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THE MYOPATHIC PROTEIN MYOTILIN IN DEVELOPING MOUSE AND IN MUSCLE FUNCTION

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Academic dissertation

To be publicly discussed, with the permission of the Medical Faculty of the University of Helsinki, in the Seminar Room 1-2 at Biomedicum Helsinki-1, Haartmaninkatu 8, Helsinki, on September 18th, 2009, at 1.30 pm

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Cover figure: whole-mount staining for ß-galactosidase activity in a Myf5-lacZ transgenic 9.5 dpc mouse embryo, showing expression of the transgene in the dorsal myotome region of somites (*Development* 130, 3297-3307 (2003)).

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To my family

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ABBREVIATIONS

ADF	actin depolymerising factors	**LGMD**	limb-girdle muscular dystrophy
ADP	adenosine diphosphate	**LIM**	Lin11, Isl-1 and Mec-3
ALP	actinin-associated LIM protein	**MAFbx**	muscle atrophy F box protein
ArgBP	Arg-binding protein	**MD**	muscular dystrophy
ATP	adenosine triphosphate	**MDM**	muscular dystrophy with myositis
BAC	bacterial artificial chromosome	**MEB**	muscle-eye-brain disease
BMD	Becker muscular dystrophy	**MEF2**	myocyte enhancer factor-2
CaMKII	calmodulin-dependent protein kinase II	**MFM**	myofibrillar myopathy
CARP	cardiac ankyrin repeat protein	**MLP**	muscle LIM protein
CCD	central core disease	**MRF**	muscle regulatory factor
CHO	chinese hamster ovary	**MuRF1**	muscle-specific RING finger 1
CM	congenital myopathy	**MyBP**	myosin-binding protein
CMD	congenital muscular dystrophy	**Myf5**	myogenic factor 5
DCM	dilated cardiomyopathy	**MyoD**	myoblast determination protein
DGC	dystrophin-glycoprotein complex	**NKX2.5**	NK2 transcription factor related locus 5
DMD	Duchenne muscular dystrophy	**NM**	nemaline myopathy
dpc	days post coitum	**nNOS**	neuronal nitric oxide synthase
DTT	dithiothreitol	**PAK**	p21 associated kinase
EBD	Evans Blue dye	**PCR**	polymerase chain reaction
EMD	Emery-Dreifuss muscular dystrophy	**PDZ**	PSD-95/Discs-large/ZO-1
ENH	Enigma-homologue protein	**PI_3P**	phosphaditylinositol-3-phosphate
ERM	Ezrin/Radixin/Moesin	**PKC**	protein kinase C
ES	embryonic stem (cell)	**POMT1**	protein-O-mannosyltransferase 1
FAK	focal adhesion kinase	**SBM**	spheroid body myopathy
FATZ	filamin-, actinin- and telethonin-binding protein of the Z-disc	**SDS-PAGE**	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
FITC	fluorescein isothiocyanate	**TMD**	tibial muscular dystrophy
FKRP	Fukutin-related protein	**TPM**	tropomyosin
FLH	four and a half LIM domains	**TRITC**	tetramethyl rhodamine isothiocyanate
FN-III	fibronectin-III	**TTID**	titin immunoglobulin domain
GST	glutathione-S-transferase	**UTP**	uridine triphosphate
HCM	hypertrophic cardiomyopathy	**VASP**	vasodilator-stimulated phosphoprotein
HLH	helix-loop-helix	**WNT**	wingless, int-1
ICM	inner cell mass	**WWS**	Walker-Warburg syndrome
IF	intermediate filament	**ZASP**	Z-band alternatively spliced PDZ motif-containing protein
ILK	integrin-linked kinase		

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following articles, referred to in the text by their Roman numerals.

I. Mologni L, Moza M, Lalowski MM, and Carpén O. *Characterization of mouse myotilin and its promoter*. **Biochem Biophys Res Commun**. 2005, 329(3):1001-1009.

II. Mologni L, Salmikangas P, Fougerousse F, Beckmann JS, and Carpén O. *Developmental expression of myotilin, a gene mutated in limb-girdle muscular dystrophy type 1A*. **Mech Dev**. 2001 May;103(1-2):121-5.

III. Moza M, Mologni L, Trocovic R, Faulkner G, Partanen J, and Carpén O. *Targeted deletion of the muscular dystrophy gene myotilin does not perturb muscle structure or function in mice*. **Mol Cell Biol**. 2007, 27(1):244-252.

ABSTRACT

Skeletal muscle cells are highly specialised in order to accomplish their function. During development, the fusion of hundreds of immature myoblasts creates large syncytial myofibres with a highly ordered cytoplasm filled with packed myofibrils. The assembly and organisation of contractile myofibrils must be tightly controlled. Indeed, the number of proteins involved in sarcomere building is impressive, and the role of many of them has only recently begun to be elucidated.

Myotilin was originally identified as a high affinity α-actinin binding protein in yeast two-hybrid screen. It was then found to interact also with filamin C, actin, ZASP and FATZ-1. Human myotilin is mainly expressed in striated muscle and induces efficient actin bundling in vitro and in cells. Moreover, mutations in myotilin cause different forms of muscle disease, now collectively known as myotilinopathies. In this thesis, consisting of three publications, the work on the mouse orthologue is presented. First, the cloning and molecular characterisation of the mouse myotilin gene showed that human and mouse myotilin share high sequence homology and a similar expression pattern and gene regulation. Functional analysis of the mouse promoter revealed the myogenic factor-binding elements that are required for myotilin gene transcription. Secondly, expression of myotilin was studied during mouse embryogenesis. Surprisingly, myotilin was expressed in a wide array of tissues at some stages of development; its expression pattern became more restricted at perinatal stages and in adult life. Immunostaining of human embryos confirmed broader myotilin expression compared to the sarcomeric marker titin. Finally, in the third article, targeted deletion of myotilin gene in mice revealed that it is not essential for muscle development and function.

These data altogether indicate that the mouse can be used as a model for human myotilinopathy and that loss of myotilin does not alter significantly muscle structure and

function. Therefore, disease-associated mutant myotilin may act as a dominant myopathic factor.

REVIEW OF THE LITERATURE

The theme of this thesis is the analysis of the sarcomeric protein myotilin, which is mainly expressed in and whose most important function associates with muscle tissue. Therefore, in this review of literature the main aspects of cytoskeleton are described with emphasis directed towards striated muscle, including its development. Since mutations in myotilin can cause myopathies, the main aspects of hereditary myogenic muscle diseases and the role of myotilin in those diseases are described.

1. The eukaryotic cytoskeleton

Eukaryotic cells maintain their shape through the cytoskeleton. Far from being a static backbone of the cell, the cytoskeleton is essential for a number of vital cellular processes, such as the internal movement of organelles, cell locomotion, cell division and muscle contraction. In order to accomplish these tasks, three types of protein filaments have evolved: microtubules, intermediate filaments and microfilaments. All these filaments are built from subunits that can be quickly added and removed, thus creating a highly dynamic network of continuously rearranged polymers.

1.1. Microtubules

Microtubules are hollow tubes, 24 nm in diameter, made up of tubulin subunits [1, 2]. They aid in several types of cellular movements, such as chromosome alignment and separation during mitosis, the transport of proteins, vesicles and organelles within the cytoplasm, neurite outgrowth and the movement of cilia and flagella. The microtubule subunit is a heterodimer of α- and β-tubulin. The longitudinal head-to-tail arrangement of subunits

forms a linear protofilament. Lateral interactions between adjacent subunits allow side-by-side association between the protofilaments, to create the wall of the microtubule. Typically, there are 13 protofilaments in each microtubule that are slightly staggered to form a spiralling structure. In some particular cases, like in centrioles or in cilia and flagella, a more complex structure is generated by the fusion of a second (doublet microtubule) and third (triplet microtubule) tubule, made of only 10 protofilaments, to the first microtubule [3]. Each tubulin subunit binds two molecules of GTP. One GTP-binding site, located in α-tubulin, binds GTP irreversibly and does not hydrolyse it, whereas the second site (called the exchangeable site), located on β-tubulin, binds GTP reversibly and hydrolyses it to GDP. Subunits containing GTP-β-tubulin polymerise into the microtubule and form the so-called GTP-cap which stabilises the structure. Until polymerisation proceeds faster than GTP hydrolysis, the microtubule grows. When GTP is converted to GDP before new subunits can be added, the microtubule becomes unstable and depolymerises rapidly. Microtubules display structural polarity: addition of subunits occurs preferentially at one end, designated as the (+) end, whereas the opposite (-) end is in close proximity with the nucleation centre, the centrosome. The centrosomes are rich in γ-tubulin, a third tubulin that is not part of the microtubules but is essential for their assembly.

1.2. Intermediate filaments

Intermediate filaments (IFs) are made from fibrous proteins encoded by a superfamily of more than 50 genes [4]. Five classes of IF proteins are recognised: one that comprises nuclear lamins, and four groups of cytoplasmic filaments. Lamins form a network of filaments on the inner surface of the nuclei (the so-called *nuclear lamina*) and create a structural scaffold for the nuclear envelop. They undergo regulated disassembly during

mitosis. Cytoplasmic IFs include acidic and basic cytokeratins, neurofilament triplet proteins, and type III proteins, such as vimentin and desmin. Many of these proteins are expressed in a tissue-specific manner: cytokeratins can be divided in "hard" and "soft" types, expressed in epithelia and hair, respectively; desmin is specifically expressed in muscle cells [5], whereas vimentin is also found in other mesenchymal cells, as well as in muscles; other muscle-associated IF proteins include synemin and nestin [6, 7]. Neurons have their own set of IF proteins. Intermediate filaments can be homo- or hetero-polymers. Subunits consist of two globular ends and a central α-helical rod that mediates dimer formation. Two such dimers associate in an anti-parallel fashion to form tetramers. Finally, tetramers associate head-to-tail and side-by-side, thus creating the final intermediate filament. The main role of IFs is to provide mechanical support to the cells, especially at cell-cell and cell-extracellular matrix contacts. Indeed, they are relatively stable and resistant to solubilisation. In skeletal muscle cells, IFs link myofibrils among them and to the sarcolemma and the nuclei. However, there is evidence that they can perform other functions as well.

1.3. Microfilaments

Microfilaments are polar, helical double strands consisting of a string of actin monomers, from few nanometres to several centimetres long. They are the thinnest filamentous structures within a cell, measuring only 5-10 nm in diameter. Actin is the most abundant protein of a eukaryotic cell, representing approximately 5% of the total protein content. Rapid polymerization and depolymerisation of actin filaments (a phenomenon known as treadmilling) occurs via binding and hydrolysis of ATP: free monomers bind ATP and are incorporated onto the barbed (fast growing) end of the filament; ATP is then hydrolysed to ADP and Pi. Dissociation of ADP-actin at the opposite pointed end causes disassembly of

the filament [8]. When association rate of G-actin at the barbed end and off rate at the pointed end are balanced, a steady-state is reached. Treadmilling is essential during cell motion: the barbed end faces the cell membrane at the leading edge, while the pointed end is at the rear. The coordinated action of a number of regulating proteins controls actin turnover [9, 10]. Capping proteins block barbed end growth. In addition, some of them possess severing activity, thus generating new filament ends. Members of the actin depolymerising factors (ADF) family can bind both F- and G-actin and induce rapid depolymerisation of filaments, by increasing the pointed-end off-rate. ADFs can severe filaments, too. The Arp2/3 complex is a seven-protein complex that is able to nucleate new branches on pre-existing actin filaments. Finally, profilin acts as a nucleotide exchange factor, by promoting the substitution of ATP for ADP on G-actin; the ATP-actin-profilin complex then associates to barbed ends, thus elongating the filament.

Actin filaments organize to form two types of higher-order structures: bundles and gel-like networks. In bundles, several actin filaments are held together by bundling proteins [11, 12]. Fimbrin is a ubiquitous cross-linking protein with two adjacent actin-binding sites, so that it bundles actin filaments in tightly packed arrays such as in filopodia, spike-like projections of the plasma membrane that allow a cell to explore the environment in the direction of migration. Looser bundles are assembled by α-actinin in stress fibres and focal contacts [13]. Stress fibres are contractile bundles of actin and myosin-II filaments ending at focal contacts underneath the plasma membrane, where they anchor the cell to the extracellular matrix through integrins. Alpha-actinin is also involved in sarcomere organisation in muscle cells. Another bundling protein, villin, helps align actin filaments in microvilli, the finger-like extensions of the plasma membrane that greatly increase the absorptive surface of many epithelial cells. Cross-linking proteins that have a long, flexible, or bent connection between their two actin-binding sites tend to form actin webs or gels,

rather than bundles. Filamin cross-links F-actin into a network of filaments oriented approximately at right angles. An example is found in lamellipodia, sheet-like extensions of the cell membrane that help the cells move over a solid substrate. Spectrin is generally found as a tetramer ($\alpha_2\beta_2$) on the inner surface of the plasma membrane, where it organises the cortical actin cytoskeleton that is needed to maintain membrane integrity. In addition to the cortex cytoskeleton, cells have a cytosolic three-dimensional actin network that is linked to the cell membrane by different anchoring proteins: filamin in platelets, dystrophin in muscle cells, ERM (Ezrin, Radixin, and Moesin) proteins in epithelial cells. This cytosolic actin web fills the cytoplasm and gives cells their shape.

The above depicted picture is a very simplified view of the cell cytoskeleton. Several other proteins are involved in its organisation and function, including motor proteins that drive transport along filaments, membrane proteins that link the intracellular to the extracellular environment, enzymes that trigger signalling cascades leading to cytoskeleton rearrangements, and so on. In addition to that, different isoforms or different members of the same protein family can exert similar or slightly different functions in a cell type-specific manner.

2. Muscle cell cytoskeleton

As noted above, contractile units (the stress fibres) are formed in non-muscle cells by assembling actin and myosin filaments with the assistance of α-actinin. A very specialised actomyosin cytoskeleton has developed in muscle cells. Vertebrate muscles are divided into three categories according to structural and functional properties: (1) smooth muscles contain a loosely organised contractile apparatus, while (2) cardiac and (3) skeletal muscles are both characterised by a striated appearance, revealing the presence of highly ordered structures, and are therefore called striated muscles. A fourth class of contracting cells is

represented by myoepithelial cells, which are found surrounding the secretory epithelium of glands. Their contractile activity forces secretions into the excretory duct of the gland.

2.1. Smooth muscle

Smooth muscles surround and control the involuntary movements of internal organs such as the large and small intestines, the uterus, and blood vessels. Smooth muscle cells contain loosely organised contractile bundles of actin (thin) and myosin (thick) filaments, non-uniform in length, which are attached to dense bodies in the cytoplasm and to dense plaques at the cell membrane [14, 15]. Dense bodies contain α-actinin and are also associated with a cytoplasmic cytoskeleton composed of desmin filaments and non-muscle actin filaments. Although highly ordered arrays of contractile units are not present, an approximately regular distribution of dense bodies has been observed [16]. Similarly, dense plaques show a characteristic, periodic banding pattern and are enriched in the proteins vinculin and talin, which bind to membrane proteins in the plaque and to α-actinin, thereby attaching actin filaments to membrane adhesion sites. Such an integrated lattice of cytoskeletal proteins provides the smooth muscle cell with the ability to adapt constantly to changes in cell length. The regulation of actin-myosin contraction in smooth muscle is regulated primarily by phosphorylation of one of the myosin light chains, called the regulatory light chain [17]. Its phosphorylation promotes the assembly of myosin into filaments and increases myosin ATPase activity, enabling contraction to proceed. The enzyme that catalyzes this phosphorylation, called myosin light-chain kinase, is itself regulated by association with the Ca^{2+}-binding protein calmodulin [18, 19]. Increases in cytosolic Ca^{2+} promote the binding of calmodulin to the kinase, resulting in phosphorylation of the myosin regulatory light chain.

2.2. Cardiac muscle

Cardiac muscle [20, 21] resembles skeletal muscle in some ways: it is striated and contains the well-organised contractile units known as sarcomeres, which will be described below. However, cardiac muscle has a number of unique features. First of all, it is made of single cells, each with a single centrally located nucleus. Secondly, the myofibrils of each cell are branched. The branches interlock with those of adjacent cardiomyocytes by adherens junctions. These strong junctions enable the heart to contract forcefully without ripping the fibres apart. Another peculiar characteristic of cardiac muscle is represented by dark lines dividing adjacent cells: the intercalated discs. At the intercalated disc the cell membranes of two adjacent cardiac muscle cells are extensively intertwined and bound together by adherens junctions and desmosomes. Through these junctions, myofibrils from the two cells are locked together. At the same time, gap junctions and other ion channels provide a cytoplasmic connection between cells [22]. All these contacts stabilise the relative positions of adjacent cells and allow ions and small molecules to move from one cell to another. This arrangement creates a direct mechanical and electrical coupling between contiguous cells, leading to a synchronised contraction of the entire tissue.

2.3. Skeletal muscle

2.3.1. Structure and function

Skeletal muscles connect the bones and are used by vertebrates to move. They are characterised by an extremely ordered assembly of contractile structures. A skeletal muscle comprises a bundle of muscle cells, or myofibres. A typical muscle cell is cylindrical, large (1 millimetre to few centimetres in length and 10-50 μm wide) and multinucleated (containing as many as 100 peripherally located nuclei), arising from the fusion of several

separate cells. A myofibre is packed with myofibrils, bundles of filaments that extend the length of the cell. Myofibrils are further subdivided into alternating light and dark bands, which are aligned along the length of the muscle cell, giving the myofibre a striated appearance under a light microscope. Closer examination reveals that the dark bands, called A-bands, are bisected by a darker region, the M-line, while the light bands, called I-bands, are bisected by a different dark line, called the Z-disc. The segment from one Z-disc to the next, consisting of two halves of an I-band and one central A-band, is termed a sarcomere [23]. A chain of sarcomeres constitutes a myofibril. Force generation is driven by sliding of actin and myosin filaments and is triggered by an electric pulse coming from a nerve cell. This signal propagates into the muscle through a series of sarcolemmal invaginations, known as T-tubules that reach and surround each myofibril. The link between the generation of an action potential in the sarcolemma and the start of contraction occurs at the triads, specialised structures composed of a T-tubule with sarcoplasmic reticulum on either side. On reaching a triad, an action potential triggers the release of Ca^{2+} from the cisternae of the sarcoplasmic reticulum into the cytosol. Ca^{2+} ions bind the thin filament-associated protein troponin, which changes conformation and causes the relocalisation of tropomyosin, thus allowing the productive interaction of myosin heads and actin filaments [24]. As Ca^{2+} concentration rapidly returns to baseline values, troponin and tropomyosin regain their original position, blocking contraction. Skeletal muscle fibres can be divided into fast and slow fibres, based on their physiology. Fast ("white") fibres are larger in size and possess high glycogen storages and high glycolytic activity. This gives them the capacity of performing extremely rapid contractions. Slow ("red") fibres are smaller, richer in myoglobin (hence the dark colour) and have high oxidative metabolism. Distinguishing features of slow and fast fibres are their respective wide and narrow Z-discs [25]. The fast muscle has a narrow, simple Z-disc that resembles a single zig-zag line connecting the ends

of the opposing actin filaments from the adjacent sarcomeres. The wide Z-discs in slow muscles comprise three to four zig-zag lines stacked axially. Two closely related genes (ACTN2 and ACTN3) encode for the main Z-disc protein, α-actinin, in humans. While ACTN2 is expressed ubiquitously in skeletal muscles, ACTN3 is only expressed by fast fibres. Intriguingly, approximately 20% of the human population is deficient for ACTN3, suggesting that its role may be redundant. However, the null genotype is strongly under-represented (6%) in sprint athletes, indicating that ACTN3 may have a role in the specification and functional properties of fibre type [26].

2.3.2. The sarcomere

The sarcomere is the structural and functional unit of skeletal muscle [27]. A low resolution view of the sarcomere structure describes alternating dark and light zones. A-bands span the length of thick filaments, while I-bands comprise the area corresponding to thin filaments alone. Thin filaments are anchored at one end to the Z-disc and interdigitate with thick filaments at the other end. Each thick filament is a bipolar structure, surrounded at both ends by six thin filaments. When the inhibitory troponin/tropomyosin complex is dislocated by Ca^{2+} binding, myosin heads interact with actin and, by changing conformation, pull thin filaments towards the centre of the sarcomere. Because actin is tethered to the Z-discs, this movement results in the shortening of the sarcomere while thin and thick filaments slide past each other. The power enabling muscle contraction comes from ATP hydrolysis [28] through a series of steps known as cross-bridge cycle: as the myosin head is bound to actin, ATP enters the catalytic site and induces a conformational change that reduces the affinity of the head for actin. This enables the head to hydrolyse ATP to ADP and P_i. Free energy from this reaction moves the myosin head to a new position, forward along the actin filament, where it binds another actin subunit. This in turn triggers the release of inorganic

phosphate which causes the myosin head to return to its starting position, pulling the actin filament. After nucleotide exchange, the head is ready for another cycle. The net result of repeated cycles is that myosin walks on the thin filament.

A plethora of accessory proteins assist in sarcomere assembly and function. According to the four filaments model (figure 1), the giant proteins titin (~3 MDa) and nebulin (800 kDa) act as rulers on which thick and thin filaments align [29, 30]. Titin, spanning half sarcomere, is tightly associated with the thick filaments via its carboxy- (C-) terminal portion. It is mainly composed of repeated fibronectin-III (FN-III) and Ig-like globular domains (overall, there are over 200 in one titin molecule) that give to the entire protein the appearance of a long necklace dotted with pearls. These repeats are organised into different patterns called super-repeats. Non-repetitive regions between FN-III and Ig domains, account for approximately 10% of the entire sequence: a kinase-like domain called titin-kinase is located near the C-terminus, in proximity of the M-line. A unique region with spring-like properties, designated the PEVK segment (as it is rich in proline, glutamate, valine and lysine residues) confers elasticity to the entire molecule [31]. Due to the PEVK domain, titin behaves as an extensible spring. This is crucial in order to allow sarcomere shortening, since titin amino- (N-) end is anchored to the Z-disc, while its C-end is attached to the M-line. Finally, four to seven alternatively spliced 45-aminoacid repeats (termed Z-repeats) in the N-terminal region are thought to be responsible for different Z-disc width in different types of muscles.

Nebulin associates with the thin filament system and is thought to be what titin is for thick filaments: a template for correct positioning. Its variable C-terminal end inserts into the Z-disc, while the N-terminal portion is located near the free end of actin, within the A-band [30]. In contrast to titin, whose Z-disc region spans the entire Z-line (thus overlapping with the corresponding titin region from an adjacent sarcomere), nebulin only enters a small

portion of the Z-disc. Similarly to titin, nebulin structure is highly modular, with 185 repeated domains (modules) arranged in super-repeats. In particular, the central modules M9-M162 are responsible for binding to actin, troponin and tropomyosin, whereas the amino-terminal M1-M8 unique repeats bind to the pointed end capping protein tropomodulin [32]. In addition to their well-established structural roles, titin and nebulin also have other functions, which have only recently begun to be elucidated. For example, there is evidence that nebulin may regulate contraction, by controlling access of myosin heads to the actin filaments. Moreover, nebulin has an SH3 domain, which may participate in signal transduction [33]. Titin, on the other hand, has a serine/threonine kinase domain that has been shown to phosphorylate T-cap/Telethonin, a Z-disc protein. It is believed that this phosphorylation regulates myofibril assembly [34].

CapZ is a dimeric Z-disc protein which binds to and caps the barbed end of F-actin, facilitating nucleation of nascent thin filaments [23]. It also interacts with α-actinin. Tropomodulin, as already mentioned, binds to the opposite (pointed) end of F-actin, where it forms a complex with tropomyosin and nebulin. Tropomyosin is a coiled-coil protein that bonds head-to-tail to form a long helical filament wrapped around F-actin [35]. Together with troponin, tropomyosin forms the Ca^{++}-sensitive molecular switch that permits muscle contraction (see above). Troponin is a three-protein complex: troponin C is the subunit that actually binds Ca^{++} ions, whereas troponin I inhibits the actomyosin ATPase activity and troponin T binds to tropomyosin, thus linking the troponin complex to the thin filament [24]. Upon Ca^{++} binding, troponin C removes troponin I-mediated inhibition and, through troponin T, releases steric hindrance determined by tropomyosin, thereby allowing the formation of functional actin-myosin cross-bridges.

Thick filaments are made of several myosin molecules grouped together to form highly regular bipolar structures, with head domains sticking out from the filament at both ends

[23]. Tails with antiparallel orientation overlap in the central bare zone. An individual myosin molecule is a six-polypeptide complex made of two heavy chains and four light chains. The heavy chains C-terminal halves are associated to form a coiled-coil rod domain, while the N-termini are folded in globular heads which are responsible for actin binding and force generation [36]. Two pairs of "regulatory" and "essential" light chains are associated to the heavy chain heads. In striated muscles, light chains do not participate in regulation of myosin enzymatic activity, rather they stabilise the structure of the complex. Removing light chains has a great impact on sliding velocity. Thick filament-associated proteins include myosin-binding proteins (MyBP) -C and –H [37]. They are composed of repeated Ig and FN-III domains and are believed to regulate the thickness of the thick filament by cross-linking myosin filaments and by interaction with titin [38].

The M-band (or M-line) is the central region of the sarcomere, where thick filaments are fastened together. It is crucial for correct alignment of the thick filament system [39]. Indeed, its main role may be to prevent or reduce misalignment of thick filaments during contraction, caused by random force imbalance between adjacent myosin filaments. Myomesin and M-protein (also called myomesin-2) are the main components of the M-band [40, 41]. They bridge myosin filaments and anchor titin at the centre, creating a complex network of stabilising interactions.

The functional equivalent of the M-line at the sarcomere ends is the Z-disc (also called Z-band or Z-line; Figure 2) named after its zig-zag appearance in electron micrographs [25, 42, 43]. It links actin and titin filaments from adjacent half sarcomeres into a lattice connected by α-actinin, a rod-shaped bipolar dimer with actin-binding sites at each end. Antiparallel actin filaments from the two adjacent sarcomeres overlap within the Z-disc, forming a square lattice such that every actin filament from one side is surrounded by four filaments from the opposite side. In addition to actin, α-actinin is also able to cross-link

antiparallel titin filaments, by binding to the Z-repeats. The best known role of the Z-disc is to transmit tension through successive sarcomeres along the myofibril. However, the presence of many other proteins argues in favour of additional functions for the Z-disc, in particular in signal transduction and in mechanical connection with the sarcolemma. Recent reports point to an active role of the Z-disc in sensing mechanical stress and inducing changes in gene expression accordingly [44]. Many Z-disc proteins belong to the family of PDZ/LIM proteins which are characterized by a PDZ and one or more LIM protein-protein interaction domains. This suggests that these proteins may be involved in the formation of large signalling complexes [43]. Five Z-disc-associated PDZ/LIM proteins have been described so far: ALP (Actinin-associated LIM Protein), ZASP (Z-band Alternatively Spliced Protein, also termed Cypher or Oracle; refs. [45, 46]), Enigma, ENH (Enigma-Homologue protein) and CLIM1 [27, 43]. A separate subfamily of LIM-only proteins that localise to the Z-disc has been identified, comprising FLH-1, -2 and –3 (Four and a Half LIM domains), Zyxin, MLP (Muscle LIM Protein). Other important signal transducers have been found within the Z-disc, including PKCε (protein kinase C epsilon), the calcium-dependent phosphatase calcineurin (also known as protein phosphatase 2B), the calsarcins [47], myopodin, phosphodiesterase 5A, PAK-1 (p21-activated kinase-1) and ArgBP2 (Arg-binding protein 2). An additional set of Z-disc proteins is involved in protein ubiquitination and degradation [43]: MuRF1(muscle-specific RING finger 1) and MAFbx (muscle atrophy F box protein) are ubiquitin ligases, whereas the calpains are intracellular non-lysosomal Ca^{++}-dependent cysteine proteases [48].

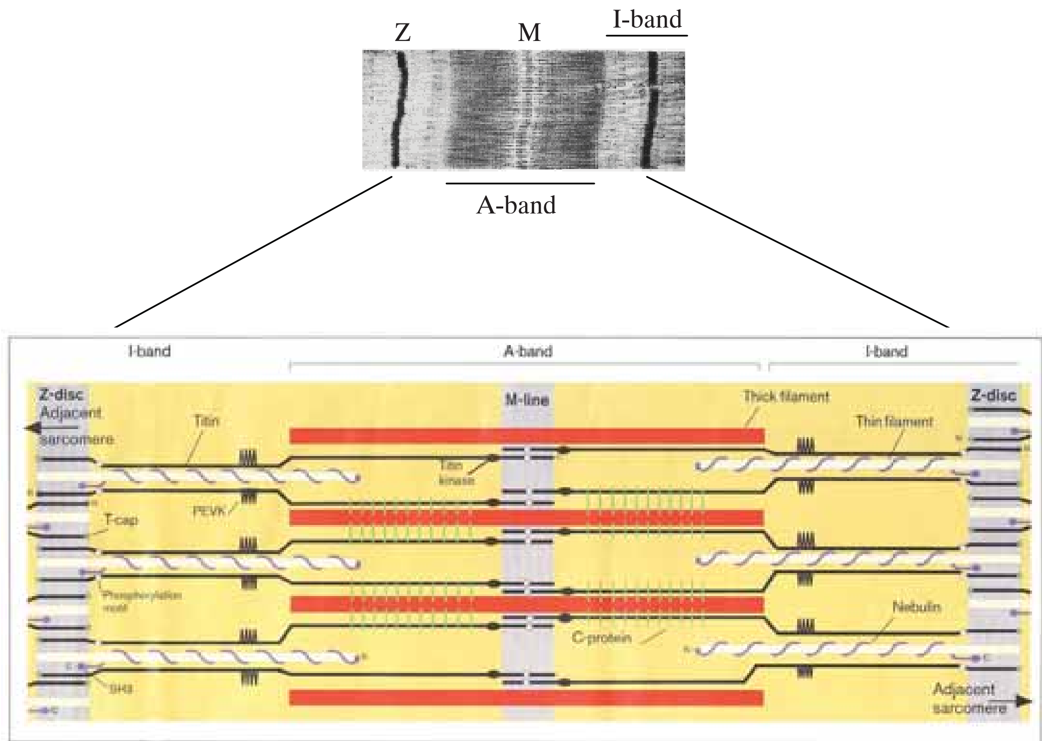

Figure 1. Electron micrograph (upper) and enlarged schematic drawing (lower) of a sarcomere, showing the four filament (actin-myosin-titin-nebulin) system and morphologic regions (A- and I-bands, M-line and the Z-disc). PEVK indicates the elastic region of titin (adapted from Gregorio et al. [29]).

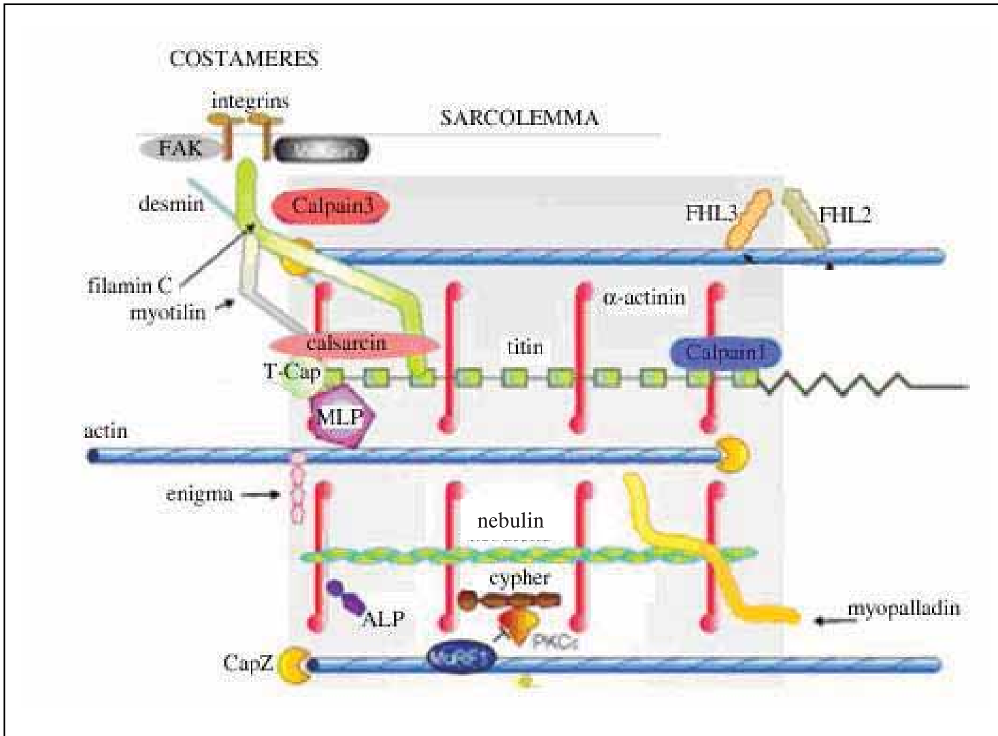

Figure 2. Schematic drawing of the Z-disc and its molecular components (adapted from Frank et al. [43]).

2.3.3. The sarcolemma connection

An IF-based network mediates the mechanical and functional linkage of the contractile apparatus to the rest of the cell, in particular to the sarcolemma. Desmin is the predominant IF protein interconnecting Z-discs between them and to the sarcolemma. Costameres are Z-disc-linked subsarcolemmal structures, representing the cortical cytoskeleton of a muscle cell [49]. They have been described as the muscle-specific equivalent of focal contacts. Indeed, both structures are enriched in vinculin, talin, α-actinin, spectrin and integrins. The available data suggest that costameres may function to laterally transmit contractile forces from sarcomeres across the sarcolemma to the extracellular matrix and ultimately to

neighbouring muscle cells [50]. This would be useful for maintaining uniform sarcomere length between adjacent cells and for minimizing shear stress imposed on the sarcolemma. A growing number of proteins forms the intricate costamere/sarcolemma network, whose importance is highlighted by the variety of muscular disorders associated with loss of its function (see section 5. *Muscular Disease*). The dystrophin-glycoprotein complex (DGC) is a transmembrane multiprotein complex that links cortical γ-actin to the extracellular protein laminin-2 [51, 52], as illustrated in figure 3. The DGC includes dystrophin, α- and β-dystroglycan [53, 54], four (α-, β-, γ-, and δ-) sarcoglycans [55, 56], α- and β-dystrobrevin, sarcospan and syntrophins. Caveolin-3 and nNOS (neuronal nitric oxide synthase) are also associated to the complex. While α- and γ-sarcoglycans are mainly expressed in striated muscle, β-, δ- and two additional (ε- and ζ-) sarcoglycans are more broadly expressed. Indeed, variants of the DGC are also present in non-muscle cells, where they connect the cortical actin cytoskeleton to the extracellular matrix [57]. Within the complex, dystrophin is the actual actin binder, through its aminoterminal actin-binding domain [58]. An additional actin binding site is located within the central rod domain, composed of 24 spectrin-like repeats. Near the C-terminus, the cysteine-rich domain binds the transmembrane protein β-dystroglycan, which in turn binds α-dystroglycan. The extreme C-terminal region mediates interaction with the syntrophins. The sarcoglycans are believed to stabilise the whole complex, as loss of any one of these proteins leads to the destruction of the entire DGC [59, 60]. The second major connection to the extracellular environment is mediated by integrins [61], a large family of ubiquitously expressed membrane proteins consisting of αβ heterodimers. Different α and β subunits are expressed in different tissues. Cell signalling originating from integrin engagement involves activation of selected protein kinases such as ILK (integrin-linked kinase) and FAK (focal adhesion kinase).

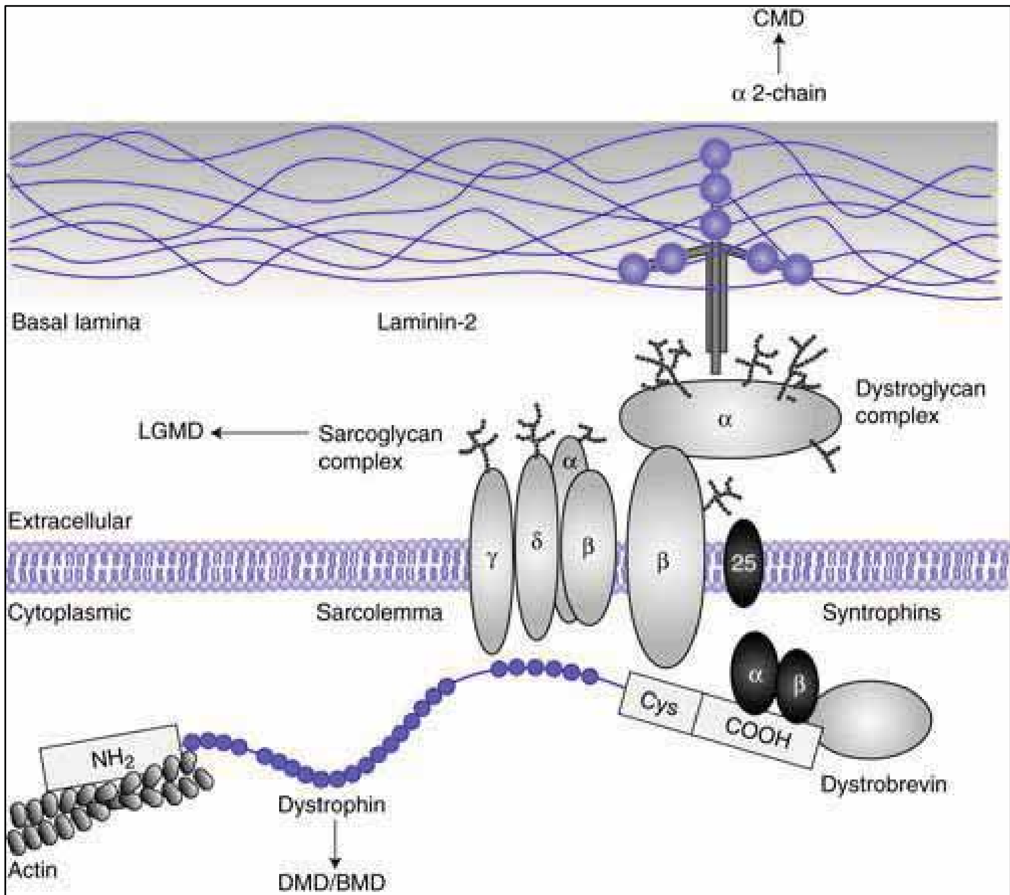

Figure 3. Schematic drawing of the dystrophin-glycoprotein complex, mediating direct connection between the cell cytoskeleton and the extracellular environment

The muscle-specific isoform of filamin, Filamin C (also known as filamin-2 or γ-filamin), is localised both at the Z-disc (where it binds FATZ-1/calsarcin-2 and myotilin) and at the sarcolemma (bound to the DGC via γ- and δ-sarcoglycan and to β_1-integrin subunits). Therefore, it provides a direct link between the sarcolemma and the myofibrils and is thought to have an important function in signalling between the two compartments [62, 63]. As all members of the filamin family, Filamin C is able to cross-link F-actin.

3. The Palladin/Myotilin/Myopalladin family

3.1. Palladin and myopalladin

Palladin, myopalladin and myotilin form a small family of highly homologous actin-binding proteins with Ig-like domains [64]. Palladin is the most widely expressed member of this family, as it is found in various epithelial and mesenchymal cell types, including smooth muscle [65, 66]. Palladin is expressed in multiple isoforms, ranging in size from 30 to 200 kDa, mainly due to alternative initiation and termination of transcription and also to alternative splicing. The longest, 200 kDa isoform is exclusively expressed in skeletal and cardiac muscle and comprises five Ig-like C2-type domains homologous to Ig-like domains of myotilin and myopalladin, and two proline-rich domains (FPXPP) responsible for the binding of Ena/VASP family proteins. VASP proteins are important regulators of actin assembly, especially at active sites of actin dynamics, such as lamellipodia, filopodia and focal adhesions in non-muscle cells [67]. This implicates palladin in the organisation of the actin cytoskeleton [68]. It is thought that palladin may recruit VASP at specific sites of actin polymerisation. As a further evidence of its role in actin dynamics, palladin co-localises with α-actinin in focal adhesions, cell-cell junctions and stress fibres. The α-actinin binding site resides in a short sequence upstream of the three C-terminal Ig-like domains [69]. This α-actinin binding sequence is highly conserved also in myotilin. Moreover, palladin interacts with ezrin, an important structural component of the cortical actin cytoskeleton [66].

Myopalladin is a 145-kDa protein most related to palladin, with five Ig-like domains and one proline-rich motif [70]. Unlike palladin, its expression is restricted to striated muscles, where it mainly localises to Z-discs and I-bands. Myopalladin binds to α-actinin, to the Z-disc region of nebulin (and its cardiac homologue nebulette) and to the cardiac ankyrin

repeat protein (CARP). Interaction with α-actinin and nebulin suggests that myopalladin may anchor nebulin to the Z-disc, thus contributing to the building of the sarcomere [33]. In line with this, overexpression of myopalladin fragments induces severe disruption of the sarcomere. The interaction with CARP suggests a role for myopalladin in mechanic stress sensing. CARP is abundantly expressed in the developing heart and is strongly induced in stressed skeletal muscle. It is localised both in the nucleus, where it regulates the expression of cardiac genes, and in the sarcomeric I-band. Therefore, myopalladin and CARP may act as a myofibril-nucleus shuttling complex that controls gene expression in response to various stimuli generated in the sarcomere. Recently, myopalladin has been implicated in the onset of idiopathic DCM: 4 of 114 patients carried missense mutations at conserved sites [71].

3.2. Myotilin

Myotilin (myofibrillar titin-like protein) also known as TTID (TiTin Immunoglobulin Domain) was the first member of this small family to be discovered [72, 73]. It was originally identified as a novel α-actinin binding partner with two Ig-like domains, that localised to the Z-disc [73]. The C2-type Ig-like domains reside at the C-terminal half, and are highly homologous to the two most C-terminal Ig domains of palladin and myopalladin [74], and more distantly related to Z-disc Ig domains 7 and 8 of titin. By contrast, the N-terminal part of myotilin is unique, consisting of a serine-rich region with no homology to known proteins. Expression in human tissues is mainly restricted to striated muscles and nerves [73], although minor expression has also been detected in other organs [72]. In muscles, myotilin is predominantly found within the Z-discs, although it has been observed at the sarcolemma as well. Myotilin forms homodimers and binds α-actinin [73], actin [75],

Filamin C [63], FATZ [76], ZASP [77] and MURF [78] proteins. Self-association is mediated by the C-terminal Ig-like domain-containing region of myotilin, whereas binding to the EF-hand motifs of α-actinin occurs via the N-terminal sequence and accounts for Z-disc localisation. The interaction with Filamin C could explain the sarcolemmal localisation of myotilin and provides a novel link between the cell membrane and the sarcomere. Filamins have a modular structure, with 24 Ig-like domains following an N-terminal actin-binding domain. The muscle-specific Filamin C isoform has a unique 78-aminoacid insertion within Ig-like domain 20, which appears to be responsible for Z-disc targeting. The interaction with myotilin was mapped to a region corresponding to domains 19-21. However, myotilin was later shown to interact with Filamins A and B, as well [76]. The filamin binding sites of myotilin have been located in the two Ig-like domains [63] and in the N-terminal half [76].

Myotilin induces the formation of actin bundles *in vitro* and in non-muscle cells [75]. The Ig-like domains most probably mediate actin binding, as a C-terminal myotilin construct showed co-localisation with the actin cytoskeleton in transfected cells. However, fragments containing Ig-like domains were not sufficient to induce the formation of large actin cables, indicating that other parts of myotilin are needed for bundling. Interestingly, a ternary complex myotilin/actin/α-actinin can be observed *in vitro* and actin bundles formed in this conditions appear more tightly packed than those induced by α-actinin alone. It was demonstrated that myotilin stabilises F-actin by slowing down the disassembly rate. Due to its properties, myotilin is likely to play a role in myofibrillogenesis in muscle cells. Indeed, ectopic overexpression of either N-terminal or C-terminal truncated myotilin causes the disruption of nascent myofibrils and co-accumulation of myotilin and titin in amorphous cytoplasmic precipitates [63, 75]. Human myotilin is normally expressed at late stages of differentiation, during myofibril alignment. In contrast, titin and α-actinin are expressed

much earlier. This suggests that premature expression of myotilin might be deleterious in differentiating myoblasts, as indicated by the dramatic reorganisation of the actin cytoskeleton in transfected non-muscle cells. In mature sarcomeres, wild-type myotilin co-localises with α-actinin and Z-disc titin, showing the striated pattern typical of sarcomeric proteins.

FATZ-1 (also known as myozenin-1 or calsarcin-2) is a Z-disc-associated protein that binds filamin, α-actinin and telethonin [79]. FATZ-1 belongs, together with FATZ-2 and -3, to a small family of proteins that interact with calcineurin. Having a similar ultrastructural distribution and common binding partners, FATZ-1 and myotilin were hypothesised to interact. Indeed, it was shown that full-length, but not truncated, myotilin binds the C-terminal domain of FATZ-1 and FATZ-2. Interestingly, while myotilin and FATZ-1 co-localise in the sarcomere, they show a different localisation in transfected CHO cells: myotilin induces and decorates actin bundles, whereas FATZ-1 shows a punctuated pattern along the stress fibres. However, when co-transfected, myotilin induces the relocalisation of FATZ-1 to actin bundles, again indicating that myotilin has a dominant effect on the intracellular distribution of its binding partners and therefore has a prominent role in the organisation of the cytoskeleton.

Very recently, our laboratory characterised a specific interaction between myotilin and various PDZ/LIM proteins [77]. Myotilin interacts with the PDZ domain of ZASP, ALP, CLP36 and RIL via its five C-terminal residues (ESEEL). This C-terminal motif is conserved in palladin and myopalladin proteins and is also present in FATZ family proteins. Accordingly, all these proteins are able to bind ZASP. Therefore, the myotilin and FATZ families form a superfamily of PDZ ligands. The interaction between PDZ domains and their ligands is often regulated by phosphorylation: myotilin was shown to be phosphorylated by CaMKII *in vitro* [77].

Considering its binding partners, *in vitro* properties, timing of expression and its activity in cells, myotilin is considered to be a stabiliser of the Z-disc structure. Of note, myotilin, Filamin C and α-actinin altogether create a formidable actin cross-linking complex at the Z-disc. Therefore, loss of normal myotilin function may have dramatic consequences for the architecture of the sarcomere. Indeed, myotilin mutations have been identified in families showing hereditary myopathies (see below).

4. Myogenesis

4.1. Stages of mouse development

As in all vertebrates, early stages of mouse development involve simple cell divisions from the fertilised egg to a 16-cell *morula*. At this stage, about 3 days post coitum (dpc; or E3), as cells continue to divide, the first specialisation occurs: an internal cavity develops and the cells from the outer layer, forming the wall of the sphere (the trophectoderm), are committed to become extraembryonic tissues. An inner mass of cells, located to one side of the cavity, will give rise to the whole of the embryo proper [80]. The cells of the trophectoderm make close contact with the wall of the uterus, initiating formation of the placenta. Meanwhile the inner cell mass (ICM) grows and begins to differentiate. Part of it (the one facing the cavity, known as visceral endoderm, endoblast, or hypoblast) gives rise to some further extraembryonic structures, such as the yolk sac, while the rest of it (the epiblast) goes on to form the embryo. As the ICM grows, a new cavity appears within it: the amniotic cavity. Now the embryo is composed of a simple bilayer of cells that separates the amniotic cavity from the yolk sac. At the beginning of the gastrulation (6-7 dpc), a linear invagination (the primitive streak) appears on the amniotic surface of the two germ layers. Complex movements of epiblast cells towards and through the primitive streak lead to the insertion of a new sheet of cells between the epiblast and the hypoblast. At the end of

gastrulation, a complete embryo with three germ layers, namely ectoderm, mesoderm and endoderm, has formed. The embryo already shows a symmetry. At this stage, the median region of the ectoderm thickens and differentiates into the neuroblast, forming a neural plate. Its margins then begin to rise (E7.5) and finally fuse along the midline, giving rise to the neural tube. Some cells that take part to this movement do not actually fuse, but form two columns of cells along both sides of the neural tube, called neural crests. They then fragment into several small cell masses, which will generate the ganglia and other cell types [81]. Proliferation and differentiation of the neural tube epithelium will eventually determine the formation of the whole nervous system. Since its first appearance, the cephalic region of the neuroblast is much larger than the rest. This difference is maintained in the neural tube, as the rostral portion of it is characterised by large vesicles rather than a simple tube. These vesicles represent the primitive brain [82]. At the same time, the digestive tract can be identified, as the most dorsal portion of the yolk sac gets engulfed by the closure of the lateral margins of the embryo. Differentiation of the various segments of the digestive tube leads to the formation of several structures, such as the pharyngeal arches (also termed branchial or visceral arches, formed between 7.5 and 9.5 dpc), and later the lungs, the liver and the pancreas. In particular, the lungs develop as an outgrowth of the foregut (the primordium of the bronchi) that serially bifurcates into progressively smaller tubes.

At E7.5 the heart starts to develop and at E8 the first somites appear in the anterior region. At 9-10 dpc the rostral domain of the neural tube closes and the forebrain vesicle subdivides into telencephalic and diencephalic vesicles; the limb and tail buds appear. By E13 all major organs, including genital and urinary systems, have started to form, and limbs with fingers are clearly visible. During late stages of development (E14-E18) the foetus increases in size,

skin derivatives (hair and nails) appear, and eyelids close upon the eyes. After 19-20 days of gestation, the newborn mouse is delivered [83, 84].

4.2. Development of striated muscles

The developmental process leading to muscle formation in vertebrates is complex and tightly regulated. The first striated muscle to form is the heart, followed by skeletal muscle, which is mainly derived from the somites.

The first sign of cardiac development is the cardiogenic plate (E7-7.5) that is first found cranially outside the embryo [85]. Pre-myocardial cells can be traced by expression of *NKX2.5*, a master gene controlling cardiac development. Then, as the embryo folds, this area is brought ventral and caudal to the head. The primitive heart develops at about 7.5 dpc from a pair of endocardic veins, which come in contact and fuse at a specific region, just caudal of the pharyngeal arches, along the embryo midline. The resultant cardiac tube, which already expresses cardiac α-actin, grows unevenly so that different regions can be distinguished in the antero-posterior axis: bulbus cordis, ventricle, atrium and the venous sinus. Then, the heart tube starts to fold in a way that brings the atrium, which is originally posterior to the ventricle, anterior and dorsal. At this stage, the ventricle region extends to the right and makes a loop with two segments that will grow out, forming the left and right ventricles, separated by a constriction that will develop as the ventricular septum. At about the same time (E11), the atrial component of the primary heart tube blows out to form morphologically distinct left and right atria, which then become separated by growth of a thin muscular wall (the atrial septum). By E12, the development of the four cardiac chambers has completed [86].

Somites appear at about E8 in the cervical region of the mouse embryo [87], as spheroid agglomerates of epithelial-like cells at both sides of the neural tube (figure 4). Formation of

new somites proceeds in the cephalo-caudal direction. Cues from the neural tube and the notochord, including Wnt, Shh and Notch signals, induce segmentation and epithelialisation of the paraxial mesoderm, leading to the formation of the somites. Presumptive muscle progenitor cells in the early somite express Pax3 and Myf5 [88, 89] which mediate activation of MyoD expression in response to muscle-inducing signals. Each somite then differentiates into two subdomains of committed cells: dermomyotome and sclerotome, which will yield skin/muscle and bone tissues, respectively. At this stage, muscle-determined cells (myoblasts) express all the four muscle regulatory factors (MRFs): Myf5, MyoD, myogenin and MRF4 (also known as Myf6). The MRFs are transcription factors characterised by a helix-loop-helix motif (HLH) that recognises CANNTG sequences (called E-boxes) on promoters/enhancers of target genes. They can roughly be divided into determination (Myf5 and MyoD) and differentiation (myogenin and MRF4) factors, as the former determine the fate of myogenic cells and the latter induce the expression of muscle-specific genes. Moreover, Myf5 and MyoD are normally considered as acting upstream of myogenin and MRF4. However, the scenario is more complex than this: there is a significant degree of redundancy among the four MRF activities, as shown by knockout and knock-in mouse models [90]. In addition, a great deal of reciprocal induction takes place and the MRFs also work in concert with MEF2 family factors. Therefore, it is the entire network, rather than a single actor, that is to be considered as the master switch of myogenesis [91]. In any case, having four different players with different spatio-temporal patterns of expression helps fine-tune muscle differentiation in the various body districts.

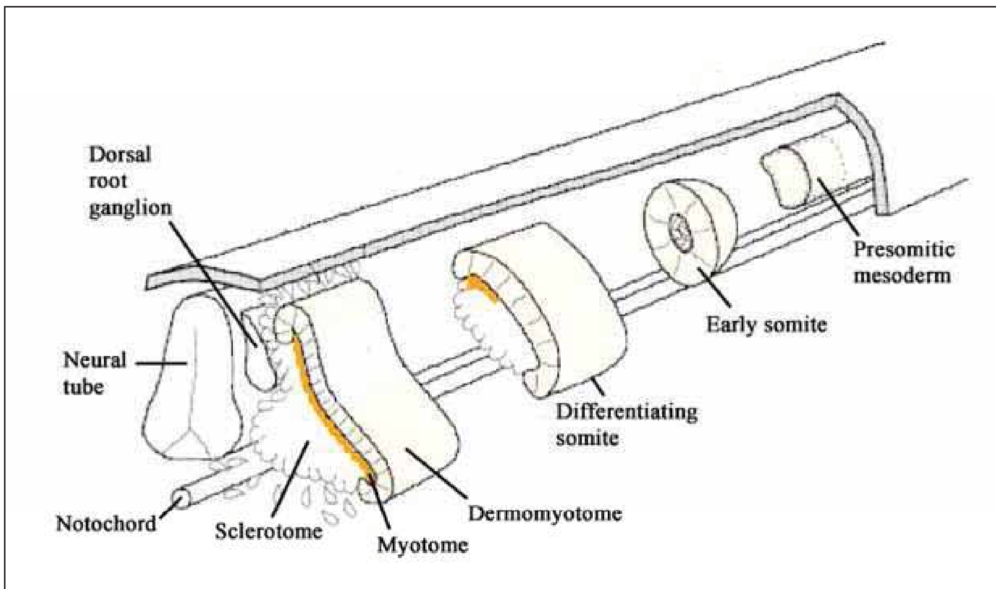

Figure 4. Schematic representation of somitogenesis. Myotome compartment is coloured orange (adapted from Buckingham et al. [92])

MRF-expressing cells in the dermomyotome still have a round shape and proliferate actively. Soon after, some cells delaminate from the ventral edge of the dermomyotome to colonise the central region of the somite and generate the myotome, the precursor of trunk muscles. Some cells migrate farther to found muscle masses of the limbs. At this point the cells (myocytes) exit the cell cycle and begin to express terminal differentiation genes. The first muscle-specific protein, detected at E9, is desmin, followed by titin, actin and myosin, and a few hours later by nebulin [5]. These proteins still show a speckled distribution within the cells. At day 11, myocytes begin to elongate and acquire a spindle shape, while a periodic staining of muscle proteins indicates the formation of the first, immature myofibrils. As myogenesis progresses, longer and more organised myofibrils fill the cytoplasm and the myocytes begin to fuse, to become multinucleated myotubes. Around E14, as myotubes grow longer and larger, myofibrillar proteins acquire the characteristic

cross-striated pattern and the nuclei are pushed aside. Mature contractile fibres have fully developed to an adult-like structure by 17 dpc, a couple of days before birth. Throughout adult life, muscle stem cells (satellite cells) are found under the basal lamina of muscle fibres. These Pax3/Pax7/Myf5-positive quiescent cells become activated in response to muscle injury, or during post-natal muscle mass growth, and provide a reserve of myogenic precursors for muscle regeneration. It has been observed that these same Pax-positive cells are present in the dermomyotome during pre-natal development and provide a source of muscle cells. Very recently, a pool of Pax7-positive endothelial cells with high myogenic potential has been observed in the same compartment as satellite cells [93]. The data suggest that this myoendothelial cell population can regenerate damaged muscle with higher efficiency than satellite cells and can also replenish the satellite cell pool. *In vitro*, myoendothelial cells show multilineage potential in the appropriate conditions and are considered as a promising tool for human therapy.

5. Muscle disease

5.1. Classification of neuromuscular diseases

Neuromuscular diseases are traditionally classified into three main categories: 1. Neurogenic muscle diseases, hereditary and acquired, 2. Disorders of neural transmission (myasthenias), hereditary and acquired and 3. Myogenic diseases, with a great number of both hereditary and acquired diseases. Given the enormous field and the scope of this thesis, only the last mentioned myogenic disease group will be described here. Even within this group, I will limit detailed description to those groups of hereditary myogenic diseases into which the myotilin-associated dystrophies/myopathies are classified. Although defects of striated muscle genes, including myotilin, also manifest themselves as cardiomyopathies, heart disease is not covered in this review. Cardiac involvement is mentioned whenever associated with skeletal muscle disease.

Defects in a large number of sarcolemmal, sarcomeric, cytoplasmic, nuclear and extracellular proteins cause muscle disease. Pathologists have traditionally divided myogenic disorders into two main classes: muscular dystrophies (MD) and non-dystrophic myopathies. While MDs show disruption of muscle integrity and necrosis, myopathies are characterised by weakness and atrophy, often with intracellular deposits. Even though this classification may still have a morphological significance, it has become more and more evident during the last decade that it does not hold from a molecular point of view, as proteins involved in both types of disorder can be found. Moreover, an overlap of phenotypic manifestations is often observed, that contribute to blur the picture of disease classification. There is nowadays a tendency to refer to a muscle disease as a specific *proteino*-pathy, and diagnosis is based, whenever possible, on genetic mutational analysis. For simplicity, I will here group the diseases by clinical denomination, according to the

official journal of World Muscle Society [94]. However, molecular relations and classification is also presented.

5.2. Muscular dystrophy

The clinical course of MD varies from mild, slowly progressing forms with late onset to severe, rapidly wasting congenital myopathies. X-linked MDs include Duchenne (DMD), Becker (BMD) and Emery-Dreifuss (EMD) muscular dystrophies. DMD and BMD are both caused by mutations in the *dystrophin* gene and are therefore allelic variants of dystrophinopathy [95]. DMD and BMD are the most common genetic muscle disorders, with an approximate rate of 1 in 3,000 newborn males. A high proportion of cases is represented by *de novo* mutations, due to the extremely large size of the *dystrophin* gene (about 80 exons spanning over 2.3 Mb). DMD patients suffer a progressive muscle weakness that typically causes inability to walk by the age of 12 and eventually leads to death because of respiratory or heart failure. BMD is usually less severe and some patients can walk throughout their lives. The majority of DMD mutations are deletions which cause a reading frameshift in the sequence, leading to a premature stop codon and the synthesis of a truncated, non-functional protein. In most cases, nonsense-mediated decay of the mRNA and/or degradation of the abnormal protein leads to almost completely negative immunostaining for dystrophin. The outcome is that not only dystrophin, but the whole DGC is missing from the sarcolemma. Thus, the link between the actin cytoskeleton and the extracellular environment is lost. This is thought to be the cause of progressive muscle damage from repeated contraction. The milder BMD phenotype is prevalently associated with in-frame deletions of the central region of the *dystrophin* gene, that lead to the production of a partially functional protein. The observed correlation between type of mutation and phenotype (the “reading frame” hypothesis) suggested that most of the central

rod-domain may not be essential for dystrophin function: the intact actin-binding (N-terminal) and dystroglycan-binding (C-terminal) domains would be enough to restore quasi-normal muscle functionality. This was indeed shown by microdystrophin transgenic mice, in which ectopic expression of miniaturised centrally deleted dystrophins restored muscle integrity in *dystrophin/utrophin* double-knockout mice [96]. The most commonly used mouse model for DMD/BMD is the naturally occurring *mdx* mouse [97], which carries a mutation in the *dystrophin* gene causing total loss of dystrophin protein. Despite striking molecular similarities with its human counterpart (fibrosis of muscle tissue, centrally nucleated fibres, elevated serum creatine kinase levels) this mouse does not show the severity that is typical of the human disease. This is possibly due to compensation by overexpression of the dystrophin-related protein utrophin. Therefore, the *dystrophin/utrophin* doubly null mouse represents a better model of DMD from a therapeutic point of view.

X-linked EMD is caused by a deficiency in emerin, an integral protein of the nuclear envelope inner membrane [98]. Emerin interacts with lamin A, one of four lamins that line the inner surface of the nuclear membrane. Notably, an autosomal form of EMD is linked to mutations in the *lamin A/C* gene. Other nuclear proteins involved in the onset of EMD are nesprin-1 and -2 (nuclear envelope spectrin repeat protein) [94]. Thus, EMD is a disease of the nuclear envelope. *Lamin A/C* knockout mice show an EMD-like phenotype [99]. Clinically, EMDs are characterised by muscle weakness, cardiac arrhythmia and contractures at the joints. Lamin defects are also associated with a number of additional muscular and non-muscular disorders, including LGMD1B (see below), Dunnigan-type familial partial lipodystrophy, and progeria. The generic terms laminopathy or nuclear envelopathy have been introduced to indicate this panel of genetically related diseases.

5.3. Congenital muscular dystrophy

Congenital muscular dystrophy (CMD) is a condition that refers to a spectrum of diseases with very early onset, normally already manifest at birth or shortly after. They have autosomal recessive inheritance and are clinically and molecularly quite heterogeneous. CMDs can be assigned to three major classes based on the genes involved:

(I) Abnormalities of extracellular matrix proteins: laminin-α2 chain of merosin (LAMA2) and collagen VI subunits COL6A1, COL6A2 and COL6A3. About one third of patients with classical CMD display LAMA2 mutations. Collagen mutations are less frequent.

(II) Defects of sarcolemmal links to the extracellular matrix: integrin α7 chain and several enzymes with glycosyltransferase activity: fukutin, POMGnT1, POMT1, POMT2, FKRP and LARGE. Thus, mutations hit, either directly (integrin $\alpha_7\beta_1$) or indirectly (loss of glycosyltransferase activity leads to defective processing of α-dystroglycan) one of the two merosin receptors. These CMDs (muscle-eye-brain disease, Walker-Warburg syndrome, Fukuyama MD) are typically associated with brain malformations and mental retardation.

(III) Mutations in an endoplasmic reticulum protein (selenoprotein N1). This relatively rare condition is also referred to as rigid spine syndrome.

Altogether, an emerging theme among factors involved in the pathogenesis of CMD is their connection to the extracellular matrix [100]. Despite obvious differences in onset and muscle involvement, several CMD forms display a certain degree of overlap with LGMD. In fact, some recessive LGMD types are allelic with CMD and the clinical boundaries between phenotypes are not so sharp.

5.4. Limb-girdle muscular dystrophy

Limb-girdle muscular dystrophies (LGMDs) are a heterogeneous group of MDs characterised by pelvic and/or shoulder girdle musculature weakness. They can be divided in two groups by mode of inheritance: there are 7 (3 known genes, 4 mapped but gene not identified) currently known autosomal dominant (type 1) and 15 recessive (type 2) LGMDs [101], as listed in Table 1. Several LGMD-associated genetic defects have also been observed in other muscle diseases, which are therefore considered as allelic disorders.

5.4.2. Dominant LGMD

Autosomal dominant LGMDs comprise seven forms, often found in only a few families. Typically, mutations lead to the expression of dominant-negative forms of the involved proteins.

LGMD1A is the limb-girdle phenotype of myotilinopathy and is discussed in a separate section [see par. 5.8]. **LGMD1B** is a mild, slowly progressive disease with cardiac conduction defects [102]. It is caused by mutations in *lamin A/C*, the same gene involved in autosomal dominant (AD)-EMD. Therefore, LGMD1B and AD-EMD are allelic disorders. Actually, lamins A/C are involved in various neuromuscular, cardiac, and lipodegenerative diseases [103]. It is not yet clear whether the type of mutation underlies the type of disease. Mutations are predicted to perturb the structure of the nuclear envelope. A single mutation was found in a family in which three phenotypes were described: EMD, LGMD1B (both with cardiac involvement) and pure DCM, suggesting the presence of additional, genetic and non-genetic, disease modifiers [104].

Caveolin-3 mutations underlie a third dominant form, named **LGMD1C**. Caveolin-3 is the muscle-specific member of the caveolin family of membrane proteins. Caveolins oligomerise in certain areas of the cell membrane, and constitute the coating of small

membrane invaginations called caveolae, involved in membrane trafficking and cell signalling [105]. Caveolin-3 is localised at the sarcolemma where it binds β-dystroglycan (it is therefore a member of the DGC) and dysferlin. Mutations in caveolin-3 lead to LGMD1C, hyperCKaemia, rippling muscle disease, and an atypical distal myopathy [106]. LGMD1C-associated mutant caveolin-3 protein acts as a dominant-negative inhibitor, oligomerising with wild-type caveolin-3 and directing these complexes to proteosomal degradation. Ultimately, this effect results in an almost complete loss of caveolin-3 expression. Both knockout and transgenic mice have been described. Lack of caveolin-3 leads to depletion of caveolae in striated muscles and induces a very mild phenotype with evidence of myofibre apoptosis in the soleus and the diaphragm [107]. Loss of caveolae seems to cause a defect in targeting of the DCG to lipid rafts, thus altering its normal sarcolemmal distribution [108]. Moreover, abnormal T-tubules were observed. On the other hand, over-expression of caveolin-3 leads to a DMD-like phenotype with an abnormal number of caveolae [109]. The similarity with DMD may be explained by a competition between caveolin-3 and dystrophin for β-dystroglycan binding.

Not all LGMD families with dominant inheritance carry mutations in myotilin, lamin or caveolin-3. At least four additional loci, designated **LGMD1D** to **-1G**, have been described, but the causing genes have not yet been identified [94]. Linkage analysis has restricted the candidate areas (6q23 [LGMD1D] - 7q [LGMD1E] - 7q32 [LGMD1F] - 4p21 [LGMD1G]).

Type	Gene/Locus	Ref.	Localisation	Allelic disorders
1A	Myotilin	[110]	Sarcomere	MFM; SBM ;Distal myopathy
1B	Lamin A/C	[102]	Nuclear membrane	EMD; Dunnigan's lipodystrophy; DCM
1C	Caveolin-3	[111]	Sarcolemma	HyperCKemia; Distal myopathy; Rippling muscle disease; HCM
1D	6q23	[112]		
1E	7q	[113]		
1F	7q32	[114]		
1G	4p21	[115]		
2A	Calpain 3	[116]	Sarcoplasm/sarcomere	Eosinophilic myositis
2B	Dysferlin	[117]	Sarcolemma	Miyoshi Myopathy; Distal anterior myopathy
2C	γ-sarcoglycan	[118]	Sarcolemma	
2D	α-sarcoglycan	[119]	Sarcolemma	
2E	β-sarcoglycan	[120]	Sarcolemma	
2F	δ-sarcoglycan	[121]	Sarcolemma	DCM
2G	Telethonin	[122]	Sarcomere	DCM; HCM
2H	TRIM32	[123]	Sarcoplasm/sarcomere	Sarcotubular myopathy
2I	Fukutin-related protein	[124]	Sarcoplasmic reticulum	CMD1C; WWS; MEB
2J	Titin	[125]	Sarcomere	TMD; DCM; HCM; Edström myopathy
2K	POMT1	[126]	Sarcoplasmic reticulum	WWS; MEB; CMD
2L	Anoctamin 5	[127]	Sarcolemma	Gnathodiaphyseal dysplasia
2M	Fukutin	[128]	Golgi	WWS; MEB; Fukuyama CMD
2N	POMT2	[129]	Sarcoplasmic reticulum	WWS; MEB; CMD
2O	POMGnT1	[130]	Sarcoplasmic reticulum	WWS; MEB

Table 1. Gene table of the Limb-Girdle Muscular Dystrophies: autosomal dominant LGMD1A-G are listed on grey background color; autosomal recessive LGMD2A-O are shown with light-blue background. Allelic disorders are shown for each disease.

5.4.1. Recessive LGMD

Recessive forms account for most LGMD cases. A growing number of genes is involved in the onset of autosomal recessive LGMDs. Mutations normally cause a loss of normal protein function.

LGMD2A (calpainopathy) is one of the most common LGMDs (approximately one third of all cases). In contrast to sarcoglycanopathies (see below) and other LGMDs, muscle degeneration in LGMD2A is not a consequence of sarcolemma disruption. The DGC is functional and no or very little Evans-blue uptake, a marker of sarcolemmal fragility, is seen [131]. Inactivating mutations of the gene coding for calpain-3 cause LGMD2A [116]. Calpains are a group of non-lysosomal calcium-dependent cysteine proteases, involved in a number of cellular processes, including remodelling of the cytoskeleton, signal transduction and apoptosis. Calpain-3 is the muscle-specific member of the calpain family. It has additional domains compared to its non-muscle homologues and is mainly localised within the sarcomere, where it binds to titin. Calpain-3 is normally present in the sarcomere in an inactive form. When activated, it cleaves itself and a number of substrates that includes titin, Filamin C, vinexin, ezrin and talin. Its activity is physiologically important for maintenance of adult myofibres, probably for turnover of sarcomeric proteins and dynamic remodeling of the sarcomere during unloading/reloading cycles of muscle activity. It was also shown that calpain-3 deficiency leads to myofibre apoptosis due to IκB accumulation and consequent loss of NFκB-dependent survival genes expression [132]. There is great heterogeneity of LGMD2A-associated calpain-3 mutations: some lead to complete lack of calpain-3 expression, while others cause loss of enzymatic activity; in other cases, the protein is active but mislocalised and yet other patients show impairment of substrate cleavage. There seem to be a correlation between type of mutation and phenotype, as null

patients tend to show a more severe disease [133]. Despite the observed variability, the common mechanism of LGMD2A is a deficiency in calpain-3 proteolytic activity. Calpain-3 knockout mice suffer muscle atrophy and show misalignment of A-bands [134].

LGMD2B is caused by mutations in dysferlin, a protein involved in sarcolemma repair [117]. In exercised fibres, contraction-induced mechanical stress leads to partial disruption of the cell membrane, even in the presence of a functional DGC. Resealing of damaged sarcolemma is induced rapidly after injury, by a calcium-dependent pathway that requires fusion of vesicles near the site of membrane disruption [135]. Dysferlin-deficient mice lack this membrane fusion-repair mechanism and develop progressive muscular dystrophy with accumulation of vesicles underneath the sarcolemma [136]. Dysferlinopathies also includes two other clinically distinct disorders termed Miyoshi myopathy and distal anterior compartment myopathy, both of which initially affect distal instead of proximal muscles. The coexistence of the three phenotypes of dysferlinopathy in one pedigree has been reported, indicating that they are separate manifestations of the same disease [137].

LGMD2C-F are also termed sarcoglycanopathies, as they affect one of the sarcoglycans [56]. As in DMD, the entire DGC is lost at the sarcolemma, whenever one of the sarcoglycans is missing. Clinically, sarcoglycanopathies show predominance of early onset, with mean age of onset within the first decade of life, and eventual confinement to a wheelchair. However, there is great variability in severity. Patients most often present with lower limb weakness and calf hypertrophy, but usually normal cardiac and intellective functions, although cardiac involvement has been reported [138]. Respiratory complications are frequent. The first animal model of sarcoglycanopathy was a Syrian hamster reported in 1962 that inherited cardiomyopathy and muscular dystrophy [139]. Loss of δ-sarcoglycan was later demonstrated to be the genetic defect of the cardiomyopathic hamster [140]. Several sublines of this hamster have been derived since then. More recently, targeted

genetic deletion of individual sarcoglycan proteins in mice has been described. α-sarcoglycan knock-out mice showed progressive muscular dystrophy similar to the *mdx* mouse, with extensive degeneration and regeneration of muscle fibres [60].

Immunofluorescence analysis demonstrated total loss of α-, β-, γ-, and δ-sarcoglycans and sarcospan. Only ε-sarcoglycan was still present at the sarcolemma. This variant, which is highly homologous to α-sarcoglycan, can form complexes with β-, γ-, and δ-sarcoglycans in smooth muscle, but its role in skeletal muscle is not clear. A later work demonstrated that enforced overexpression of ε-sarcoglycan led to the formation of functional sarcoglycan complexes and rescued *SCGA-/-* mice [141]. For what pertains the other components of the DGC, only a minor reduction of dystrophin and α-dystroglycan was observed in these mice. Nevertheless, association of both α- and β-dystroglycan to the sarcolemma was greatly affected. Similarly to *mdx* mouse, utrophin was up-regulated in α-sarcoglycan null mice. Similar findings were reported for β-, γ- and δ-sarcoglycan-deficient mice [59, 142, 143]. These data altogether demonstrate that the integrity of the whole DGC is dependent on the expression of each sarcoglycan.

The Z-disc protein telethonin is the target of frameshift mutations that create a premature stop codon and cause **LGMD2G** [122]. Telethonin, also known as T-Cap, is a titin capping protein at the Z-disc of the mature sarcomere, where it also interacts with FATZ-1, MLP and other proteins. During myofibrillogenesis, telethonin co-localises with titin M-band region and is phosphorylated by the titin kinase domain [43]. LGMD2G is a mild form of MD with mixed proximal/distal presentation and age of onset between 2 and 15 years. Ultrastructural analysis of LGMD2G muscle indicated that sarcomeric architecture integrity was maintained. Interestingly, mutations that alter telethonin binding to titin are associated with cardiac diseases [144]: impaired binding causes dilated cardiomyopathy (DCM), whereas stronger interaction leads to hypertrophic cardiomyopathy (HCM). No knockout

model for telethonin has been reported yet. However, MLP-null mice were shown to have mislocalised telethonin in the Z-discs of the myocardium and were afflicted by both skeletal muscle and heart performance deficit [44].

LGMD2H was recently linked to mutations in the *TRIM32* gene, which encodes for a protein involved in the ubiquitin-proteasome degradation pathway. Trim32 is an E3-ubiquitin ligase with anti-apoptotic properties, its overexpression is associated to cancer development [145]. Hence, it is possible that inactivating mutations in LGMD2H affect survival signals in myocytes. Moreover, Trim32 ubiquitinates actin, suggesting its involvement in myofibrillar protein turnover. The same missense mutation (D487N) was identified in patients suffering from sarcotubular myopathy, which therefore can be considered as a clinical variant of LGMD2H [123].

LGMD2I belongs to the group of diseases caused by loss of the linkage between the extracellular matrix and the actin cytoskeleton, such as the sarcoglycanopathies. Mutations in the gene encoding fukutin-related protein (FKRP) cause LGMD2I as well as various forms of CMD with mental retardation [124, 146], of which CMD1C (congenital muscular dystrophy type 1C) is the most severe disorder, with very early onset. FKRP is a glycosyltransferase involved in the O-linked glycosylation of α-dystroglycan. Accordingly, patients have a marked reduction of α-dystroglycan at the sarcolemma [147]. The same deficit of α-dystroglycan processing has been observed in Fukuyama CMD (FCMD) and muscle-eye-brain disease (MEB), both caused by mutations in glycosyltransferases. Biopsies can also reveal a secondary deficiency in laminin-α2. The data in literature suggest a correlation between the reduction in α-dystroglycan and the clinical phenotype in MDC1C and LGMD2I, supporting the hypothesis that dystroglycan plays a central role in the pathogenesis of these disorders. Additional LGMD forms with mental retardation have been recently shown to be allelic to CMDs with defective dystroglycan O-glycosylation. In

particular, **LGMD2K** is caused by mutations in the POMT1 gene [126], **LGMD2M** is characterised by fukutin mutations [128], while **LGMD2N** and **LGMD2O** are associated with mutations in POMT2 and POMGNT1 proteins, respectively [129, 130]. Together with LGMD2I, these disorders are now collectively referred to as dystroglycanopathies.

The giant sarcomeric protein titin is mutated in **LGMD2J** and in tibial muscular dystrophy (TMD), an autosomal dominant distal myopathy [125]. Furthermore, individuals with Edström disease were shown to carry a mutation in titin , therefore all three these diseases are considered allelic [148]. Mutations causing TMD/LGMD2J (also referred to as titinopathy) normally cluster at the M-line portion of titin, and affect one of the two calpain-3 binding sites of titin. Indeed, secondary deficiency of calpain-3 is a common feature of titinopathies. Integrity of the sarcomere is preserved, but myocytes show signs of apoptosis, suggesting that the sensor/signalling function of titin may be damaged in this disease. Defects in titin are also associated with cardiomyopathy, but in this case the mutations are located more N-terminal, before the M-line region. A natural model of titinopathy is the muscular dystrophy with myositis (*mdm*) mouse, harbouring a deletion of 83 aminoacids from the first calpain-3 binding site in I-band titin [149]. The *mdm* mouse shows the same loss of calpain-3 seen in human patients. Titin knockouts die embryonically, indicating that functional titin is indispensable during development. In contrast, conditional deletion of M-line exons in adult striated muscle leads to severe myopathy, with progressive disassembly of the sarcomeres in skeletal muscle and widened M-line in cardiomyocytes. Consequently, the mice show both skeletal and cardiac involvement and die within 5 weeks of age [150]. In line with these studies, expression of a truncated titin in a myofibroblast cell line leads to defective myofibril assembly [151].

Finally, **LGMD2L** was recognised in 2007 as a later evolution of patients initially diagnosed with quadriceps myopathy [152] and has recently been linked to Anoctamin 5

[127], also known as TMEM16E or GDD1. Anoctamins are Ca^{++}-activated Cl^{-} channels with eight transmembrane segments. In particular, Anoctamin 5 is highly expressed in cardiac and skeletal muscle as well as in bone and was previously found to be mutated in patients affected by a bone fragility syndrome called gnathodiaphyseal dysplasia [153].

5.5. Congenital myopathy

Congenital myopathies (CM) include a series of both dominant and recessive, slowly progressive disorders, usually apparent at birth, that cause general muscle weakness and loss of muscle tone and bulk [154]. The distinctive feature of CMs is the accumulation of protein aggregates in the cytoplasm [155]. The aggregated material can have tubular, filamentous or other morphologies, depending on the type of disease. Major CM forms include nemaline myopathy (NM), central core disease (CCD), actin myopathy, and myotubular myopathy.

Typical NM is characterised by intracellular thread- or rod-like bodies. NM presents with early weakness of the facial, neck, respiratory and proximal limb muscles, with a later distal involvement. More severe forms are rare. Seven loci for NM have been established [94, 156]: *TPM2* (β-tropomyosin), *TPM3* (α-tropomyosin), *NEB* (nebulin), *ACTA1* (α-actin), *TNNT1* (slow troponin T), *CFL2* (cofilin-2), and an unknown gene located on chromosome 15q. It clearly appears that NM is a thin-filament-associated disease. NM-associated aggregates, called nemaline rods, are composed essentially of filamentous actin, but also contain α-actinin, nebulin and myotilin.

CCD causes skeletal deformities and diffused weakness and is caused by mutations in the gene encoding for ryanodine receptor 1, a sarcoplasmic reticulum Ca^{2+} release channel. It is characterised pathologically by the presence of so-called central core lesions, regions of sarcomeric disorganization, absence of mitochondria and of oxidative activity [157]. A

variant of CCD, known as multi-minicore disease, is caused by alterations in the same gene or in selenoprotein N1, and is characterised by multiple, smaller core lesions.

Myotubular myopathy is caused by defects in myotubularin, a lipid phosphatase that acts on phosphatidylinositol 3-monophosphate (PI_3P) and has been shown to be essential for skeletal muscle maintenance in mice. Myotubular myopathy patients generally have a poor prognosis and suffer an early death, due to diminished respiratory capacity, unless ventilated [158].

Centronuclear myopathy includes both dominant and recessive forms, with mutations in the genes encoding dynamin-2 and amphiphysin, respectively [94].

Additional CM variants are known to be caused by defects in myosin heavy chain, MyBP-C, tropomyosin 2, contactin, titin, TRIM32.

5.6. Myofibrillar myopathy

Myofibrillar myopathy (MFM) is a relatively broad term comprising various muscle diseases that show a variety of intracellular inclusions that appear to be of Z-disc origin and are associated with myofibrillar degeneration [159]. In fact, the unifying feature of MFM is the dissolution of myofibrils and the abnormal aggregation of disorganised sarcomeric material in the sarcoplasm. Protein deposits may appear as amorphous, granular, spherical, or polymorphous hyaline structures. These disorders are also termed desmin-related myopathies, as they were initially described by accumulation of desmin-positive inclusions [155, 160]. However, several additional myofibrillar proteins are found in MFM aggregates, including actin, myosin, nebulin, titin, α-actinin, myotilin: basically, all major myofibrillar components, reflecting the destruction of the sarcomere [161]. At present, mutations in the genes encoding for desmin, selenoprotein N1, αB-crystallin, four-and-a-half-LIM protein 1 (FHL1) and the sarcomeric proteins myotilin [162], ZASP [163] and Filamin C [164], are

known to cause MFM. The majority of patients, however, carry mutations in other unidentified loci. Clinically, MFM is heterogeneous, often showing late onset and distal muscle involvement, although proximal weakness has been reported in many cases. In addition, cardiac and neurological symptoms are frequent in MFM. Other symptoms are specific to the gene involved: spine rigidity is associated to selenoprotein N1 mutations, whereas cataract is often found in patients carrying αB crystallin defects.

In 2005, Sharma and Goebel [155] proposed to unify MFMs and CMs under the general class of protein-aggregate myopathy (PAM), since they share morphological hallmarks (intracellular inclusion bodies).

5.7. Distal myopathy

Mutations in several genes are known to be the causative defect in various non-congenital myopathies with predominant distal involvement and presenting with variable severity. Many of these disorders are allelic forms of other muscle diseases described in this review, such as LGMD (mutated genes include myotilin, dysferlin [Miyoshi myopathy], titin [TMD] and caveolin-3), CM (nebulin, dynamin-2) and MFM (ZASP). Unique types of distal myopathy are Nonaka myopathy, caused by mutations in *N*-acetylmannosamine kinase, and Vocal Cord and Pharyngeal Distal Myopathy (VCPDM), which maps to 5q31 and was therefore thought to be caused by myotilin defects, but no mutations have been found [165].

5.8. Myotilinopathy

The first mutation in *myotilin* gene was discovered in a large American kindred with LGMD1A [110] and caused the substitution of an isoleucine for a threonine in the N-

terminal serine-rich region of the protein (T57I). This mutation does not affect binding to α-actinin, nor myotilin localisation within the sarcomere. It is likely that the mutation causes a gain of function, since LGMD1A shows dominant inheritance. This hypothesis is supported by the finding that truncated myotilin constructs have dominant-negative effects on myofibril structure in transfected myoblasts [63]. A second mutation was subsequently observed in another family [166], affecting the same serine-rich region (S55F). Interestingly, both substitutions change a polar, potentially phosphorylable residue (Ser or Thr) to a hydrophobic one (Ile or Phe). Emphasising the importance of the Ser-rich domain, additional mutations (S60C, S60F, S95I, as well as the previously known S55F) were later identified in MFM [162]. LGMD1A and myotilin-related MFM share common molecular features, such as Z-line streaming, rimmed vacuoles and accumulation of fibrillar material in the myofibres (Figure 5). Clinically, although similar, the two diseases remain distinct entities. LGMD1A patients usually present with proximal weakness that can progress to involve distal limb muscles and have nasal dysarthric speech. In contrast, MFM patients present with more distal than proximal involvement. Moreover, cardiomyopathy is rare in LGMD1A individuals, whereas it is often associated with MFM [64]. Furthermore, MFM patients are frequently affected by peripheral neuropathy. However, the discovery of myotilin mutations (including novel mutations K36E and Q74K) in patients showing both LGMD1A and MFM phenotypes led to a common molecular classification under the name of myotilinopathy [167]. Another family with mixed proximal and distal myopathy has been recently shown to carry a S60F myotilin mutation [168]. The affected members have no other phenotypic sign of LGMD1A or MFM, indicating that myotilinopathy has a broader clinical spectrum than previously thought. Confirming this finding, a family with myotilin S55F substitution was shown to have almost exclusive distal involvement in all affected members [169].

Very recently, a novel mutation in the second immunoglobulin-like domain was described in a Japanese patient with classical LGMD1A [170], carrying an R405K mutation that causes defective homodimerisation and weaker α-actinin binding.

Another neuromuscular disease, called spheroid body myopathy (SBM), was shown to harbour a mutation in the serine-rich domain of myotilin (S39F). SBM has been described in only one family [171]. Affected individuals show late onset symptoms quite similar to classical LGMD1A. However, a unique pattern of spheroid bodies within the cytoplasm of type I fibres was found, which have not been reported in LGMD1A. Rather, they are consistent with the amorphous deposits observed in MFM biopsies. In this regard, SBM can be viewed as a bridge between other myotilinopathies, as it shares clinical features with LGMD1A but shows morphological abnormalities typical of MFM.

More recently, strong myotilin immunoreactivity was found in nemaline rods and central core lesions, the large protein aggregates that are typical of nemaline myopathy and central core disease [172]. No mutations have been detected in *MYOT* gene in these patients. The presence of myotilin in both types of protein accumulation may suggest a role in the formation or maintenance of such material.

To recapitulate the phenotype of myotilinopathies, a transgenic mouse model has been generated, expressing human myotilin carrying the LGMD1A-associated mutation T57I. These mice showed many of the features observed in patients, such as Z-line streaming, muscle weakness and myofibrillar aggregation [173]. Moreover, centrally located nuclei were observed, indicating regeneration and replacement of damaged myofibres. Protein aggregates derived from degenerating myofibrils contained transgenic myotilin, as well as endogenous α-actinin, γ-filamin, desmin, titin and myosin proteins. A similar pattern of protein aggregation has been described in muscles from MFM and SBM patients.

An interesting observation derives from a non-pathological situation. Exercise-induced myofibrillar damage leads to severe pain in non-diseased muscles. This is normally associated with Z-disc streaming. Interestingly, myotilin accumulation has been observed at sites of active myofibrillar remodelling in exercised muscles, once again indicating a prominent role of myotilin in sarcomerogenesis [174].

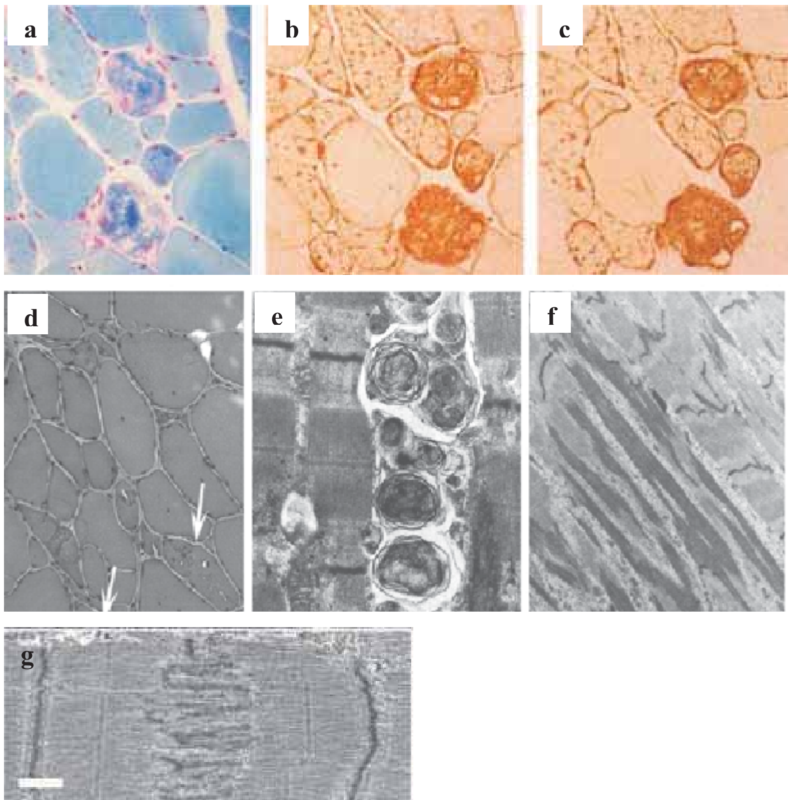

Figure 5. Morphological features of myotilinopathy. Intracellular deposits are revealed by Gomori trichrome stain (a) of myofibres from a MFM patient [162]. Desmin (b), αB-crystallin (c) and other myofibrillar proteins (not shown) accumulate in regions of abnormal trichrome staining. (d) Section of LGMD1A muscle [110] showing variations fibre size, fibre splitting, centrally located nuclei and rimmed vacuoles (arrows). Electron-microscopy images show the vacuoles (e) and characteristic Z-line streaming (f). In (g) another electron micrograph showing Z-disc smearing.

AIMS OF THE STUDY

The sarcomere is a highly ordered structure that maintains skeletal muscle integrity and is essential for force transmission throughout the muscle fibre. As a consequence, the sarcomere is composed of hundreds of proteins, whose expression and localisation is tightly regulated. Whenever one of its components is altered or missing, a pathogenic condition ensues. Myotilin is an actin cross-linking sarcomeric protein that is mutated in LGMD1A, MFM and SBM. Despite growing knowledge of its biology, its role in muscle development and function is still poorly understood. This work was undertaken in order to elucidate the mechanisms that underlie myotilin-related diseases.

Specific aims of these studies were:

1. To characterise myotilin gene and its promoter in mouse.
2. To analyse the expression pattern of myotilin during mouse development and compare it with that in human.
3. To generate a myotilin-null genetic model in the mouse and analyse its phenotype.

The result obtained should give important hints on the biological role of myotilin within the sarcomere.

MATERIALS AND METHODS

Antibodies used in the study

Three myotilin-specific antibodies were raised in rabbits in our laboratory and used for these studies. Polyclonal antibody #353 was generated using a peptide corresponding to residues 353-369 (within the second Ig-like domain) of human myotilin as an antigen. The antibody #231 was raised against a GST-tagged fragment spanning amino acids 231–342 (corresponding to the first Ig loop) of human myotilin. These two antibodies against human sequences cross-reacted with mouse myotilin. Finally, antibody #151 was raised against purified His-tagged N-terminal mouse myotilin (amino acids 1–150). All antibodies were affinity purified using mouse myotilin coupled to Sepharose 4B resin. Titin-specific antibody was provided by Prof. Jacques Beckmann (Généthon Laboratory, Evry, France; [175]). Anti-α-actinin monoclonal antibody (clone EA-53) was purchased from Sigma-Aldrich; anti-myosin heavy chain antibody (MF20) was a kind gift of Dr. Marianne Tiainen, University of Helsinki. Other antibodies employed were: mouse polyclonal anti-telethonin, rat polyclonal anti-FATZ, mouse anti-ZASP, mouse monoclonal anti-β-tubulin clone B-5-1-2, anti-actin (AC40), and anti-palladin rabbit antibody, (E1a, kindly provided by Markus Moser from Max Planck Institute, Germany).

Cloning of mouse myotilin

To isolate the mouse orthologue of myotilin, the human full-length cDNA was used as a probe to screen a mouse cDNA library. A positive clone representing the partial mouse sequence was identified and used for second-round screening at higher stringency. Finally, full-length mouse myotilin cDNA was isolated and sequenced. RACE analysis was

performed in order to detect the 5'-end of mRNA, using a myotilin-specific reverse primer for first strand cDNA synthesis.

Long-range touchdown PCR was used to determine the genomic structure of mouse myotilin gene, based on homology with the human locus. Genomic DNA was extracted using a standard method [176] and amplified using the following protocol: after initial template denaturation (5 minutes at 94°C) four miniprograms of four cycles each were run (94°C, 30 sec; annealing step, 30 sec; 68°C, 4 min) with annealing temperatures 62, 60, 58, and 56°C, followed by 32 standard cycles (94°C, 30 sec; 55°C, 30 sec; and 68°C, 4 min). PCR products were purified from agarose gel and sequenced to determine exon/intron boundaries.

In situ hybridisation

A non-radioactive method was employed to study myotilin mRNA levels in developing and adult mouse tissues. Two fragments of myotilin cDNA (nucleotides 531-1056 from the coding region and 1614-2150 from the 3'-untranslated region) were amplified by PCR and cloned into a pBluescript-based vector, with T3 and T7 promoters on either side of the insert. In vitro transcription from T3 and T7 sites in the presence of digoxigenin-UTP allowed the synthesis of sense and antisense digoxigenin-labelled RNA probes. Antisense probes were used to detect myotilin RNA, while sense transcripts were used as negative controls. Pregnant female mice were sacrificed and embryos and adult organs were dissected according to approved procedures and fixed in 4% paraformaldehyde, dehydrated in ethanol and xylene and embedded in paraffin. Four micrometer sections were placed on glass slides, paraffin was removed and tissues rehydrated. Subsequently, the samples were treated with 0.3% Triton X-100 and proteinase K and hybridised overnight with RNA probe

in hybridisation buffer (40% formamide, 10% dextran sulfate, 1X Denhardt's solution, 4X SSC, 10 mM DTT, plus yeast tRNA and salmon sperm DNA as blocking agents). After extensive washes, the probe was detected by alkaline phosphatase-conjugated anti-digoxigenin antibody. In some cases, samples were counterstained with hematoxylin. A titin-specific probe was obtained from Prof. Siegfried Labeit (University of Heidelberg, Germany) and used as a control for muscle-specific staining.

Immunochemistry

For immunohistochemistry, tissue specimens were snap-frozen in isopentane chilled with liquid nitrogen and stored at -80°C. Cryostat sections were mounted on glass slides, air-dried and stored at -80°C until needed. C_2C_{12} cells were directly grown on glass coverslips, induced to differentiate by shifting the culture to medium containing 2% horse serum and finally fixed with methanol and mounted onto glass slides.

Tissue sections and cell specimens were stained with the specific antibody, or the corresponding pre-immune serum as a control, in the presence of 3% BSA as a blocking agent, and then incubated with a species-specific secondary antibody. Bound antibodies were detected with Vectastain Elite ABC kit and the slides were counterstained with haematoxylin and eosin.

Myofibrils were isolated from skeletal muscles as described [177], centrifuged on glass slides, fixed with methanol and incubated with specific primary antibody. FITC- or TRITC-conjugated secondary antibodies were visualised by fluorescence microscope.

For Western blotting, tissues were homogenised in reducing Laemmli buffer using an Ultra-Turrax® homogeniser and the lysates were run on SDS–PAGE, transferred to nitrocellulose membranes and probed with the various antibodies.

Promoter characterisation

A Bacterial Artificial Chromosome (BAC) comprising the mouse myotilin locus was isolated from a mouse genomic library prepared from 129/SvJ mouse DNA (Research Genetics) using a full-length cDNA as a probe. The promoter region was cloned from the BAC DNA and sequenced. Analysis of putative transcription factor binding sites within the promoter sequence was performed with MatInspector software and with the TESS database. A number of progressively deleted fragments from this region were cloned into the pGL3basic vector, upstream of the firefly luciferase gene. To study promoter activation during myoblast differentiation, C_2C_{12} cells were seeded in 6-well plates, induced to differentiate, and co-transfected with the reporter constructs and the pRL-TK control plasmid (for internal sample normalisation). The cells were then lysed at different times and assayed for luciferase activity.

Generation of knockout mice

Conditional deletion of myotilin gene was achieved by replacing the endogenous locus with a floxed allele, in which exon 3 was flanked by loxP sites, that can be induced to recombine by expression of Cre recombinase, thus deleting the flanked sequence. The targeting vector carries the Neomycin resistance gene for selection of homologous recombination events, and the Herpes simplex virus thymidine kinase (HSV-tk) marker for negative selection of incorrect recombination. The construct was electroporated into R1 embryonic stem (ES) cell line, of 129Sv genetic background. After electroporation, G418- and ganciclovir-resistant ES clones were tested for correct targeting by Southern blotting, using 5’ and 3’ external probes. Chimeric embryos were produced by aggregation of targeted ES cells with morula stage wild-type embryos of ICR strain. Two chimaeras were identified that passed the

floxed allele through the germ line. Heterozygous floxed mice (myo$^{fx/+}$) were crossed with Cre-mice, ubiquitously expressing Cre recombinase under the cytomegalovirus promoter, to generate myo$^{-/+}$ offspring carrying the deleted allele, which were then interbred to obtain homozygous myotilin knockout mice (myo$^{-/-}$). The mice were subsequently bred on a mixed 129SvJ/ICR background for analysis.

Phenotypic analysis of myo$^{-/-}$ mice

For morphological analysis, muscle specimens were dissected and frozen in nitrogen-chilled isopentane. Five µm cryostat sections were placed on silanized glass slides, fixed with acetone and stained with haematoxylin and eosin. For ultrastructural studies, muscles were fixed in 4% phosphate buffered glutaraldehyde, dehydrated and embedded in epoxy resin. Ultra-thin sections were post-stained with uranyl acetate and lead citrate.

Mice were weighed weekly from birth for 11 weeks and then monthly until 38 months of age. Spontaneous behaviour was monitored throughout life. Muscle strength was tested using a grip strength meter. The mouse is placed in front of a grasping bar. The animal instinctively grabs the bar as it is pulled backwards. The instrument records the peak pull force, when the mouse releases its grip. The strength was normalised according to the weight. Voluntary running was measured in cages containing small metal running wheels connected to digital magnetic counters. The mice were allowed free access to the wheel. Running activity was recorded for a total period of 5 weeks. The parameters collected were daily running time and distance. At the end of the exercise, the mice were sacrificed and skeletal muscles were collected and prepared for cryosectioning.

For analysis of sarcolemma integrity, the mice were injected intraperitoneally with 1 mg of Evans Blue dye (EBD, a fluorescent dye that cannot enter intact cells) in 0.1 ml PBS per 10 g of body weight and euthanized by cervical dislocation 6 hours later. The muscles were

dissected and rapidly frozen in nitrogen-cooled isopentane. Cryostat sections were fixed and observed with a fluorescence microscope.

RESULTS AND DISCUSSION

Mouse myotilin gene characterisation (I)

Analysis of the mouse *myotilin* gene demonstrated a high degree of conservation between human and mouse. The coding sequences are 80% identical and amino acid sequences show 90% identity and 94% similarity, when considering conservative changes (I, Fig. 1). The mouse genomic locus is localised in a syntenic region (murine 18 B3 corresponding to human 5q31) and its overall exon/intron structure is conserved; the first exon comprises only untranslated sequence, while start and stop codons are located on exons II and X, respectively (I, Fig. 1, Table 1). PCR analysis of the transcript revealed three isoforms in mouse skeletal muscle tissue, two of which are predicted to encode for the same polypeptide. The third, smaller mRNA isoform lacks the upstream translation start codon: although another in-frame ATG is present, no smaller protein form was detected by Western blotting. The significance of such deleted mRNA *in vivo* is not clear. Northern blot analysis confirmed the presence of one major myotilin mRNA band, mainly in skeletal and cardiac muscle (I, Fig. 2). Minor expression was also found in other tissues, such as lung, kidney and liver. Localisation of myotilin transcript within the tissues was further studied by in situ hybridisation. Apart from strong staining of striated muscles, expression was identified in the bronchial epithelium, the cortical region of the kidneys, as well as various structures of the central nervous system, such as the hippocampus, the olfactory bulb and Purkinje cells (I, Fig. 3). A particularly high signal was detected in peripheral ganglia. Human myotilin has also been shown to be expressed in nerves [73]. These data may explain peripheral neuropathy observed in MFM patients. The fact that this was not found in LGMD1A families raises interesting questions concerning the phenotypic variability of myotilinopathy. However, absent or diminished tendon reflexes in LGMD1A patients could

suggest an incipient peripheral neuropathy. Also, cardiomyopathy is not a constant manifestation of myotilinopathies.

Interestingly, no myotilin was detected in smooth muscles, in keeping with its suggested role in the organisation of the sarcomere. Strong association of myotilin with sarcomeric/cytoskeletal structures was also indicated by its high resistance to detergent-mediated extraction (I, Fig. 4), and its ability to induce actin bundles when over-expressed in non-muscle cells.

Analysis of myotilin regulation showed that it is transcribed relatively late in the maturation of mouse C_2C_{12} myoblasts: while α-actinin and myosin heavy chain are already up-regulated 48 hours after induction of differentiation, myotilin expression was not observed until day 8 (I, Fig. 5). At this stage, however, myotilin is already correctly positioned along the myofibrils, showing the classical cross-striated staining pattern. In agreement with its predominant muscular expression, several muscle-specific responsive elements could be identified within the promoter region of mouse myotilin gene (I, Fig. 6). The importance of such putative transcription factor binding sites was studied by luciferase reporter assays, using progressive deletion constructs derived from the promoter sequence. Reporter activity was recorded during C_2C_{12} differentiation. Analysis of the data suggested a crucial role for one MEF2 binding site and a canonical myogenin/myoD E-box site (both situated approximately 1000 bp upstream of the transcriptional start site) which appeared to be required for full transcriptional activity (I, Fig. 6). However, we did not observe an all or nothing effect of any single region of the promoter. Rather, progressive loss of reporter activity was caused by successive deletions. Consistent with late up-regulation of endogenous myotilin in differentiating myocytes, highest luciferase activity was detected at day 8 post-induction.

These data altogether suggest that myotilin function is extremely conserved between human and mouse. Gene structure, amino acid sequence, pattern of expression and regulation are indeed very similar in the two species. In particular, the residues that are mutated in human disease are identical in murine sequence. In addition, this study indicates that myotilin is expressed, albeit at very low levels, in various tissues other than striated muscle. This finding is corroborated by data from embryonic tissues (see next section) and also by database searches (www.ncbi.nlm.nih.gov/UniGene) and previous work [72]. The role of myotilin in these cell types is unclear, but is not unprecedented among sarcomeric proteins, as exemplified by cellular-titin [178]. Non-muscle myotilin might be involved in the organisation of stress fibre structures, since it has potent actin bundling activity and binds to stress fibres-associated proteins α-actinin, filamin-A and -B, and CLP-36. However, this hypothesis has not yet been experimentally validated.

Expression of myotilin during mouse development (II)

We studied the spatial and temporal pattern of myotilin expression during embryonic development by in situ hybridisation and immunohistochemistry. As noted above, myotilin is mainly expressed in adult striated muscles; however, low levels of myotilin are also detected in other tissues, suggesting a broader function. In mouse embryos, its expression is even more widespread, in particular during mid-stages of gestation: at E13, strong myotilin staining was observed in many tissues including lung, liver, skin, cartilage and most of the nervous system (II, Fig. 2 and 3, Table 1). Later (E16-E18), expression in various organs declined to lower levels, as observed in the adult. As control experiments, non-specific probes or pre-immune serum did not give any signal, while a titin-specific probe only labelled skeletal muscles. A similar perinatal down-regulation of expression has been described previously, for β-sarcoglycan and palladin [65, 175].

Looking at earlier stages of development, between E8 and E11, myotilin mRNA and protein were found in the heart (the first organ to show myotilin expression), in the somites and in neural epithelium (II, Fig. 1). In line with *in vitro* observations (see I, Fig. 5 and [75]), expression in the somite was delayed compared to other sarcomeric proteins: while titin is already expressed at day 9 post coitum [5], myotilin could not be detected before day 10. Interestingly, myotilin in the somite was not restricted to the myotome compartment, as normally observed for sarcomeric proteins, but appeared as a more diffuse staining reaching the surrounding areas, corresponding to the dermatome and sclerotome, although myotomal cells showed the strongest signal. In addition, in cross-sections, the neural tube showed myotilin expression. These features were also observed in human embryos, where parallel staining with a titin-specific antibody allowed a direct comparison: while titin was exclusively detected in the myotome, myotilin was clearly present in a broader area (II, Fig. 4). Thus, myotilin is similarly regulated in mouse and human embryos, again suggesting that the mouse should be a good model for myotilinopathy.

Deletion of myotilin gene in vivo *(III)*

Mutation of myotilin in LGMD1A/MFM patients leads to disruption of the Z-discs, illustrating the importance of myotilin function in the maintenance of sarcomere architecture. Also, its widespread developmental expression suggested a relevant role in mouse development. To study the consequences of myotilin loss *in vivo*, we generated a conditional knock-out mouse model (*myo*$^{-/-}$) using Cre-LoxP-mediated gene targeting. We chose an inducible system in order to circumvent possible embryonic lethality, which we might have expected from the available data. The targeting construct was designed to cause Cre-inducible loss of the entire exon 3, which was predicted to create a reading frameshift, leading to a premature stop (III, Fig. 1). This would cause nonsense-mediated decay of the

mutant mRNA, or drive the synthesis of a non-functional polypeptide missing most of myotilin sequence including the two Ig-like domains. Indeed, while exon 3-specific primers did not amplify any PCR product from *myo*$^{-/-}$ mice, primers flanking the targeted exon revealed the presence of a deleted mRNA (III, Fig. 1). However, by Northern blotting, no myotilin transcript could be detected in null mice, even using an exon 4-5-6-specific probe (III, Fig. 2). This is probably due to a lower sensitivity of Northern blotting compared to RT-PCR, and suggests degradation of the deleted myotilin transcript. In agreement with Northern data, no myotilin protein was expressed in *myo*$^{-/-}$ skeletal muscle, as assessed by Western blotting and immunofluorescece staining (III, Fig. 3, 4).

Surprisingly, *myo*$^{-/-}$ mice not only were born at normal mendelian ratio, but appeared healthy throughout their lives. Myotilin null mice grew normally, showing no statistically significant difference in weight compared to myo$^{+/+}$ and myo$^{+/-}$ littermates (III, Fig. 5). Moreover, no alteration was observed in muscle force and running performance.

Ultrastructural and morphological analysis of skeletal muscles did not reveal major consequences of myotilin absence (III, Fig. 4). The fibres appeared normal, with peripheral nuclei; no sign of Z-disc streaming and no cytoplasmic deposits were noted in knock-out muscles. No damage in sarcolemma integrity was present. Similarly, we did not see any overt abnormality in other organs. Anti-α-actinin and anti-titin immunostaining of myofibrils showed a normal cross-striated pattern in both wild-type and null mice, indicating that the general structure of the sarcomere is preserved (III, Fig. 4).

Seeing that there is no obvious phenotypic repercussion of myotilin deletion, we thought that subtle compensatory changes in the organisation of the sarcomere might have occurred and vicariate the loss of myotilin. Analysis of Z-disc proteins expression revealed a significant up-regulation of telethonin, in both skeletal and cardiac muscles, in *myo*$^{-/-}$ mice

(III, Fig. 3). Interestingly, both telethonin and myotilin bind to titin within the Z-disc. Thus, telethonin might compensate for myotilin loss in *myo*-null mice. This may explain why absence of myotilin is never observed in human disease. However, the reverse is not true, as lack of telethonin leads to LGMD1G. Whether this increase in telethonin expression is required for maintenance of muscle integrity in the absence of myotilin, is not clear at the moment. Alternatively, telethonin up-regulation could be a sign of stress, as telethonin has been shown to be part of a sensor machinery within the Z-disc. Neither palladin nor myopalladin, the two closest homologues of myotilin, showed any change in expression levels.

The targeting of other Z-disc proteins, such as FATZ-2 and ALP, did not cause any clear skeletal muscle perturbation in mice [179, 180], suggesting that the Z-disc is a highly stable structure that is able to sustain specific protein losses.

Our results indicate that myotilin is not required for mouse development or for adult muscle tissue integrity. Combining these data with the current knowledge of myotilin function and the pathobiology of myotilinopathies, we suggest that disease-associated myotilin forms carry a dominant pathogenic activity, rather than a loss of their normal physiologic role. This is in agreement with cellular data, showing that over-expression of a truncated myotilin induces the destruction of nascent sarcomeres in differentiating myoblasts [63]. Furthermore, *in vivo* data from transgenic myo^{T57I} and double transgenic wt/T57I mice [173, 181] suggest that mutated myotilin may act as a super-active centre of protein aggregation, so that, while its normal function stabilises the Z-disc, its hyperactivity (or overexpression) destroys it.

CONCLUSIONS

In this work I describe the cloning and characterisation of the mouse myotilin gene. Myotilin was identified in our laboratory as an α-actinin binding protein in human striated muscle [73]. It was later found to be involved in the onset of muscular disease [110], now termed myotilinopathy. This finding prompted us to initiate studies on the mouse orthologue, in order to gain insights into the function of myotilin *in vivo*, using murine models.

Myotilin is highly conserved and similarly regulated between human and mouse (I), which is consistent with its role in sarcomere building. Its expression begins at late stages of muscle cell differentiation (I-II), suggesting that myotilin is involved in the final alignment of myofibrils, rather than in initial assembly of the Z-disc. Over-expression of truncated myotilin leads to Z-disc collapse and dominant mutated myotilin in patients causes Z-disc streaming and misalignment. Within the Z-disc, myotilin interacts with α-actinin, filamin C, actin, FATZ and ZASP.

Both human [73, 110] and mouse (I) studies identified the presence of two major transcripts, and other minor isoforms, but their role is not clear at this stage. It is possible that shorter forms of myotilin may regulate localisation and function of the full-length isoform. Future work may help elucidate the role of alternative transcripts of myotilin. In addition, post-translational modifications of myotilin have not yet been studied. Both in mouse and human, myotilin antibodies detected minor bands at higher and lower molecular weights. In preliminary experiments, we did not find evidence of N- or O-linked glycosylation, nor tyrosine phosphorylation of mouse myotilin (data not shown). However, disease-associated mutations very frequently hit serine or threonine residues, strongly

suggesting a possible regulation by phosphorylation, which was indeed verified at least *in vitro* [77]. This might open a new line of investigation in myotilin studies.

The fact that myotilin expression is not tightly restricted to striated muscle, but is found in other tissue types (I-II), although at lower levels, indicates that it may have additional functions, for example in focal adhesions. The biological meaning of the wide expression pattern during development (II) is not understood. It may reflect a function in tissue modelling. However, targeted disruption does not prevent normal development of *myo*-null mice (III). Therefore, myotilin does not seem to have a fundamental role in mouse development, not even in skeletal muscle formation. Alternatively, its task is backed up by other Z-disc proteins. However, even double mutant myotilin-null/200kDa-palladin-hypomorph animals do not show an overt phenotype, only developing a mild myopathy at old age [182]. It will be interesting to engineer a myotilin/palladin/myopalladin triple knockout. Indeed, no myotilin loss-of-function mutations have been described in muscle disease. Patient-derived mutant T57I, when introduced into transgenic mice, caused a LGMD1A/MFM-like phenotype [173], confirming that myotilinopathy is a gain-of-function disorder.

The accumulating knowledge of the functions of myotilin in muscle physiology should help understand its role in muscle pathology.

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