / **V3**	225
PAPPA_ex.19F	CCTGAGCTGCGCCTCATGC	PAPPA_ex.19R	GGTGGGTAGATGTCCCTGG	*PAPPA* exon 19	170
PAPPA_ex.20F	CAAGCCCATCTGACCTTTC	PAPPA_ex.20R	CAGGGAACTGAGGAGTGC	*PAPPA* exon 20	142
PAPPA_ex.21F	CAGGATGCTAACAAGGCCTC	PAPPA_ex.21R	GTGGGCAGGAGGCAGCTG	*PAPPA* exon 21	187
PAPPA_ex.22F	GACCAAATCACCTGATTCAC	PAPPA_ex.22R	CACAGATGGGCACACTCTAG	*PAPPA* exon 22	474
P14_BsmA1F	GCTGGCTGTGGAGAATGCgT	P14_BsmA1R	AGCACCTGCACATACCTGtC	**V2**	145
PAPPA16/17	AACGGGCCTTCAAGACTCAG	PAPPA19/20	ACAACTCTCTCTACCCGTG	*PAPPA* cDNA	439
ASTN2_ex.6F	CAGGGCTTCTAGCAGGTG	ASTN2_ex.6R	GTCTCTGGGGTTTAACCATC	*ASTN2* exon 6	253
ASTN2_ex.7F	TCCCTCACTTCTGTAAATGG	ASTN2_ex.7R	CCATAACTACCCTGTTGC	*ASTN2* exon 7	250
ASTN2_ex.8F	GACTCAGACAAGCAATGTGC	ASTN2_ex.8R	GATGTGAAGCTAATTCTGAGC	*ASTN2* exon 8	264
ASTN2_ex.9F	GACTCGGTGTGTGGTCAGG	ASTN2_ex.9R	CAGCAGTCTCCACTTCCTCG	*ASTN2* exon 9	227
ASTN2_ex.10F	GTGCTAAATTATTCCAAGTCC	ASTN2_ex.10R	GTGAACAATGTGGTGTGAAGC	*ASTN2* exon 10	214
ASTN2_ex.11F	GGCAGCTCAGTTGTCTCC	ASTN2_ex.11R	CCACCGTATAATTTGCTAGC	*ASTN2* exon 11	328
ASTN2_ex.12F	GACAAAGGGTCCACAAGG	ASTN2_ex.12R	GTTTACCTTTCGCTCTTCC	*ASTN2* exon 12	378
ASTN2_ex.13F	AGGACAGGAGTGTGAGTCC	ASTN2_ex.13R	CGATGGCTCACATCATGGC	*ASTN2* exon 13	286
ASTN2_ex.14F	CTTCCCAAGACAGATGAGACC	ASTN2_ex.14R	GTGCTGGCTGCCAAACAC	*ASTN2* exon 14	290
ASTN2_ex.15F	CTCATGCCATTCCTCTTAGG	ASTN2_ex.15R	CTCACTATGGCCTGAGCTG	*ASTN2* exon 15	263
ASTN2_ex.16F	GATTAGTGACTGTTGGGTGC	ASTN2_ex.16R	CTTCCCTGCAACTCCCAG	*ASTN2* exon 16	245
ASTN2_ex.17F	GATAAAGCCTAGTCTCAGC	ASTN2_ex.17R	GAAGCCCATGCTTATATCC	*ASTN2* exon 17	350
ASTN2_ex.18(1)F	GATCTGGACTTCTTTAATCATGG	ASTN2_ex.18(1)R	CCATGGAGAGTCTCTGTGC	*ASTN2* exon 18 (1)	304
ASTN2_ex.18(2)F2	CGTGAGCACAGAGACTCTCC	ASTN2_ex.18(2)R2	GTCCATGGCAGGAAGAAAGC	*ASTN2* exon 18 (2)	359
ASTN2_ex.18(3)F	AGGCAGAAGTTTTCAGGTGG	ASTN2_ex.18(3)R	GGTCTCTCAAGCACATTCC	*ASTN2* exon 18 (3)	380
HT2A_ex.2(1)F	GACTGAATGACTGGTCATAGC	HT2A_ex.2(1)R	CTTGCTGCAAAAGGGACAGC	*TRIM32* exon 2 (1)	377
HT2A_ex.2(2)F	CAGTAGCATCAATGGTGTCC	HT2A_ex.2(2)R	TATACCTTGCCTGAAGGTCC	*TRIM32* exon 2 (2)	386
HT2A_ex.2(3)F	CTTGGAAGGTGTCTCCAAGG	HT2A_ex.2(3)R	GTAACAGAGGTAGAGGCAGC	*TRIM32* exon 2 (3)	456
HT2A_ex.2(4)F2	GCTGCCTCTACCTCTGTTAC	HT2A_ex.2(4)R2	CATAGCTGTCAGTCACACC	*TRIM32* exon 2 (4)	403
HT2A_ex.2(5)F	GGTGTGACTGACAGCTATG	HT2A_ex.2(5)R	GATGAGATCACCACGAGC	*TRIM32* exon 2 (5)	444
HT2A_ex.2(6)F	GCTGCATTGCTGGCATGTG	HT2A_ex.2(6)R	CATAGGCTGAGTCTATTCTGC	*TRIM32* exon 2 (6)	333
HT2A_ex.2(7)F	GGTTGTTAGTGGCACATGC	HT2A_ex.2(7)R	GGAAACAATGGATCAAGGG	*TRIM32* exon 2 (7)	326
-	-	H2_HpyR	AGCTTTCCACCTTCCACgT	**V4** (w/ HT2A_ex.2(5)F)	152
HT2A_Nde1F	GGAATTCCATATGGCTGCAGCAGCAGCTTCTC		-	Cloning TRIM32, full	-
HT2A_Nde2F	GGGAATTCCATATGAAAGGCAGCACTCCAGGAA		-	Cloning TRIM32, C-term	-
New_ex1F	ATTTGATCCTAGGCTGGACAG	New_ex1R	TGGAGGCCAAGGCTTTGG	*Novel Gene* exon 1	583
New_ex2F	TTTGGGAAACTGGGTTTGTC	New_ex2R	TGGGATGCAAAACCTTCCTTG	*Novel Gene* exon 2	525
New_ex3F	GTGCAAGAGCTCTTTGAATCC	New_ex3R	ACAGGTGGACAGCAGTCAC	*Novel Gene* exon 3	588
New_ex4F	CTCCCTGCCTCCCTTTCTTC	New_ex4R	GAAAGAAGCTTGGACAAGTG	*Novel Gene* exon 4	569
FKRP_ex4(1)F	CTGCCTTCCCTTTCGTCC	FKRP_ex4(1)R	CCGAGAGGTTGAAGAGGT	*FKRP* exon 4 (1)	702
FKRP_ex4(2)F	CCTGGACGGAGATGCTGT	FKRP_ex4(2)R	GGTAGAAGGGCCACAGGT	*FKRP* exon 4 (2)	686
FKRP_ex4(3)F	GGAGAAGGCGGTCGAGG	FKRP_ex4(3)R	CCAAAACTCTGCCCCTGC	*FKRP* exon 4 (3)	694
FKRP_ex4(4)F	CTGCCCAAGATTCCGAGAGC	FKRP_ex4(4)R	GAGCTGCGATTCAGAAGTCG	*FKRP* exon 4 (4)	675
FKRP_ex4(5)F	GAGGAGGAAATGCTCAGAGC	FKRP_ex4(5)R	GCAAAAGTCATTGGGATGAGG	*FKRP* exon 4 (5)	804
FKRP_ex4(6)F	GGTATTTGGGCATGTATTCCC	FKRP_ex4(6)R	GGCTGATCTTGAACTCTTGG	*FKRP* exon 4 (6)	776
FKRP_ex4(1)F2	TCAGGGAGTGCTTCCTGGAGAAG	FKRP_ex4(3)R2	CCCTCTAGTGGCGGAAGCAAC	*FKRP* exon 4	2235
rs3810288_F	TCCAACCTGCACCTGGCTAGG	rs3810288_R	AGCTGGAGGGGTCTGGGAGAtCT	*FKRP* promoter SNP	118
FKRP52_F	TCTCCAAAAAACAACAACAAC	FKRP52_R	CTAGTGTTCTGGGACCTTT	*FKRP52* STR	92-128
FMS2_F	GGAGGGAATAGGGGATCTGG	FMS2_R	CACCATGCTGGTTATTTTGGC	*FMS2* STR	140-152

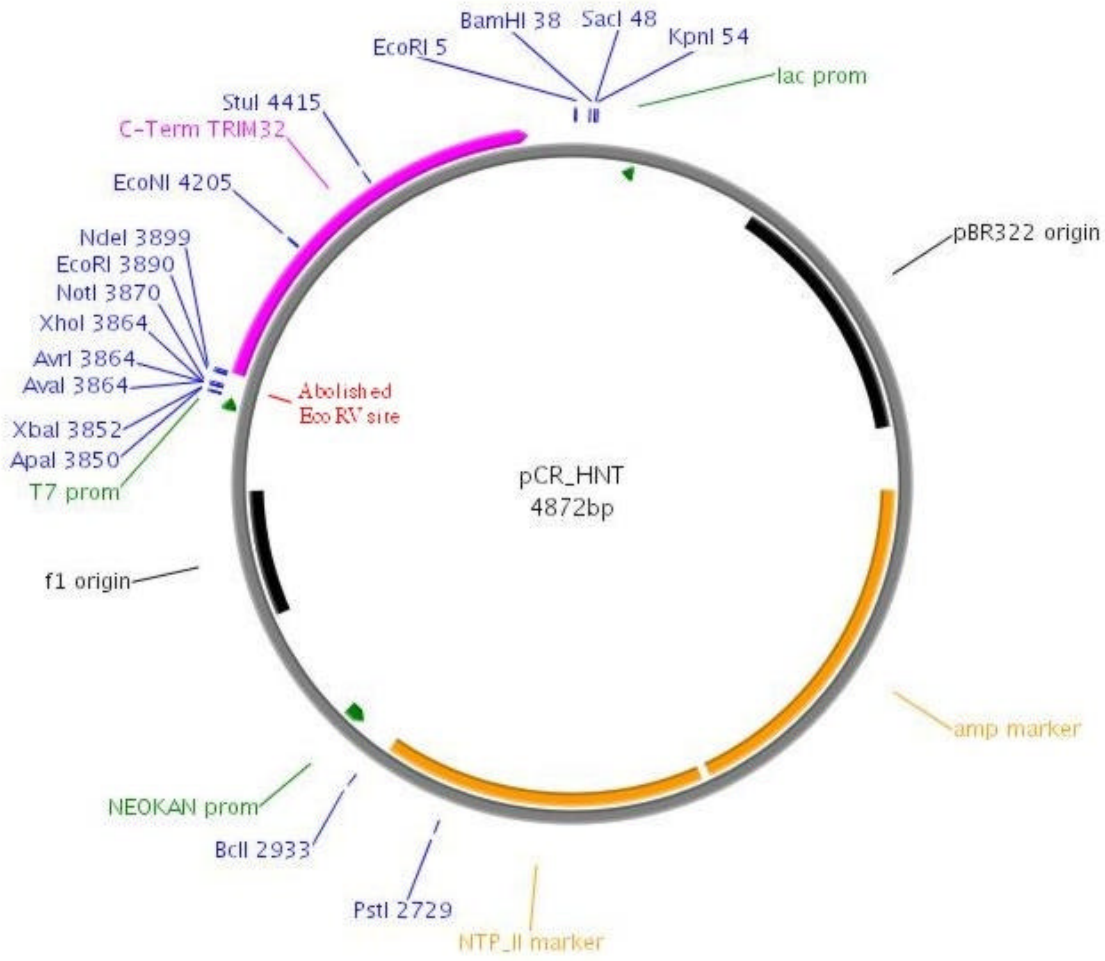
Appendix 4



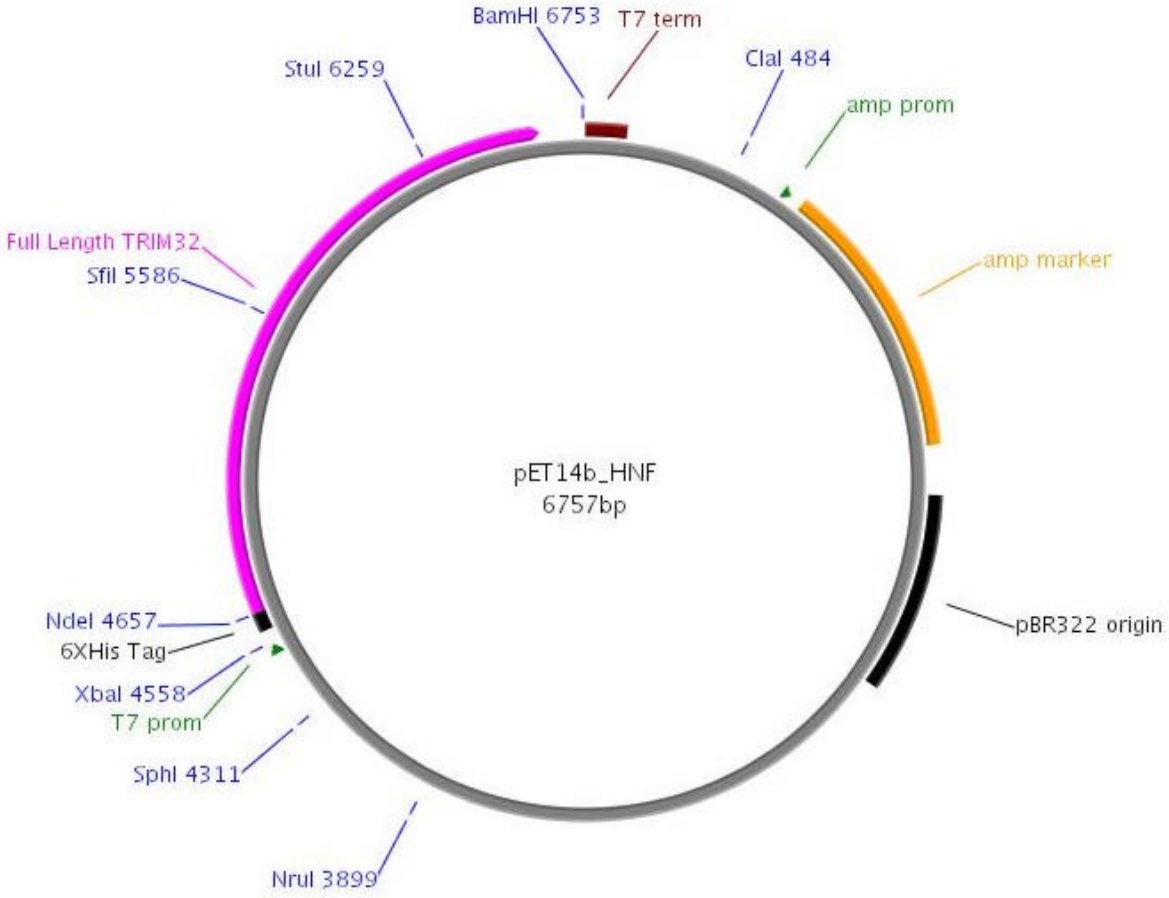
pBR322 origin: 459-1078 bp
Ampicillin resistance: 1233-2093 bp
Kanamycin resistance: 2114-2902 bp
F1 origin: 3330-3636 bp
TRIM32 full length coding sequence: 4084-6045 bp



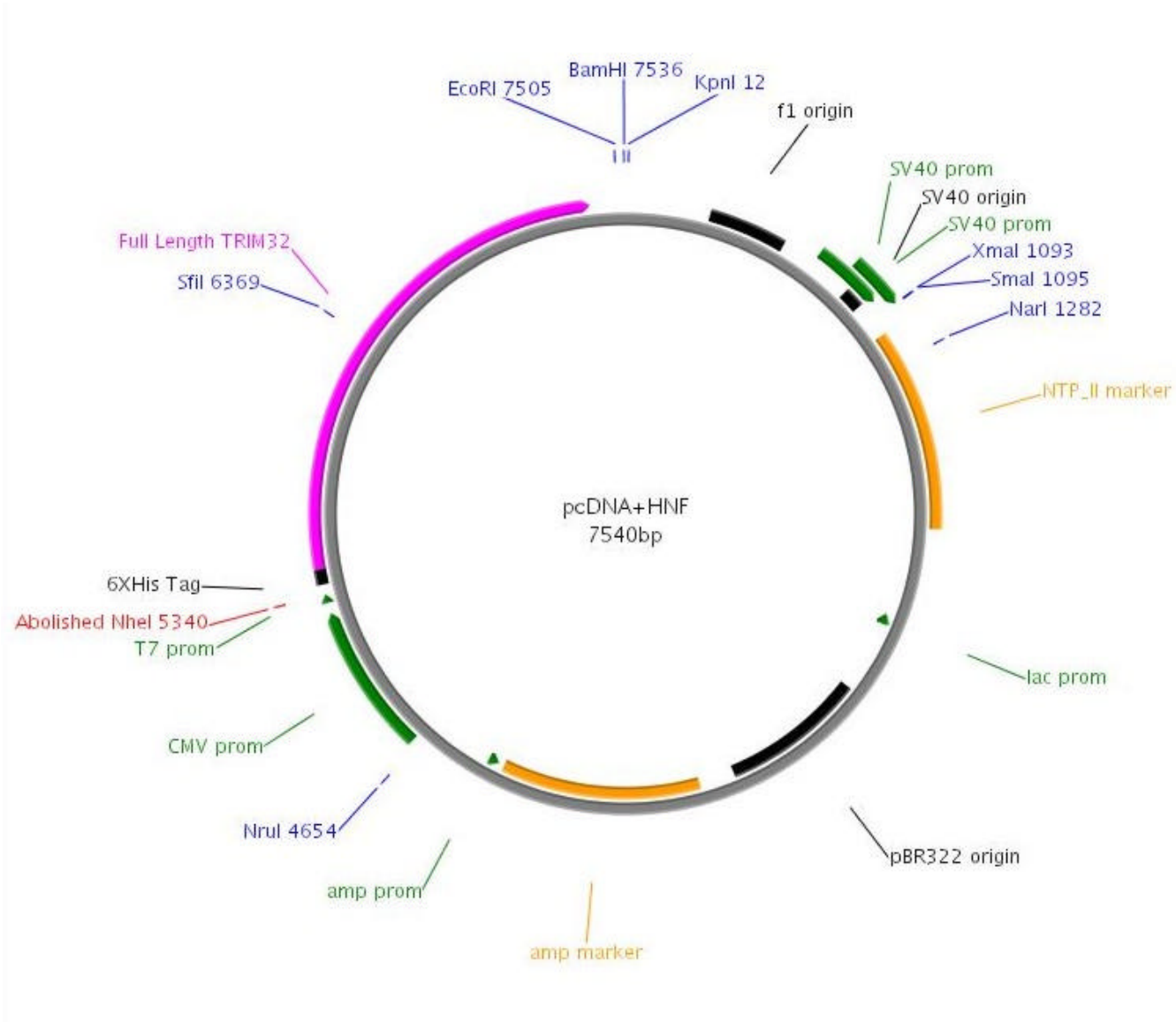
pBR322 origin: 460-1079 bp
Ampicillin resistance: 1234-2094 bp
Kanamycin resistance: 2115-2903 bp
F1 origin: 3331-3637 bp
TRIM32 full length coding sequence: 3887-5860 bp



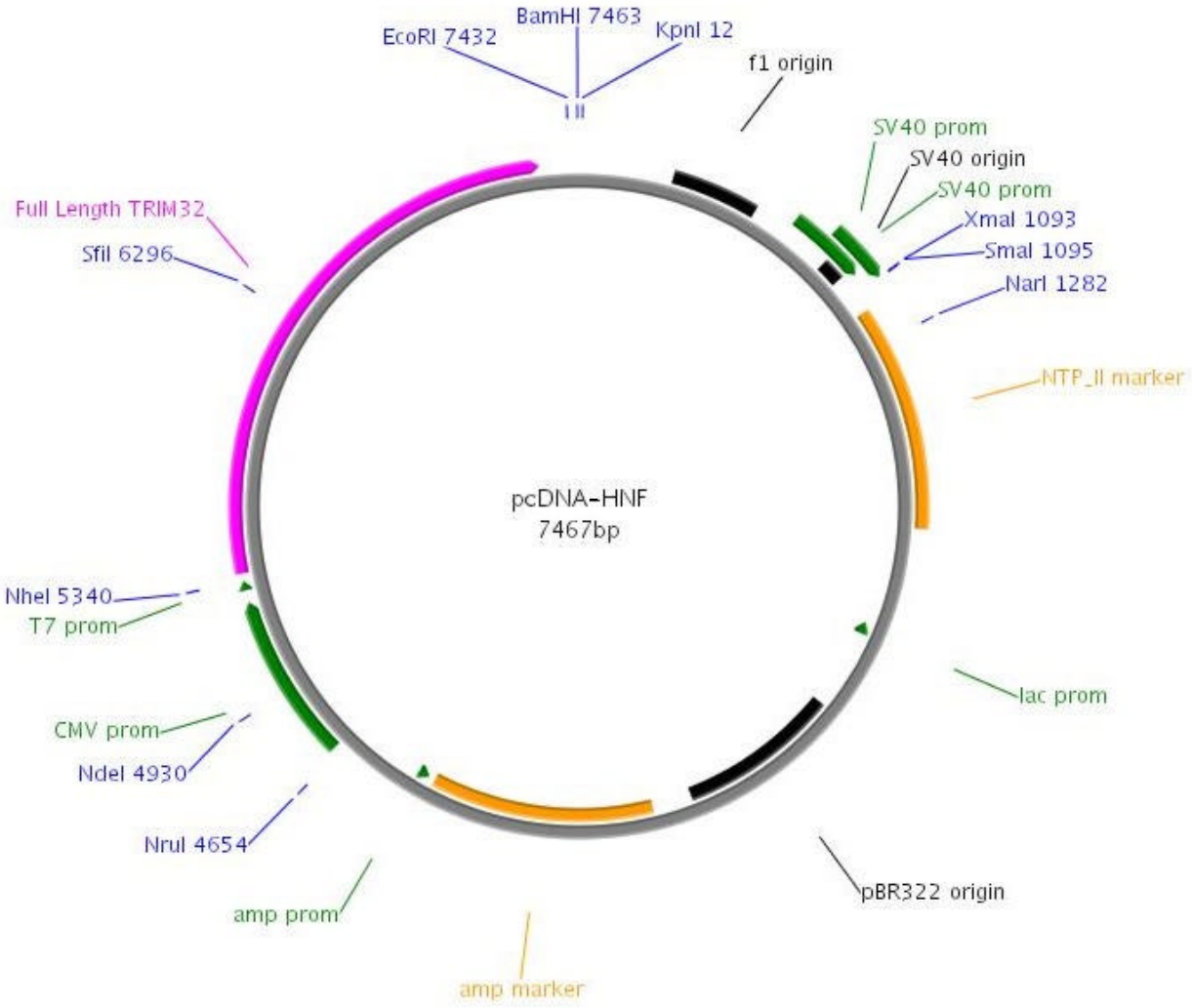
pBR322 origin: 460-1079 bp
Ampicillin resistance: 1234-2094 bp
Kanamycin resistance: 2115-2903 bp
F1 origin: 3331-3637 bp
TRIM32 C-terminal coding sequence: 3900-4775 bp



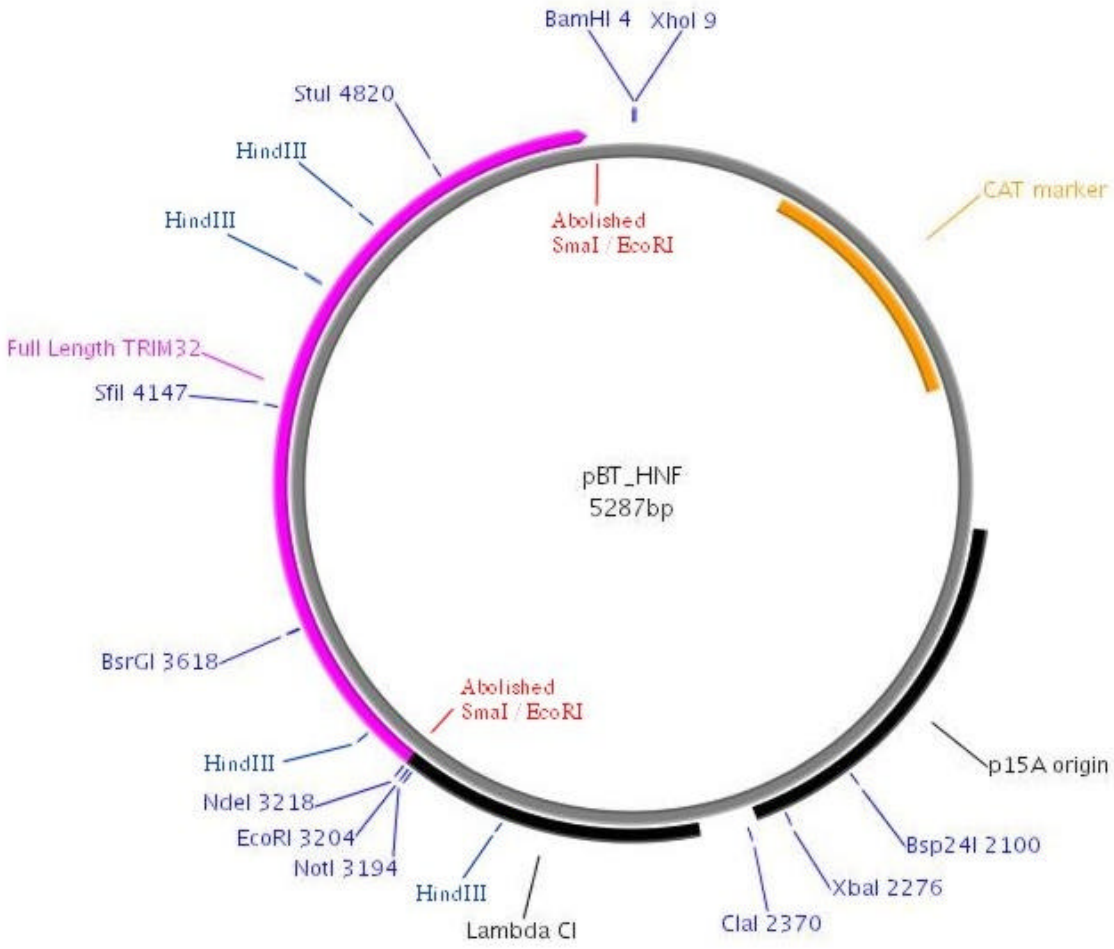
Ampicillin resistance: 718-1578 bp
pBR322 origin: 1733-2352 bp
6XHis_TRIM32 ORF: 4598-6619 bp
-6XHis Tag: 4598-4657 bp
-TRIM32 (full): 4658-6619 bp



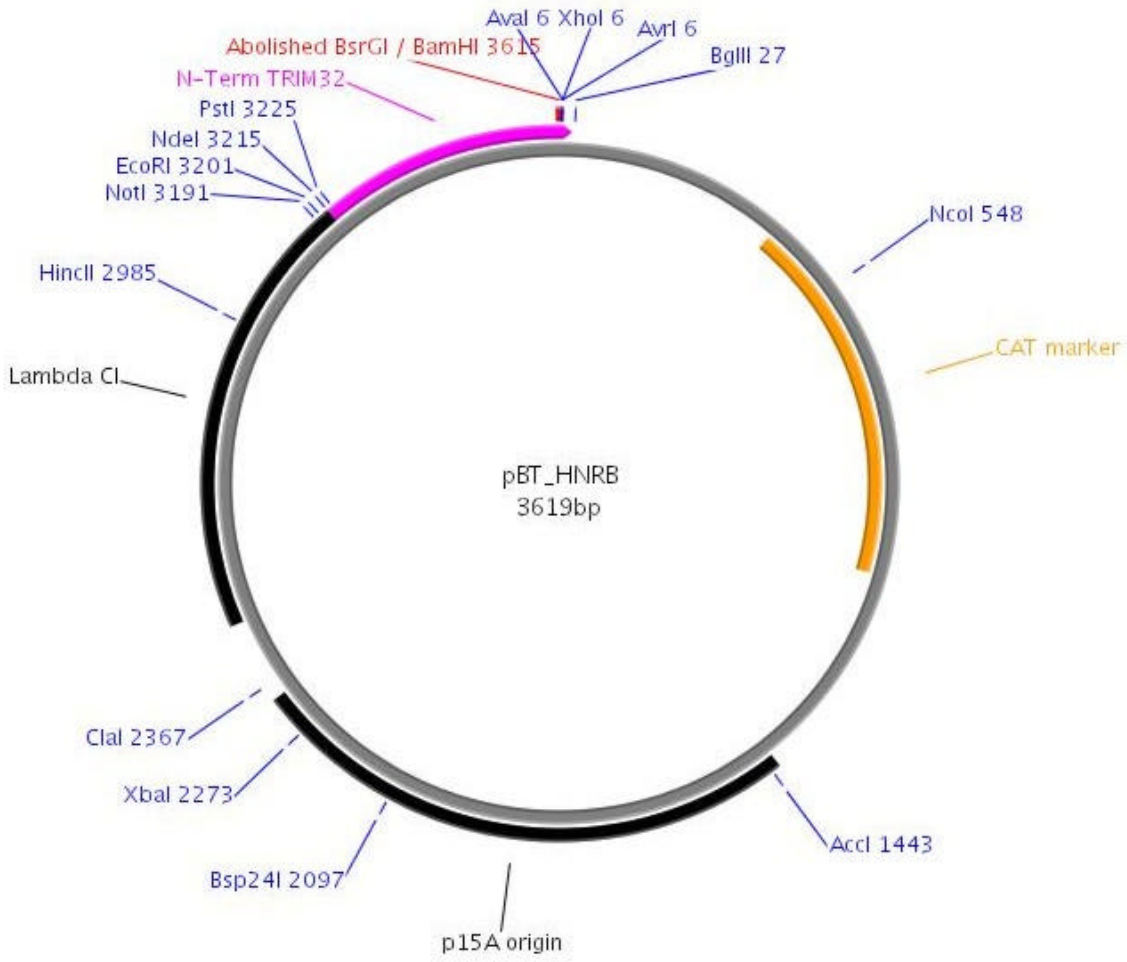
F1 origin: 332-638 bp
Neomycin resistance: 1156-1944 bp
pBR322 origin: 2678-3294 bp
Ampicillin resistance: 3449-4309 bp
6XHis_TRIM32 ORF: 5381-7402 bp
-6XHis Tag: 5381-5440 bp
-TRIM32 (full): 5441-7402 bp



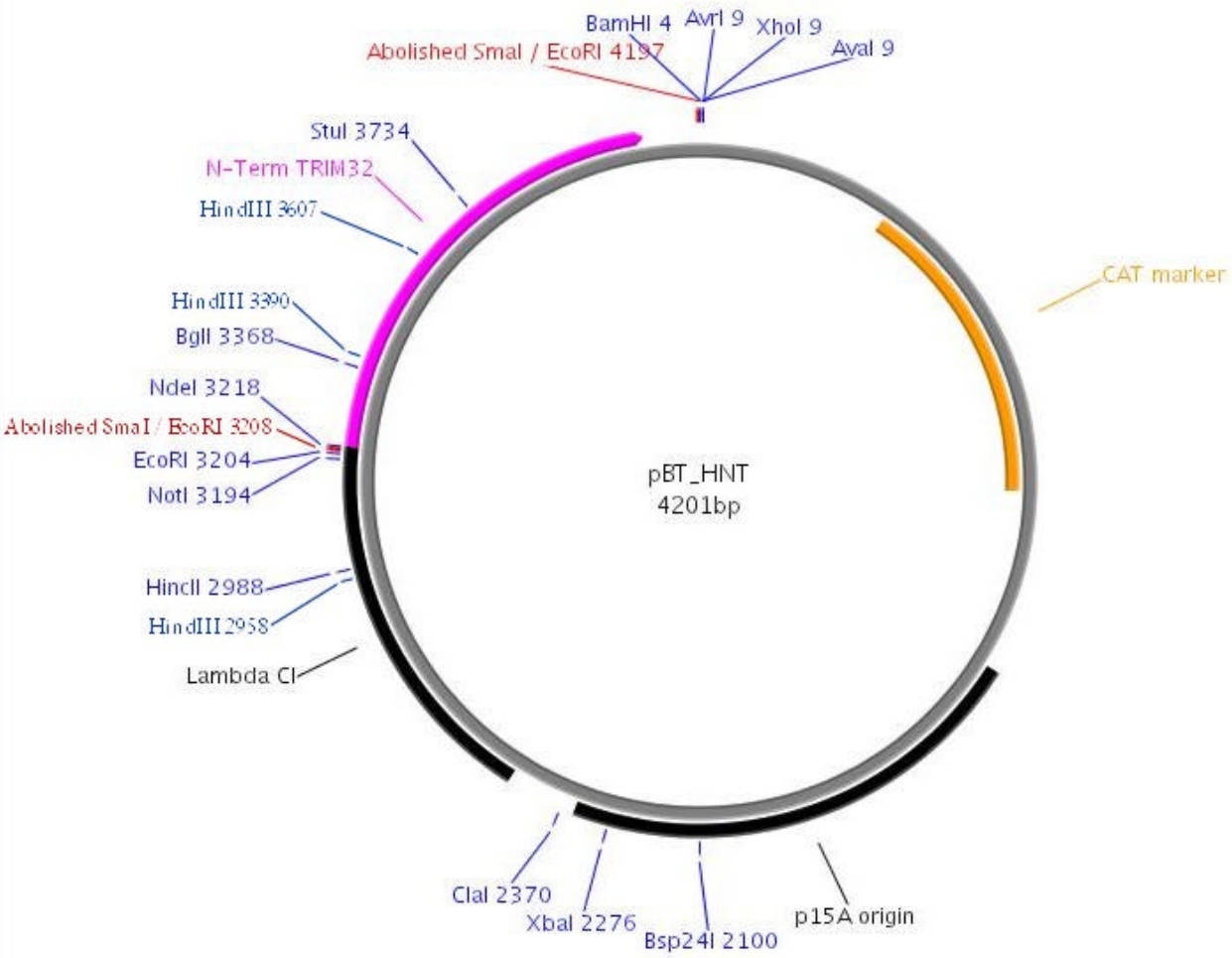
F1 origin: 332-638 bp
Neomycin resistance: 1156-1944 bp
pBR322 origin: 2678-3294 bp
Ampicillin resistance: 3449-4309 bp
TRIM32 full length coding sequence: 5368-7329 bp



Chloramphenicol resistance: 410-1069 bp
p15A origin: 1431-2343 bp
λCI_TRIM32 ORF: 2481-5180 bp
-λCI DNA-binding domain: 2481-3218 bp
-TRIM32 (full): 3219-5180 bp



Chloramphenicol resistance: 407-1066 bp
p15A origin: 1428-2340 bp
λCI_N-TRIM32 ORF: 2478-3617 bp
-λCI DNA-binding domain: 2478-3215 bp
-TRIM32 (N-terminal portion): 3216-3617 bp



Chloramphenicol resistance: 410-1069 bp
p15A origin: 1431-2343 bp
λCI_C-TRIM32 ORF: 2481-4094 bp
-λCI DNA-binding domain: 2481-3218 bp
-TRIM32 (C-terminal portion): 3219-4094 bp