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# **ROLE OF INTERMEDIATE FILAMENT DESMIN IN DEVELOPMENT OF DESMIN- RELATED MYOPATHY**

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# ROLE OF INTERMEDIATE FILAMENT DESMIN IN DEVELOPMENT OF DESMIN-RELATED MYOPATHY THESIS FOR DOCTORAL DEGREE (Ph.D.)

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*To my Babusya and Dedusya*

## ABSTRACT

Desmin is a major intermediate filament of muscle cells, serving to transmit mechanical forces and propagate mechanochemical signals, to coordinate contraction and relaxation cycles, and to stabilize the positioning of cellular organelles, e.g. mitochondria. Around 70 desmin gene mutations have been reported in conjunction with desmin-related myopathy. Desmin-related myopathy can be described as pathophysiological complex, accompanied by desmin intracellular aggregate accumulation and impairment of desmin interactions with structural proteins, signal molecules, and cell organelles. However, the precise molecular mechanism underlying desmin-related myopathy have not been described yet. There are speculations if it is connected with toxic effects of desmin aggregates or with violation of desmin mechanotransduction functions.

The general aim of the present PhD project was to extend existing knowledge about the molecular machinery on how desmin gene mutations lead to the development of desmin-related myopathy, with an emphasis on development of cardiomyopathies. To address this aim the following research questions were stated: (i) genetic study of a group of patients with cardiomyopathies in order to describe novel mutations in the desmin gene, and to assess the frequency of *DES* A213V; (ii) genetic study by means of next-generation sequencing approach of a group of patients with idiopathic restrictive cardiomyopathy in order to describe novel genetic variants associated with disease; (iii) functional study of desmin gene point mutations effect on mitochondrial properties.

The main findings regarding genetic background were: (i) *DES* A213V represents a disease-modifying polymorphism, rather than disease-related mutation, since it was found both in patients and healthy donors; (ii) combination of disease-related–disease-modifying or disease-related–disease-related genetic variants, rather than single disease-related mutation, determined the development of idiopathic restrictive cardiomyopathy. Most proteins of these combinations belonged to four functional groups: sarcomeric contractile proteins, mechanosensing Z-disc proteins, nuclear membrane, and outer mitochondrial membrane proteins. Functional studies of the impact of desmin mutations on mitochondria showed that aggregate-prone mutations decreased mitochondrial calcium uptake, as well as depressed maximal oxygen consumption rate and spare respiratory capacity. In contrast, non-aggregate-prone mutations did not disturb mitochondrial calcium. They did, however, result in the reduction of maximal oxygen consumption rate and affected spare respiratory capacity.

To conclude, (i) distortion of desmin mechanotransduction functions plays an important role in desmin-related myopathy onset, affecting mitochondrial properties; (ii) combination of mutations in genes encoding sarcomeric contractile and mechanosensing proteins, rather than a single mutation, predisposes to the development of cardiomyopathy. These data facilitate understanding of molecular pathways underlying desmin-related myopathy development, and increase existing knowledge of intracellular interactions within the muscle cell.

## LIST OF SCIENTIFIC PAPERS

- I. Gudkova A, Kostareva A, Sjoberg G, Smolina N, Turalschuk M, Kuznetsova I, Rybakova M, Edstrom L, Shlyakhto E, Sejersen T. **Diagnostic challenge in desmin cardiomyopathy with transformation of clinical phenotypes.** *Pediatr Cardiol.* 2013 Feb; 34(2): 467–20. doi: 10.1007/s00246-012-0312-x.
- II. Kostareva A, Sjoberg G, Gudkova A, Smolina N, Semernin E, Shlyakhto E, Sejersen T. **Desmin A213V substitution represents a rare polymorphism but not a mutation and is more prevalent in patients with heart dilation of various origins.** *Acta Myol.* 2011 Jun; 30 (1): 42-5.
- III. Kostareva A, Kiselev A, Gudkova A, Frishman G, Ruepp A, Frishman D, Smolina N, Tarnovskaya S, Nilsson D, Zlotina A, Khodyuchenko T, Vershinina T, Pervunina T, Klyushina A, Kozlenok A, Sjoberg G, Sejersen T, Shlyakhto E. **Genetic spectrum of idiopathic restrictive cardiomyopathy uncovered by next-generation sequencing.** *Submitted manuscript.*
- IV. Smolina N, Kostareva A, Bruton J, Karpushev A, Sjoberg G, Sejersen T. **Primary murine myotubes as a model for investigating muscular dystrophy.** *BioMed Research International*, vol. 2015, Article ID 594751. doi: 10.1155/2015/594751.
- V. Smolina N, Bruton J, Sjoberg G, Kostareva A, Sejersen T. **Aggregate-prone desmin mutations impair mitochondrial calcium uptake in primary myotubes.** *Cell Calcium.* 2014 Oct; 56(4): 269 – 75. doi: 10.1016/j.ceca.2014.08.001.

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## LIST OF ABBREVIATIONS

AD	Autosomal dominant inheritance
ANT	Adenine nucleotide translocator
AR	Autosomal recessive inheritance
CmC	Chloro-m-cresol
CMP	Cardiomyopathy
DES	Desmin gene
DRM	Desmin-related myopathy
FCCP	Carbonyl cyanide p-[trifluoromethoxy]-phenyl-hydrazone
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
IF	Intermediate filament
IMM	Inner mitochondrial membrane
iPS	Induced pluripotent stem cells
LV	Lentivirus
MCU	Mitochondrial calcium uniporter
MFM	Myofibrillar myopathy
MICOS	Mitochondrial contact site complex
MyHC	Myosin heavy chain
NGS	Next-generation sequencing
OCR	Oxygen consumption rate
OMM	Outer mitochondrial membrane
RCMP	Restrictive cardiomyopathy
RFLP	Restriction fragment length polymorphism
RyR	Ryanodine receptor
SNP	Single nucleotide polymorphism
SR	Sarcoplasmic reticulum
TG	Transgenic mouse
TMRE	Tetramethylrhodamine, ethyl estere, perchlorate
ULF	Unit-length filament
VDAC	Voltage-dependent anion channel
WHO	World Health Organization
WT	Wild-type

# 1 INTRODUCTION

## 1.1 INTERMEDIATE FILAMENT

### 1.1.1 Intermediate filament overview

The cytoskeleton is composed of three classes of protein filaments—microtubules, intermediate filaments, and microfilaments, named after their diameter size: 25 nm, 10 nm, and 7 nm, respectively. The intermediate filament (IF) protein family is usually subdivided into five principal types according to primary amino acid sequence, net acidic charge, assembling properties, and tissue expression pattern. The types I and II IF comprised of acidic and basic cytokeratins that form only heterodimers and are found in epithelial cells. In contrast, the type III IF, encompassing vimentin, desmin, glial fibrillary acidic protein (GFAP), and peripherin form homodimers and are present in fibroblasts, muscle cells, some neural cells. The type IV IF includes low-, middle-, and high-molecular weight (MW) neurofilaments,  $\alpha$ -internexin, and nestin that are mainly expressed in neural cells. Type V IF is constituted of nuclear lamins, considered to stand apart from the other types due to the presence of nuclear localization signal in its primary sequence, and its ubiquitous expression pattern (Table 1) (1,2). It is important to note that IF of different types can co-exist in one cell, e.g. keratin and vimentin in human alveolar carcinoma cells (2). Moreover, some of them display ability to co-polymerize, e.g. during embryonic development vimentin and desmin co-expressed in early muscle, however vimentin is not found in mature muscle cells (3,4).

### 1.1.2 Intermediate filament structure and assembling

Despite the differences in amino acid sequences and expression sites, all IF share common tripartite structural traits. IF consist of a central  $\alpha$ -helical rod domain flanked by non-helical head and tail domains. The rod domain is composed of four  $\alpha$ -helical segments that retain repeating heptad motifs (1A, 1B, 2A, 2B). These segments are interrupted by linker sequences (L1, L12, L2). The rod domain is  $\approx 310$ - $350$  amino acid residues in length. This domain governs the process of filament dimerization and subsequent formation of coiled-coil structure. 1A and 2B segments are considered to be the most conserved part of IF. Whereas 1A is responsible for filament assembly, 2B mediates lateral filaments association (5,6). Assembly of IF occurs in three stages. During the first stage filament molecules form parallel dimers due to the rod domain interactions. Two antiparallel half-staggered dimers assemble to form tetramers. Eight tetrameres organize into unit-length filament (ULF)  $\approx 17$  nm in diameter and  $\approx 60$  nm long by means of lateral association. In the second stage ULFs undergo longitudinal annealing, resulting in formation of non-compact long filaments,  $\approx 17$  nm in diameter and hundred nanometers long. In the final assembly stage these loosely packed structures reorganize into mature tightly packed intermediate filaments  $\approx 10$  nm in diameter (Fig. 1) (6,7). It is noteworthy that rearrangements cause neither loss of the protein mass nor gain of the protein length (8,9).

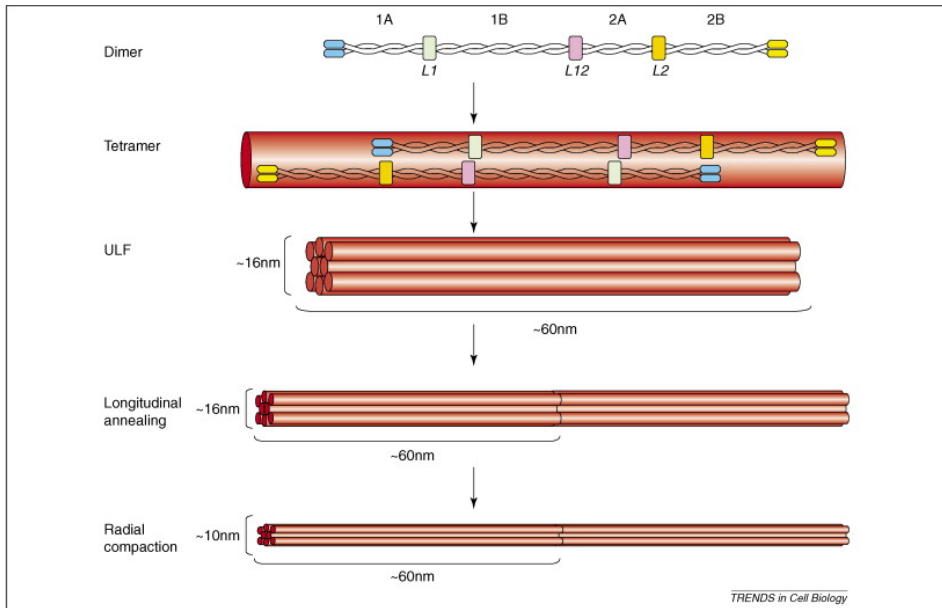


Figure 1. Schematic image of typical intermediate filament structure and process of intermediate filaments assembling (adapted from Godsel et al., Trends Cell Biol. 2008 Jan; 18(1): 28–37, (10); republished with permission).

### 1.1.3 Intermediate filament functions and interaction partners

For a long time cytoplasmic IF were considered to serve only as structural proteins, with a predominant role in maintaining cellular architectural integrity. Since IF were isolated as intact filaments and almost did not exist in soluble form, they were counted as a static element of the cytoskeleton, in contrast to actin and tubulin proteins that depend on pool of soluble subunits (11,12). However, over the past decades this conception was greatly revised due to experiments with microinjections of exogenous soluble proteins and fluorescent recovery after photobleaching (FRAP) analysis (13–16). It was established that exogenous keratin and vimentin freely incorporated into endogenous filamentous network via dynamic subunit exchange processes (17,18). FRAP analysis confirmed that subunit exchange took place along the filament length (16,19,20). Therefore, it was determined that IF are remarkably dynamic structures. Moreover, it was demonstrated that dynamic actions are under control of numerous molecules, mostly signaling, rather than structural, e.g. kinases (21, 22), phospholipases (23), GTPases (24). Subsequently, IF were proposed to be engaged in signal transduction processes affecting participants of signaling pathways, altering their intracellular localization, hindering or promoting their translocation between organelles (25). Besides, IF might interact with the other intracellular components, organelles, and molecules by means of linker proteins belonging to the plakin family. Plectin is one of the most important members of this family since it mediates crosstalk between all cytoskeletal components (26), as well as interconnects IF and mitochondria (27). Vimentin was recently showed to contribute to the cellular mechanics by providing intracellular stiffness and

maintaining spatial content organization (28). Subsequently, other studies unveiled role of vimentin also in cell adhesion and migration (23). Nuclear lamins reside in nuclear lamina, between the inner nuclear membrane and chromatin. Apart from maintaining nuclear structure lamins are also involved in gene expression, chromatin organization, cell cycle regulation, embryonic development, and cell differentiation (29–31). To sum up, IF are immensely dynamic and interactive molecules, accomplishing diverse functions, not limited to structure maintenance.

### **1.1.4 Intermediate filament mutations and diseases**

Due to the high variability of IF family members, their functions, and interaction partners IF gene mutations often result in severe disorders. The causative role of keratin K14 genes mutations in developing epidermolysis bullosa simplex was confirmed at the end of the 1980s (18). Keratin aggregates served as a hallmark of affected tissues, and were detected in cell culture expressing dominant-negative mutant form of the keratin gene (32). Cells devoid of keratin network lost their resilience and resistance to mechanical stress—for this reason it was deduced that the primary role of keratin filaments is to provide mechanical support of epithelial cells. Up to date, mutations in ~20 keratin genes have been described in connection with monogenic diseases. Nevertheless, some of them do not result in a failure of IF polymerization process. Detailed examination of these mutations instead revealed impairment of pigment uptake and transport mechanisms within keratinocytes (33). Type III IF mutations are also linked to clinical phenotypes. Desmin was the first non-keratin IF characterized to play a causative role for the development of desmin-related myopathy (DRM). DRM is a variant of myofibrillar myopathy, featured by the presence of desmin-positive aggregates within the cardiac and skeletal muscle cells (34–36). Subsequently, non-aggregate as well as aggregate desmin mutations were described in patients with cardiac and skeletal myopathies (37), suggesting impairment of nanomechanical properties of desmin, rather than structural entirety (38). Alexander disease, characterized by white-matter degeneration and the formation of astrocytic cytoplasmic inclusions, is caused by mutations in GFAP, another member of type III IF. Accumulation of cytoplasmic aggregates containing GFAP is demonstrated as histological sign for this illness (39). A number of neurodegenerative disorders are now known to be related to mutations in type III and IV IF, e.g. amyotrophic lateral sclerosis, Charcot-Marie-Tooth disease, Parkinson disease. Similar to earlier described phenotypes, these pathologies can be identified by the presence of abnormal protein aggregates in neurons and glia of the central nervous system. Experimental data revealed that mutations result in alteration of neuronal IF expression pattern and biochemical functions (For review see 40). Laminopathies is a broad group of diseases associated with mutations in LMNA/C gene and commonly affect tissues of mesenchymal origin (41). A wide range of clinical phenotypes are linked to nuclear lamin mutations, including cardiomyopathies and distal myopathies (42), lipodistrophies (43), progeria (44), and mandibuloacral dysplasia (45). It has been discovered that lamin mutations impair cell differentiation processes, probably altering gene expression pattern. However, precise molecular mechanisms remain to be elucidated. For some mutations aggregates residing within the nuclei have been detected (46). Taken

together, despite the majority of IF associated disorders being accompanied by accumulation of protein aggregates, development of the disease could not be attributed to structural defects only. IF non-mechanical properties, e.g. organelle positioning, signaling, cellular transport, gene expression are here plausible factors for the pathogenesis process.

## **1.2 DESMIN**

### **1.2.1 Desmin overview**

Desmin is a major IF of cardiac, skeletal, and smooth muscle cells. It was originally isolated from chicken smooth muscle, and characterized as a 100Å protein due to its size (47). Desmin is a 53 kDa protein expressed in mature muscle cells, and serves as an important early marker for myogenic cells (48). Like all myogenic proteins desmin expression is under control of basic helix-loop-helix (bHLH) transcription factors—MyoD, myogenin, and MRF4 (49). Moreover, desmin can be found in undifferentiated satellite cells, however at low levels (50). Along muscle development, desmin is upregulated, while vimentin pre-existing in immature myoblasts undergoes downregulation. Desmin filaments associate with vimentin filaments to form elongated strands. Upon myofiber development, these strands rotate transversely to take the position across myofibrils at the level of Z-discs (51). It has been demonstrated in vitro that desmin filaments indirectly mediate myoblast fusion via interaction with MyoD (52). On the other hand, desmin knockout mice displayed normal muscle maturation and were viable, thus disproving the essence of desmin in embryogenesis (53). This phenomenon might be accounted for large compensatory effect of cytokeratins. In fast twitch skeletal myofibers desmin is co-expressed with keratins K8 and K19 in the area of Z-discs and M-line, cooperating to interconnect neighboring myofibrils (54). Further investigations have broadened existing knowledge regarding desmin-keratin relations. To conclude, these data have shown that keratins might act independently, complementary or adversely to desmin activity (55).

### **1.2.2 Desmin functions and interaction partners**

#### *1.2.2.1 Desmin localization*

Desmin filaments are mainly localized in the area of Z-discs, under the sarcolemma at the myotendinous and neuromuscular junctions in skeletal muscle cells. Desmin is found at intercalated discs and Purkinje fibers in cardiac muscle (Fig. 2). Desmin filaments interconnect Z-discs to each other and to the sarcolemma at the level of costameres, thus integrating the process of muscle contraction (56).

#### *1.2.2.2 Desmin functions and interaction partners*

It is noteworthy that a bulk of existing data about desmin functions and interaction partners were obtained from desmin knockout mouse model (57). As was stated before, desmin ablation did not effect the muscle development, and knockout

animals were viable and fertile. At the same time, mice lacking desmin displayed multisystem disorders involving all muscle types. Analysis of affected tissues revealed severe disruption of cell architecture and massive degeneration of tissue, especially in the myocardium. Cardiomyocytes underwent progressive degeneration and calcification. On the structural level lateral alignment of myofibrils was missed and mitochondrial organization was disrupted (57). Therefore, the desmin knockout mouse model verified that desmin expression is pivotal for maintaining structural integrity of striated muscles. Thereafter, these null mice were studied to assess desmin impact on physiological properties of myofibrils. The absence of desmin resulted in more rapid fatigue and less isometric force. Myofibrillogenesis during regeneration was abnormal, and displayed signs of disorganization. However, vimentin was not upregulated. It is remarkable that in desmin-null mice weight-bearing muscles, e.g. soleus, or continually used muscles, e.g. diaphragm or myocardium, were mostly affected (53). Recently, elevated levels of inflammation response and muscle regenerative processes, as well as adipocyte infiltration were reported for the desmin deficient muscles responding to notexin treatment (58). To expand the previously obtained knowledge the desmin knockout model was used to estimate the effect of desmin on mitochondrial spatial orientation and bioenergetic functions. It was disclosed that desmin filaments play a key role in mitochondrial anchoring within the myofibers. Slow twitch skeletal myocytes lacking desmin filaments demonstrated aberrant accumulation of mitochondria in the subsarcolemmal space, while cardiomyocytes demonstrated additional extensive mitochondrial proliferation after work overload. To estimate mitochondrial activity ADP-stimulated respiration was measured in situ and in isolated mitochondria. Interestingly, isolated mitochondria did not show any differences in respiration rates between knockout and wild-type (WT) animals, in contrast to in situ measurement, where desmin-null myocardium and soleus muscle exhibited reduced ADP-stimulated oxygen consumption rate (59). Thus, it was concluded that desmin affects not only mitochondrial positioning, but also mitochondrial functional properties. Subsequently, it was found that plectin, a member of cytolinker proteins, provides a desmin–mitochondria link (27). Later on, desmin deficient muscles were tested for response to isometric exercise and to susceptibility to mechanical injury. Desmin knockout muscles were shown to generate lower force and be less exposed to injury in comparison to WT muscles (60). These data demonstrate that lack of desmin IF reduces isometric force production, and that the desmin knockout muscle is less vulnerable to mechanical injury. Very recently desmin was found to be involved in mechanical response to high isometric stress by modifying the nuclei shape, and by participating in stress-mediated JNK signaling within myofibers (61). Altogether, desmin filaments similar to other IF execute interactions with signaling proteins, cytolinkers, shaperones, components of cytoskeleton (62,63), and various membranous organelles, e.g. nucleus (61,64), mitochondria (59), lysosomes (65), and probably sarcoplasmic reticulum (66,67). Therefore, like other IF, desmin provides cellular integrity, supports crosslinks between structural and signaling proteins, maintains spatial localization of cellular organelles, and participates in mechanotransduction of extracellular stimuli. Desmin and its counterparts establish a cellular scaffold for

adjusting mechanochemical signaling and trafficking processes within the cell, thus controlling cell fate. However, the precise molecular machinery underlying most of this networking remains to be elucidated.

<i>Type</i>	<i>Protein</i>	<i>Size of protein</i>	<i>Site of expression</i>	<i>Specific features</i>	<i>Associated diseases</i>	<i>Reference</i>
<b>I</b>	Acidic keratins	40–60 kDa	Epithelial cells	Heterodimers	Epidermolysis bullosa simplex diseases; Keratoderma disorders; Meesmann corneal dystrophy; White sponge nevus of cannon	(68)
	Basic keratins	50–70 kDa				
<b>II</b>	Vimentin	54 kDa	Fibroblasts	Homopolymers	Dominant cataract	(69)
	Desmin	53 kDa	Muscle cells		Desmin-related myopathy; Dilated cardiomyopathy; Restrictive cardiomyopathy	(68)
	GFAP	51 kDa	Glial cells		Alexander disease	(68)
<b>III</b>	Peripherin	57 kDa	Peripheral neurons	Neurons	Amyotrophic lateral sclerosis 1	(68)
	Neurofilaments (Light-Medium-Heavy)	67–150–200 kDa	Neurons		Amyotrophic lateral sclerosis; Parkinson disease; Neuronal IF inclusion disease	(68)
	$\alpha$ -internexin	66 kDa				
	Nestin	200 kDa			Neuroepithelial cells	
<b>IV</b>	Nuclear lamins	60–75 kDa	Nuclear lamina	Nuclear localization signal	Laminopathies	(30,70,71)

Table 1. Types of intermediate filaments and associated diseases.

### 1.2.3 Desmin gene and mutations

#### 1.2.3.1 *Desmin* gene

Nucleotide sequence of the human desmin coding gene (*DES*) was deciphered in 1989 by Li et al. (72). It was determined that *DES* is a single copy gene that spans a region of 8.4 kb and encompass nine exons separated by introns. *DES* has one 2.2 kb mRNA transcript found in all types of muscle cells (72). Further investigations allowed to perform gene mapping and to assign *DES* to 2q35 band in the human karyotype (73). In accordance with its protein structure, the *DES* gene coding sequence might be organized in three domains corresponding to non-helical head and tail domains, and  $\alpha$ -helical rod domain. The latter, the  $\alpha$ -helical core, is the most conserved, and is composed of four segments—1A, 1B, 2A, 2B, maintaining heptad repeats of hydrophobic and hydrophilic amino acids. These repeats drive the formation of desmin dimers (74). Detailed structural analysis of the rod domain unveiled the presence of so-called «stutter» element in the sequence of 2B segment, which is equivalent to the deletion of three amino acids residues, and «stammer» corresponding the deletion of four amino acids residues (75). Further, it was convincingly proved that «stutter» is an obligatory trait of all IF and its position is strictly defined. Moreover, addition of the missed three amino acids to the «stutter» sequence had a deleterious effect on the filament polymerization process (5). Thus, it was concluded that «stutter» elements control desmin IF assembly. Another structural sequence found in 2B segment is 405-YRKLLERGEESRI-416 motif. This element is responsible for the coiled-coil untwisting and serves as starting point to  $\alpha$ -helices dissociating from each other (76). Non-helical head and tail domains are mainly composed of  $\beta$ -sheets, while the latter displays mostly random structure (77), and plays a distinct role in the regulation of ULF width during tetrameres formation (78). Furthermore, the tail domain was discovered to interact with other components of the cytoskeleton (79). In conclusion, all structural elements of desmin participate in processes crucial for protein organization and functioning.

#### 1.2.3.2 *Desmin* mutations

Up to date, nearly 70 *DES* mutations have been described in patients with affected cardiac and skeletal muscles (Fig. 3a). In general, *DES* mutations display autosomal dominant (AD) inheritance pattern accompanied with intracellular aggregate formation (80). Three families with autosomal recessive (AR) inheritance with either homozygous or compound heterozygous *DES* mutations have been described (81–83). De novo *DES* mutations have also been reported (84–86). The most frequent are missense *DES* mutations resulting in amino acid substitution (87). In some families with AR inheritance a small in-frame deletion leading to p.Arg173\_Glu179del has been described (81,83). In other families larger deletion of 22 bp in exon 6 resulted in occurrence of premature stop codon and a complete lack of desmin has been reported (88). A series of mutations impairing splicing process have also been identified, for instance deletion of exon 3 splice sites resulted in complete ablation of 96 bp region corresponding to exon 3 (89). An insertion of a single nucleotide resulted in premature termination of translation process (90).



Some *DES* mutations are less pathogenic than others, and appear often to depend on localization site of the mutation. Comparative analysis of the pathogenic potential of various *DES* mutations unraveled that mutations residing in 2B segment of the rod domain exerted more severe effect on filaments architecture in contrast to tail domain mutations (91). In addition, previous studies indicated a phenomenon of incomplete penetrance for tail mutation I451M. In a family carrying this mutation, only a subset of carriers developed a cardiac phenotype (92). Later on, in another family with progressive skeletal myopathy there were no evidence of cardiac involvement for three members carrying the I451M mutation (93). Recently, it was discovered that rare *DES* single nucleotide polymorphism (SNP) result in only mild alterations in filament structure during development of skeletal and cardiac muscle disorders (38). Based on these data it can be concluded that *DES* rearrangements play a causative role in development of striated muscle disorders. The extent of its negative effect depends on the type of rearrangement as well as the site of its localization.

### **1.3 DESMIN-RELATED MYOPATHY**

#### **1.3.1 Desmin-related myopathy overview**

The term myofibrillar myopathy (MFM) was first introduced in 1996 by Nakano et al. to cover a number of various non-inflammatory pathological muscle conditions specified by common denominators—abnormal foci of desmin positivity, focal dissolution of myofibrils, accumulation of myofibrillar degradation products, and ectopic expression of multiple proteins in the abnormal fiber regions (94,95). A skeletal muscle biopsy is always utilized to establish the diagnosis of MFM. Electron microscopy shows disintegration of myofibrils starting from the Z-disc. Gomori trichrome staining indicates the presence of a mixture of amorphous, granular or hyaline deposits in abnormal myofibers. Immunohistochemical studies discover focal accumulation of desmin,  $\alpha$ B-crystallin, dystrophin, and myotilin in abnormal muscle fibers. Typical histological features include focal areas with reduction or total loss of ATPase and oxidative enzyme activity in irregular fiber areas (96). Desmin-related myopathy (DRM) (OMIM 601419) has been described as a distinct subtype of MFM, characterized by intracytoplasmic accumulation of desmin-positive deposits in striated muscle cells (97–101). However, initial investigations regarding the genetic nature of DRM did not attest involvement of *DES* mutations in development of this disorder (102). Only later, a number of clinical cases have been reported confirming a role of *DES* in DRM evolution (34,82,81,103). Since then, many new affected families have been identified. Novel data provide a more detailed analysis of clinical manifestation and pathogenesis of DRM.

#### **1.3.2 Desmin-related myopathy clinical manifestation**

At the present time a key limitation of studying DRM incidence and prevalence is the lack of epidemiological data. As discussed by Clemen et al. (87) DRM belongs to a class of rare diseases with no more than 5 affected in 10000 individuals. Much research on DRM epidemiology has been done in meta-analysis study encompassing

159 cases with 40 different *DES* mutations (36). DRM was reported in diverse ethnic groups and affected both males and females. A gender impact on DRM incidence was clearly shown by two studies, where males suffered from sudden death of cardiac origin, while females exhibited a more benign myopathy of distal onset and slower progression (36,104). The time of the first disease onset is wide ranging from the 1st up to 8th life decades, with more frequent cases in the period from 2nd up to 4th decades. Disease manifestation in the 1st decade was emanated by rare recessive mutant desmin forms (82,83,88,99). A meta-analysis described that more than 70% of patients exhibited myopathy or muscular weakness, with elevated plasma creatine kinase levels in more than 60% of the cases. Among these patients 67% have combined distal and proximal muscular weakness, while isolated distal weakness was found in 27% and isolated proximal weakness in 6%. The distal muscle weakness generally precedes the proximal muscle impairment with muscle weakness in lower and upper limbs. Muscle weakness can progress to trunkal, neck, and facial muscles. A combination of signs of striated muscle pathologies was found in 49%. Neurological symptoms were found in 74% and cardiological symptoms in 74% of patients, while both neurological and cardiological symptoms presented in 49% of subjects, isolated neurological signs in 22%, and isolated cardiological signs in 22%. Up to 50% of carriers had cardiomyopathy and around 60% had cardiac conduction disease or arrhythmias, with atrioventricular block as a definitive indicator. Respiratory insufficiency was presumed in 26% of carriers, since death was reported in 26% of carriers at a mean age of 49 years. Sudden cardiac death was recorded for 7 patients. Five patients harboring *DES* mutations did not show any clinical phenotype (36). According to this meta-analysis, DRM is a heterogenous group of disorders with greatly varying clinical phenotypes with predominance of skeletal and cardiac muscle involvement, at times clinical forms can overlap (Fig. 3b, 3c).

#### *1.3.2.1 Skeletal muscle involvement in desmin-related myopathy*

Initially DRM was associated with progressive skeletal myopathy, mostly affecting distal extremities, and especially legs. Subsequently, *DES* mutations were shown to be cosegregated with scapuloperoneal distal myopathy (105), limb girdle myopathy (106), and generalized myopathy phenotype (107). In 2007 Fischer et al. addressed a question about comparative analysis of affected skeletal muscles in DRM (108). They performed a retrospective muscle imaging assessment in a series obtained from 19 DRM patients ranging from mild to severe muscle involvement. In all of these patients the semitendinosus muscle was at least equally affected as the biceps femoris muscle, and the peroneal muscles were similarly involved as the tibialis anterior. More detailed analysis revealed the following muscle characteristics: in pelvic muscles—the gluteus maximus muscle was significantly more involved than the gluteus medius and minimus muscles; in thigh muscles—semitendinosus, sartorius, and gracilis were the most affected muscles exceeding the involvement of the adductor magnus, biceps femoris, and semimembranosus; in anterior compartment—rectus femoris, vastus lateralis, intermedius, and medialis were relatively spared in most patients; in lower legs—the peroneal muscles displayed significantly more lipomatous changes than the tibialis anterior and muscles of the posterior compartment (soleus, medial, and lateral gastrocnemius). Distal muscle

involvement was slightly increased in comparison to proximal muscles (thigh and pelvis) (108).

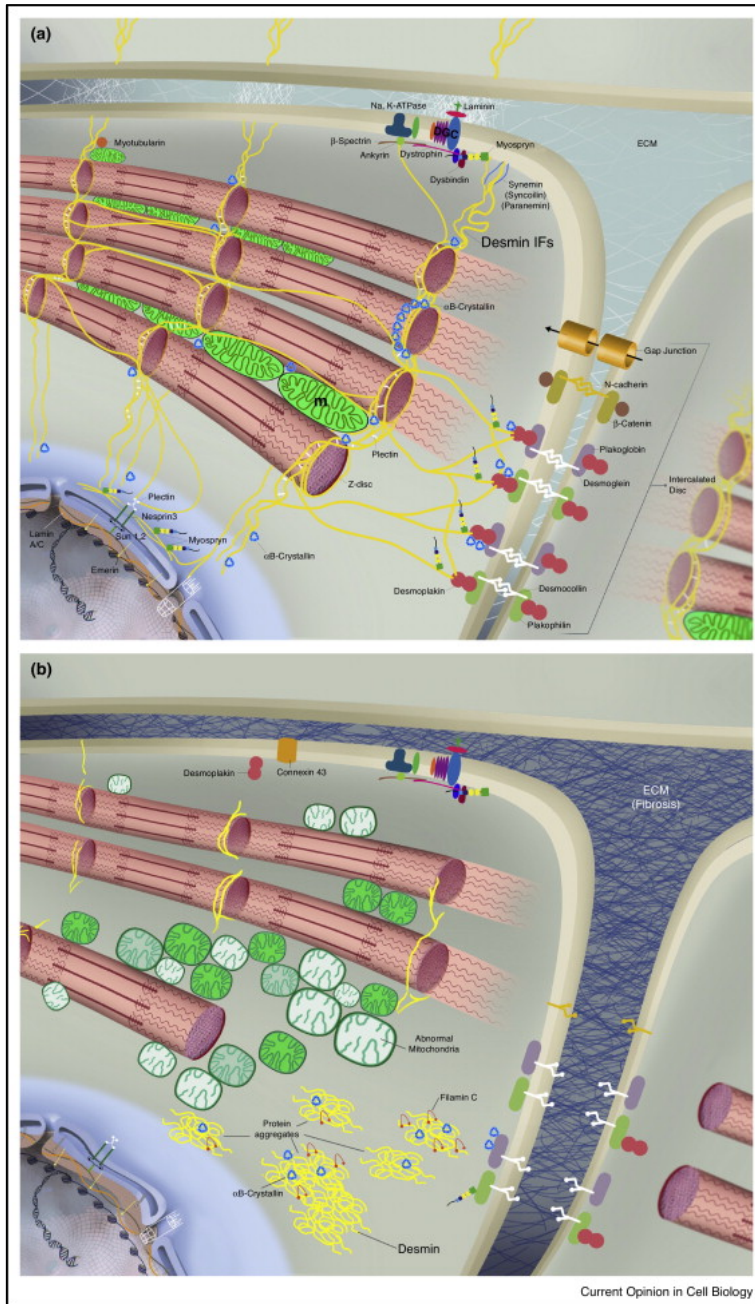


Figure 2. Schematic representation of the desmin filaments scaffold with its direct and indirect interaction partners in (a) cardiomyocyte and in (b) cardiomyocyte harboring mutant form of desmin (adapted from Capetanaki et al., *Current Opinion in Cell Biology*. 2015; 32:113–120, (109); republished with permission).

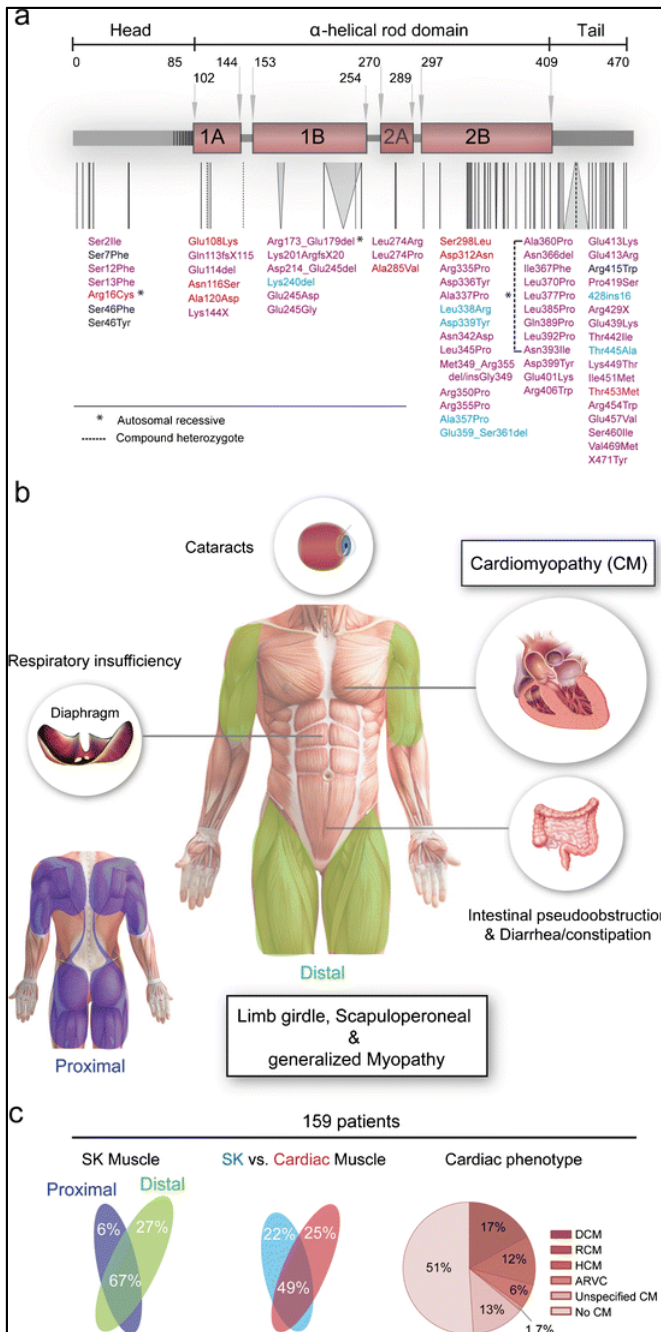


Figure 3. Desmin mutations and pathophysiology of desmin-related myopathies (a) schematic representation of described mutations. Mutations are subdivided into three groups depending on related phenotype: skeletal (blue), cardiac (red), both skeletal and cardiac (purple); (b) the major phenotypes associated with mutations—distal myopathy and cardiomyopathy; (c) meta-analysis of 159 patients with desmin-related myopathy. Dilated (DCM), restrictive (RCM), hypertrophic (HCM), arrhythmogenic right ventricular (ARVC) cardiomyopathies (adapted from Hnia K. et al., Cell Tissue Res. 2015; 360:591–608, (110); republished with permission).

### *1.3.2.2 Cardiac muscle involvement in desmin-related myopathy*

Cardiac muscle involvement in DRM can precede, coincide or succeed skeletal myopathy (103). Some cases have also been reported with isolated cardiac phenotype, namely dilated cardiomyopathy, without affecting skeletal muscles (92,111). Cardiac disease manifestation comprise all forms of cardiomyopathies—dilated (111), restrictive (112), hypertrophic (89), and arrhythmogenic right ventricular cardiomyopathy (113), as well as cardiac conduction disease (34,85,114) with atrioventricular block (89). The earlier mentioned meta-study revealed that 74% of all DRM patients developed a cardiomyopathy. The dilated form of cardiomyopathy was observed in 17%, hypertrophic cardiomyopathy in 12%, restrictive cardiomyopathy in 6%, and arrhythmogenic right ventricular cardiomyopathy in 1% of the patients. Cardiac conduction defects were observed in 62% of patients, while two thirds of them displayed cardiomyopathy as well. Atrioventricular block was the most frequent manifestation of cardiac conduction defects—almost 50% usually requires pacemaker implantation (36).

### *1.3.2.3 Other muscle involvement in desmin-related myopathy*

Apart from skeletal and cardiac muscle engagement, respiratory muscle weakness was reported in 26% of analyzed patients, being the leading reason of patient's death (36). Several evidence of smooth muscle involvement were uncovered by Ariza et al., who showed intestinal pseudo-obstruction (99), Goldfarb et al. demonstrated swallowing insufficiency (82), and Olive et al. described nuclear and cortical cataracts (115).

## **1.3.3 Desmin-related myopathy pathogenesis**

### *1.3.3.1 Cellular alterations*

Pathological changes of genetically proven DRM include the presence of intermyofibrillar, subsarcolemmal or perinuclear desmin-positive aggregates both in skeletal and cardiac muscles, additionally in myocytes Z-disc streaming resulting in loss of tension transmission between sarcomeres (116). Analysis of patients skeletal muscle biopsies exhibited irregular shape of muscle fibers and abnormal mitochondrial enzyme staining (90,117). Cardiomyocyte hypertrophy and disarray, accompanied with misshaped nuclei and degenerating mitochondria were described in desmin deficient mice (118,119). Overall, it is considered that the severity of muscle pathophysiological changes reflects the DRM stage. The following approaches are commonly used to verify the presence of myopathological alterations: light microscopy, immunostaining, and electron microscopy. Histological analyses of muscle biopsies have shown a myopathic pattern with rounding of muscle fibers, increased fiber size, abundance of necrotic and regenerating fibers, internalization of nuclei, and an increase of connective tissue content (For review see 87). In addition, haematoxylin-eosin and Gomori trichrome staining revealed multiple fibers with cytoplasmic and subsarcolemmal basophilic inclusions (101,120). Analysis of succinate dehydrogenase and

cytochrome c oxidase expression demonstrated multiple muscle fibers with focal areas of attenuated or devoid of enzyme expression indicating a focal depletion of mitochondria (106). Another study demonstrated a vacuolar myopathy with single or multiple round or rimmed vacuoles. The majority of these vacuoles contained PAS-positive material. Multiple fibers displayed dots of increased acid phosphatase reactivity within the cytoplasm and partially at the rim of larger vacuoles. In addition to membrane- and non-membrane-bound vacuoles, the ultrastructural analysis revealed multiple fibers containing granulo-filamentous material (107). It was also shown that «rubbed-out» fibers were more numerous in DRM than in other types of MFM (121). Desmin immunostaining is performed as a diagnostic tool to illustrate protein inclusions in subsarcolemmal or sarcoplasmic regions. However, these inclusions also exhibit immunoreactivity to other miscellaneous cytoskeletal and signaling proteins. For instance,  $\alpha\beta$ -crystallin (90), filamin-C (122), and myotilin (123) positive staining is seen in aggregates in DRM as well as in other form of MFM (120,124). Electron microscopy discovered accumulation of granulo-filamentous material in the subsarcolemmal space and between neighboring myofibrils, as well as cytoplasmic bodies and autophagic vacuoles (125,126). These intracellular changes were concomitant with structural Z-disc alterations—streaming, irregularities, and total loss of structure (125). Typical signs of accompanying mitochondrial pathology with normal or abnormal morphology were their accumulation in the subsarcolemmal area, or depletion of mitochondria (116).

### 1.3.3.2 Phenotype-genotype correlation

As was previously discussed, mutations in *DES* are the leading genetic cause of DRM development. Since the beginning of 2000s due to the genetic testing achievements it became possible to link clinical phenotypes and different *DES* mutations. Numerous original studies and reviews summarize available data regarding phenotype-genotype correlations (35,36,80,87,93,111,115,127). Mutations have been found in all *DES* domains—non-helical head and tail domains, as well as in all segments of the  $\alpha$ -helical rod domain, where the majority of mutations affect the 2B segment. According to meta-analysis data mutations in 2B segment are more prevalent in patients with skeletal myopathy (in conjunction with or without cardiac involvement), whereas mutations in head and tail domains were found in patients with isolated cardiological or combined phenotypes (36). I451M is among the first-described tail mutations leading specifically to dilated cardiomyopathy without any skeletal muscle phenotype. Some carriers of the I451M mutation did not develop any phenotype, implying incomplete penetrance (92,93). Further studies of *DES* variants underlying dilated cardiomyopathy disclosed that disease-causing mutations can reside out of the tail domain, as demonstrated by mutation in the highly conserved segment of the 1A rod domain (111). It is important to note the study from 2008 by Strach et al. arguing the concept of phenotype-genotype correlations. Eleven patients with heterozygous E245D, D339Y, R350P and L377P *DES* mutations corresponding to the rod domain without cardiac symptoms were reported. Clinical evaluation revealed a marked variability of skeletal, respiratory, and cardiac muscle involvement between patients harboring identical mutations,

ranging from asymptomatic to severely deteriorated. Therefore, the authors concluded an absence of distinct genotype-phenotype correlations in patients with genetically proven DRM (127).

### *1.3.3.3 Mechanism of DES mutation action*

#### *Aggregate toxic effect*

*DES* A337P and A360P were the first described missense mutations associated with familial cardiac and skeletal myopathy (82). Thirteen of up to date described *DES* mutations introduce the amino acid proline (110). Proline normally does not present in the desmin amino acid sequence, since it can act as a potent helix breaker (128). Forced introduction of proline in desmin leads to disturbance of polymerization resulting in formation of short, thick, and kinked abnormally-assembled filaments in vitro (129). Functional analysis has been performed for a *DES* homozygous deletion of 21 nt found in a patient with severe generalized myopathy (81). This study provided evidence of greatly affected assembly process in vivo and in vitro in the presence of this deletion. Filaments totally lost their ability to assemble in vitro, while in vivo short rodlets of irregular diameter were observed and tended to form aggregates (81). Since then, every year new *DES* mutations have been reported in conjunction with DRM, thus it was crucial to identify the precise molecular mechanisms of *DES* mutations action. Bär et al. presented one of the first comparative investigations of  $\alpha$ -helical rod domain mutations impact on the recombinant protein assembly in vitro and the filament-forming capacity of IF-free cells transfected with *DES* cDNA (130). It was demonstrated that *DES* rod domain mutations could violate filament assembly in vitro at diverse stages. Mutations associated with the same disease phenotype, nevertheless showed different abilities to form filaments—from non-affected to severely compromised. Moreover, it was demonstrated that the assembly process could be interrupted at almost all stages: tetramer formation, ULF formation, filament elongation, and filament maturation. According to their assembly properties, mutants behave within the transfected IF-free cells: variants disabling to polymerize in vitro presented as dot-like aggregates in the cell cytoplasm (130). Further analysis was dedicated to describe the performance of these mutations in different cell types, IF-free and expressing vimentin (131). A great variability in mutation action was discovered. In vitro filaments-forming mutants were able to assemble into filamentous network in cells lacking IF, as well as integrate in pre-existing vimentin network in vimentin-positive cells. Mutants that failed to polymerize in vitro caused destruction of vimentin network prompting its aggregation in perinuclear space. Exogenous desmin in these cells was completely sequestered from vimentin filaments (131). Subsequent work by Bär et al. was focused on biophysical properties of mutant desmin proteins (132). Mechanistic details of how mutations affect the filament assembly process were measured by employing analytical ultracentrifugation, quantitative scanning transmission electron microscopy, and viscometric studies. It was shown that the soluble assembly intermediates of the mutant proteins exhibit unusually high s-values, compatible with octamers. Several of the filament-forming mutant variants

deviated considerably from WT desmin with respect to their filament diameters and mass-per-length values. In the heteropolymeric combination with WT desmin four of the mutant variants caused a prominent «hyper-assembly» assessed by viscometry. These results indicate that the various mutations found mostly in the 2B segment of the rod domain may cause distortion of filament formation by the mutant protein at distinct stages, and that some of them preclude the assembly of WT desmin (132). On the other hand, it has been explicitly demonstrated that mutations residing in the 1B segment of rod domain, *DES* A213V and E245D, form filaments in vitro (130). Transfected in IF-free cells these mutants were able to produce filamentous network similar to WT desmin. In vimentin expressing cells mutants integrated to pre-existed vimentin filaments. Comparable results were obtained for *DES* N393I and D399Y mutations, resided in the area closed to the tail region (131). Therefore, desmin aggregate formation cannot be attributed as the only reason promoting the DRM development. It is here more reasonable to propose the concept of loss of desmin function, rather than gain of aggregates toxic function. Data obtained on desmin deficient mouse model also favour this perception. Mice completely lacking desmin still developed skeletal and cardiac myopathies (57,118,119). It is thus important to focus on desmin functions and interactions that might be perturbed due to the loss of proper desmin filament structure.

#### Nanomechanical property distortion

One of the first functional studies of desmin in *DES* knockout mouse unveiled its role in the force transmission (53). Moreover, desmin filament elasticity was later confirmed in experiments with atomic force microscope approach (133). One year later, the same group corresponded another study about IF viscoelastic properties, where they demonstrated that IF exhibit a very pronounced increase in elastic properties or «stiffness» upon increases in shear stress, a phenomenon referred to as «strain hardening» or «strain stiffening» (134). Skeletal and cardiac muscle cells constantly undergo mechanical stress due to their contraction ability, while desmin filaments participate in cell protection against this stress. Mutations in 2B segment tail end, namely *DES* Q389P and D399Y, previously reported to be filaments assembly-competent (130,131), were investigated by aforementioned atomic force microscope approach (135). Despite the presence of virtually regular filament network, local variations in tensile properties along the filament length were exhibited in comparison to WT. From this fact the authors concluded that *DES* Q389P and D399Y may cause muscle disease by altering particular biophysical properties of the desmin filaments and compromising their nanomechanical properties (135). Tail *DES* mutations exhibited a decrease in «strain stiffening» compared to *DES* WT and promoted non-affine network deformation, thus significantly modifying the response to applied load (136). Another confirmation of *DES* mutations impact on cells vulnerability to mechanical stress was obtained on the culture of myoblasts isolated from a patient carrying *DES* R350P. Compared to WT cells, these myoblasts demonstrated increased contractile prestress and were more susceptible to stretch-induced programmed cell death, implying a higher baseline stiffness (137). To sum up, mechanotransduction properties of the muscle



cells are ensured by desmin filaments, while the expression of mutant desmin contributes to an increased biomechanical cell vulnerability. Increased vulnerability of muscle fibers in response to shear or pulling force during contraction-relaxation cycles has been reported as a central mechanism for muscle fiber degeneration (8).

### Violation of desmin-protein interaction

Intracellular protein aggregates are considered as a molecular hallmark to diagnose DRM. Immunostaining analysis revealed that desmin is not the only component of aggregates (90,120–124). High-throughput proteomic approach was applied to describe protein content of aggregates found in patients with DRM (138). Various structural and signaling proteins showed to be over-represented in aggregates, thus confirming their direct or indirect links with desmin filaments, e.g.  $\alpha\beta$ -crystallin, synemin, syncoilin, nestin, plectin, filamin C, myotilin, dystrophin, utrophin, dystroglycans, sarcoglycans, caveolin, dysferlin, actin, actinin, N-CAM, NOS, collagen VI, laminin, b-spectrin, and ubiquitin. The majority of these proteins were described as an aggregate component for the first time by Maerkens et al. in 2013 (138). Some of these proteins are known to be localized in the Z-disc area, thus supporting the Z-disc involvement in desminopathy pathogenesis. However, not only aggregate formation disarranges desmin-protein interactions. Two non-aggregate *DES* mutations resided in coil 1B segment of the rod domain were reported to play a causative role in impairment of desmin-nebulin interaction, and destabilizing actin filaments (139,140). Myocytes expressing exogenous *DES* E245D displayed presence of abnormal actin filaments. Detailed analysis uncovered that actin filament polymerization is under control of nebulin, a Z-disc protein directly linked to the 1B desmin region. Thus, *DES* E245D mutation negatively affects microfilament architecture (139). Another mutation *DES* K190A was also accompanied with irregular actin filamentous network, confirming that actin organization is dependent on desmin-nebulin crosslink. These data are in line with the perception of desmin being a Z-disc stabilizer, which provides steadiness during myocytes contraction (140). Taken together, desmin possesses numerous interaction partners; the majority of them are located in the Z-disc. Therefore, collapse of desmin filaments regardless presence of aggregates affects the whole communication network, thus confirming desmin implication in DRM pathogenesis.

### Mitochondrial dysfunction

The first observations of desmin-mitochondrial crosstalk were obtained on *DES* knockout model. Electron microscopy of cardiac muscles from desmin deficient mice showed highly disorganized mitochondria and swollen mitochondrial matrix (57). A following study was aimed to assess mitochondrial distribution and functional activity in skeletal and cardiac muscles lacking desmin (59). Slow twitch skeletal muscle demonstrated abnormal accumulation of mitochondria bundles in the subsarcolemmal area, while cardiac muscle additionally displayed extensive mitochondria fission in response to work overload. Mitochondrial functional

alterations mostly concerned depressed ADP-stimulated respiration. It is of interest to note that maximal rates of respiration in isolated cardiac mitochondria from desmin-null and WT mice were similar. However, in situ respiration rates were significantly reduced in cardiac and slow twitch skeletal muscles lacking desmin, whereas fast twitch muscles were unaffected (59). Examination of transgenic mouse model expressing low level of the *DES* L345P discovered mitochondrial swelling and vacuolization in muscle from TG mice, as well as significant increase in mitochondrial  $Ca^{2+}$  level in skeletal and cardiac myocytes during and after contractions (141). To broaden the existing knowledge regarding mitochondria in desmin-null cells, investigations of mitochondrial proteome were performed (142). Many proteins had a differential expression in WT and desmin deficient cells, the majority of which were enzyme subunits. However, cytoskeletal, calcium-binding proteins, and others were found as well. Proteome analysis unveiled a diversity predominantly in expression of proteins involved in ketone body and acetate metabolism, amino-acid metabolism; NADH shuttle proteins, and respiratory enzymes; and with less extension in apoptosis, calcium homeostasis, and fibrosis (142). The first described cytolinker for IF and mitochondria was plectin 1b, a ubiquitously expressed isoform of plectin, capable to tether mitochondria with the IF network system (143). Recently, myotubularin was also reported to control desmin IF architecture and mitochondrial dynamics in skeletal muscle (66). Some assumptions exist that desmin-mitochondrial interactions take place in the particular contacting sites rich in the proteins important for mitochondrial biogenesis, morphology, and function, e.g. VDAC channels, ANT, and the MICOS complex, where mitochondria-sarcoplasmic reticulum cross-talk occurs (144). Any distortion of these interactions can affect mitochondrial permeability transition pores and mediate apoptosis pathways, thus developing the heart pathology observed in DRM animal models (145). Bcl-2 is a well-known antiapoptotic protein, which overexpression in desmin-null mice rescued the cardiomyopathy phenotype. It was shown that bcl-2 corrected mitochondrial defects, reduced fibrotic lesions, and prevented from developing hypertrophic cardiac muscle. Overexpression of bcl-2 increased the ability of mitochondria to resist calcium exposure, lost due to the desmin absence (146). All of these studies indicate that desmin IF play a significant role in mitochondrial positioning and respiratory function in striated muscles. Desmin can influence mitochondrial function by regulating mitochondrial shape by stretching, and by contraction of the mitochondrial membrane, through direct or indirect interactions with mitochondrial membrane proteins. Defective mitochondrial homeostasis is a common pathological trait found in patients with DRM.

This section dedicated to putative mechanisms of *DES* mutations action could be much longer, including for instance mutation effect on protein post-translational modifications, cell differentiation and development, autophagy and apoptosis processes. However, it was limited by the framework of the current thesis and covered the issues outlined in this book.

#### 1.3.3.4 *Animal models to study desmin-related myopathy*

Progress in studying the impact of *DES* mutations on muscle cell structure and functions has been achieved due to the availability of various animal models.

##### *Desmin knockout mouse model*

*DES* knockout mouse model was reported independently by two groups in the middle of 1990s (53,57). Examination of these animals demonstrated intriguing findings. Despite expectations of a more drastic phenotype, desmin-null mice were viable and fertile, reproduced normally, and did not show anatomical defects, indicating that desmin is not essential for myofibrillogenesis (53). However, the desmin-null animals were prone to develop myopathy and cardiomyopathy (57,147), and displayed reduced ability to endure chronic and acute bouts of running exercise (148). Characterization of electrophysiological cardiac properties deciphered that desmin deficiency decreased atrial, but prolonged ventricular refractory periods, resulting in ventricular conduction slowing, enhanced inducibility of atrial fibrillation, and a reduced susceptibility to ventricular arrhythmias (149). The heart function of desmin deficient mice was examined by a cardiac MRI study. This demonstrated significantly reduced left and right ventricular ejection fractions and cardiac output, an increased left ventricular mass, segmental wall thinning, and akinesia (150). Histological analysis of both skeletal and cardiac myocytes uncovered the perturbation of myofibrils, Z-disc streaming, and subsarcolemmal accumulation of mitochondria. Cardiac tissue examination showed areas of fibrosis, ischemia, and calcification (53,57,147). Ultrastructural analysis of cardiomyocytes showed increased volume and alterations in intercalated disc morphology (118,119). To elucidate molecular pathways underlying cardiomyopathy and enhance *DES* mutant phenotype, *DES* knockout mice were crossbred with transgenic mice, carrying *DES* mutations. For instance, expression of I451M mutation in the desmin-null background helped to describe crucial role of head and tail domains in the formation of IF scaffold around Z-discs (151).

##### *Desmin transgenic mouse model*

Transgenic (TG) mouse models have been used to elucidate the role of particular *DES* mutations in the development of myopathy and cardiomyopathy. The first TG mouse model was established in 1996 (152). Chimeric protein composed of desmin and vimentin amino acid sequences was expressed under the desmin promoter. Despite the low level of expression, this truncated protein had a dominant-negative effect on the desmin IF network. Typical loss of cross-striation pattern and accumulation of intermyofibrillar deposits were observed in skeletal and cardiac samples (152). Next TG model expressing p.Arg173\_Glu179del was reported in 2001 by the group of Jeffrey Robbins and referred as D7-des (153). Examination of D7-des TG mouse heart showed aberrant intrasarcoplasmic and electron-dense granular filamentous aggregates. The desmin filamentous network was significantly distorted, and myofibril alignment was greatly compromised. Systolic function in D7-des TG hearts under baseline conditions and during maximal  $\beta$ -adrenergic stimulation was significantly reduced (153). These D7-des TG mice were crossed

with  $\alpha\beta$ -crystallin TG mice to amplify the DRM phenotype (154), or with animals harboring genes engaged in autophagy (155,156) to characterize this process in conjunction with aberrant desmin. Myocardium from double TG exhibited significantly more desmin aggregates than from D7-des TG mice. Moreover, double TG mice displayed a significantly stronger cardiac hypertrophic response, with the mice dying of congestive heart failure before 7 weeks (154). Studies of autophagy unraveled that abnormal desmin aggregation impairs the proteolytic function of the ubiquitin-proteasome system in the heart (155). It was recently shown that autophagic flux is increased in desminopathic hearts, since it serves as an adaptive response to overexpression of misfolded proteins (156). Our group also established *DES* TG mouse model. The peculiarity of this model was co-expression of endogenous *DES* WT and exogenous *DES* L345P. Despite the low expression level of exogenous *DES*, reduced contractile function and recovery from fatigue in soleus muscle were reported. Moreover, cardiac alterations, particularly a hypertrophic left ventricular posterior wall and decreased left ventricular chamber dimension were described. Mitochondrial pathology characterized by swelling and vacuolization and increased calcium levels were found as well (141). Recently, another TG mouse model was reported, namely *DES* R349P knock-in mice. This model recapitulates the characteristics of human missense mutation *DES* R350P. Examination of this model revealed that the distortion of IF network integrity, rather than aggregate toxic effect plays a causative role in DRM development (157).

### Zebrafish model

Over the last 20 years, zebrafish have become a trendy animal model. The first paper describing desmin spatial distribution during zebrafish embryonic development revealed its concentration at Z-bands and intercalated discs (158), while analysis of adult organisms sharply localized desmin to the Z-bands of myofibrils (159). Desmin in zebrafish is encoded by two genes, *desma* and *desmb*, sharing 68.7% and 72.0% similarity with the human desmin protein respectively (160). Knockdown of *desma* via morpholino antisense oligonucleotide approach exhibited alteration of Z-disc structure, as well as reduced content of sarcomeres for both skeletal and cardiac muscle (161). A recent study reported simultaneous knockdown of *desma* and *desmb* that resulted in muscle disorganization, reduction of larvae size, and declining in fish swimming activity (162). The cardiac phenotype has not been studied so far due to the unavailability of fish model possessing DRM phenotype with aggregate accumulation.

## 2 AIMS OF THE STUDY

The general aim of the present PhD project was to extend existing knowledge about the molecular machinery on how desmin gene mutations lead to the development of desmin-related myopathy, with an emphasis on development of cardiomyopathies.

To address this general aim several specific research questions were stated:

1. To perform genetic study of a group of patients with cardiomyopathies in order to describe novel mutations in the desmin gene and to assess the frequency of *DES* A213V.
2. To perform genetic study using next-generation sequencing approach of a group of patients with idiopathic restrictive cardiomyopathy in order to describe novel genetic variants associated with disorder.
3. To perform functional study of aggregate-prone and non-aggregate-prone desmin gene point mutations and compare their effects on mitochondrial properties.

## **3 MATERIALS AND METHODS**

### **3.1 PATIENT MATERIAL**

Studies were performed according to the Helsinki declaration, and ethical permits were obtained from local ethic committees in Stockholm and Saint-Petersburg. All patients involved in the study signed written informed consent prior to the enrollment.

Genetic study aimed to search novel *DES* mutations and assess *DES* A213V frequency involved 108 patients with heart failure and cardiac dilation with signs of heart failure and enlarged left ventricle dimensions of various etiologies. Clinical data and case history were obtained by direct physical examination and from medical records. The control group included 300 healthy donors with the same Caucasian background.

Genetic study by next-generation sequencing (NGS) approach included 24 individuals with restrictive cardiomyopathy (RCMP) hospitalized or treated in the clinic of Federal Almazov Medical Research Centre, St. Petersburg, or Astrid Lindgren's Children Hospital, Karolinska University Hospital, Stockholm. The RCMP diagnosis was based on the WHO/International Society and Federation of Cardiology Task Force clinical criteria, and classified according to the European Society of Cardiology classification of cardiomyopathies. To compare the spectrum of genetic variants, six patients with early onset ventricular arrhythmias without diastolic dysfunction, ischemic heart disease, or structural cardiac abnormalities were examined using the same panel of genes.

### **3.2 SEQUENCING OF DESMIN GENE**

#### **3.2.1 Sanger sequencing**

Patient genomic DNA was extracted from peripheral blood by using QiAmp DNA Blood Mini Kit (52304, Qiagen, USA). All encoding exons of *DES* were analyzed. Detailed parameters of the PCR reactions are available upon request. After amplification, PCR fragments were cleaned with a GeneJET PCR Purification Kit (K0702, Thermo Fisher Scientific, USA), and cycle sequencing was performed using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied biosystems, USA) according to the manufacturer's instructions. Sequence analysis was performed on ABI PRISM 3130 genetic analyzer, and the data were interpreted using BioEdit sequencing analysis program.

#### **3.2.2 Restriction fragment length polymorphism**

All found *DES* A213V substitutions were confirmed by restriction fragment length polymorphisms analysis. *DES* A213V substitution introduced a restriction site for AccBsi, thus in the presense of substitution the original 384 bp PCR product cleaved to 103+281 bps fragments.

## 3.2.3 Next-generation sequencing

### 3.2.3.1 Design of the target gene panel

A list of cardiomyopathies- and channelopathies-associated genes was compiled from the literature covering 108 genes that are either known causes or candidate genes for RCMP development. To ensure a comprehensive coverage of the target genes, all annotated coding regions were extracted based on genes and tracks data from RefSeq, Ensembl, CCDS, Gencode, VEGA, SNP, CytoBand. The resulting target region covered 426332 bp and was used as input for SureSelect (Agilent Technologies, USA) to design the custom capture-oligonucleotides for in-solution target enrichment. Manual optimization was applied to readjust capture oligonucleotides in regions with lower capture efficiency. In total, 19956 capture probes mapping to 424430 bp were synthesized (BED file with target region is available upon request).

### 3.2.3.2 Gene enrichment and next-generation sequencing

For all samples alignment was performed using Burrows-Wheeler Aligner (BWA MEM) and called with SNPPEP tool (<http://www.eposters.net/pdfs/snppet-a-fast-and-sensitive-algorithm-for-variant-detection-and-confirmation-from-targeted.pdf>) as a part of SureCall software (Agilent Technologies, USA). As an alternative, output BAM files obtained from BWA was processed using GATK (V.3.3-0) (<http://www.broadinstitute.org/>) pipeline to increase reliability. BAM files were sorted and indexed with Picard tools (V.1.128) (<http://broadinstitute.github.io>). Restriction enzyme fingerprints was clipped with GATK ClipReads, then BAM files were realigned and recalibrated versus dbSNP 138 (NCBI) with GATK tools. Coverage metrics files were produced with SAMtools depth and analyzed with a custom R script. All samples were annotated using Annovar. Sanger sequencing was applied to validate results.

### 3.2.3.3 Variant classification

To express the pathogenic role of the identified genetic variants we utilized the classification proposed by Haas et al. (163) with modifications. All disease-relevant and disease-modifying variants were classified as damaging (D) or neutral (N) by SNPs&GO (164).

### 3.2.3.4 Protein network analysis

A disease interaction network was generated by manual curation using the CIDEr database (165). Text-mining tools such as iHop, Chilibot, and EvidenceFinder were used for literature mining of Pubmed abstracts and PMC full text articles (164,166,167). Proteins with RCMP mutations were analyzed with regard to their physical and regulatory interactions. All interactions from the protein network were manually curated and supported by experimental evidence from the scientific literature. If available, we used information from cell types that are related to cardiac cells.

### **3.3 PRIMARY MAMMALIAN CELL CULTURE**

#### **3.3.1 Muscle fiber isolation**

Young (8–16 weeks old) C57BL/6 male mice were supplied by B&K Universal (Sollentuna, Sweden). All studies were approved by Stockholm North Local Animal Ethics Committee. Mice were sacrificed by cervical dislocation. Muscles were removed and placed in DMEM with 1% penicillin/streptomycin (Gibco, USA). Single muscle fibers were isolated from flexor digitorum brevis muscle. Isolated muscles were cleaned of the connective tissue and tendons and placed in 2 mL of filtered 0.3% collagenase I (Co130, Sigma, Germany) dissolved in DMEM (Gibco, USA) supplemented with penicillin-streptomycin (Gibco, USA) for 2 h at 37°C. After digestion, muscles were washed with DMEM supplemented with 20% FCS (Gibco, USA) to remove the residual enzyme. Muscles were gently triturated in 2 mL of DMEM supplemented with 20% FCS. After trituration, fiber suspension was incubated for 10 min in plastic dishes, which was found to be optional for reduction the amount of nonmuscle cells contamination. After the 10 min incubation, the fiber suspension was plated on Geltrex-coated (Gibco, USA) glass bottom Petri dishes (P35G-0-20-C, Mattek, USA), 500 µL of suspension per one dish. Geltrex was diluted in cold DMEM (1:100) and the glass bottoms of the dishes were coated and incubated at 37°C for one hour, after which the dish was washed with PBS several times to remove excess Geltrex. The fiber suspension was plated on the dish and left for 10 min to allow fibers to attach to the glass bottom before the addition of 2 mL of incubation media (DMEM supplemented with 20% FCS). The incubation media was renewed every two days by replacement of half of the medium. Cells were cultured in an incubator at 37°C under a 5% CO<sub>2</sub> atmosphere.

#### **3.3.2 Satellite cell isolation, cultivation and differentiation**

Satellite cells were isolated via two strategies. In the first strategy, satellite cells were allowed to branch out of muscle fibers and attach to the dish bottom. In the second strategy, satellite cells were isolated as a «pure» culture by enzymatic dissociation of muscle fibers (168–170). For the first strategy, muscle fibers were isolated from soleus and flexor digitorum brevis muscles by incubation in collagenase and subsequent trituration as described above and incubated until the satellite cells appeared in the dishes. For the second strategy, satellite cells were isolated enzymatically according to the protocol of Yablonka-Reuveni et al. (171) with minor changes. In brief, isolated muscles were placed directly into enzyme solution, without any additional mechanical disruption with scissors. Digestion was done using collagenase type I instead of pronase. Muscle mincing was done using sterile blue pipette tips instead of glass Pasteur pipettes or serological pipettes; we did not filter the cell suspension through a strainer, since in our hands it decreased cell yields. The resultant satellite cells were plated on dishes coated with Geltrex instead of Matrigel. Thus, soleus and flexor digitorum brevis muscles were digested for 90 min at 37° C in 2 mL filtered 0.1% collagenase I (Co130, Sigma, Germany). To remove collagenase and cell debris after digestion, the cell suspension was centrifuged for 5 min at 400 ×g and the supernatant containing enzyme solution was discarded. To release



satellite cells from the fibers the pellet was resuspended in 2.5 mL of washing media (DMEM supplemented with 10% horse serum (HS) (Gibco, USA)). After the resuspension the fibers were let to settle for 5 min and then the supernatant containing satellite cells was replaced to a fresh tube. To increase satellite cells yield purity this step was repeated twice. The double-collected supernatant was centrifuged for 10 min at 1000 ×g, and the resultant supernatant was discarded and the pellet of cells was redissolved in 0.5 mL of proliferation media (DMEM supplemented with 20% FCS, 10% HS, and 1% chicken embryo extract (C3999, USBiological, USA)). Cells were plated on Geltrex-coated glass bottom petri dishes and cultured in proliferation medium until 80% confluence was reached. Fusion of some cells without external stimuli (differentiation media) was observed usually after 7 days of cultivation and served as a reliable indicator, after which we induced differentiation. To induce satellite cell differentiation, the proliferation media was removed, cells were washed once with prewarmed PBS, and then differentiation media was added (DMEM supplemented with 2% HS). The differentiation media was renewed every other day by replacement of half of medium. Cells were cultured in an incubator at 37°C under a 5% CO<sub>2</sub> atmosphere.

### 3.4 LENTIVIRAL TRANSDUCTION

Murine desmin (*Des*, NM 010043.1) cDNA was cloned into a lentiviral plasmid vector pBK RSV. It was amplified via polymerase chain reaction to create restriction sites for *AscI* and *SpeI* on 5'- and 3'-ends. These sites were used for subsequent subcloning in a LVTHM plasmid. Site-directed mutagenesis was performed by means of polymerase chain reaction with primers containing the desired mutation. A set of plasmids carrying mutated copies of *Des* was obtained and the following mutations were chosen for subsequent functional investigation: Leu345Pro, Ala357Pro, Leu370Pro, Asp399Tyr.

The pLVTHM (20 µg), pMD2G (5 µg), and packaging pCMV-dR8.74psPAX2 (5 µg) plasmids were cotransfected into HEK-293T cells by a calcium phosphate method. The resultant production of lentivirus was concentrated by an ultracentrifugation method (20000 ×g for 2 h at 4°C), resuspended in 1% BSA, and frozen in aliquots at -80°C, titered using HEK-293T cells as described previously (172) (<http://tronolab.epfl.ch/>).

Several different approaches were tested to successfully transduce primary muscle fibres. To facilitate transduction, polybrene (Sigma, Germany) at a final concentration 8 µg/mL was added to all transduced cells. We used (i) nonconcentrated virus and DMEM supplemented with 20% FCS as solution for muscle trituration and (ii) nonconcentrated and concentrated viral suspension as transduction agent and varied (iii) the incubation time with viruses and (iv) the type of plating surface. For satellite cells transduction concentrated viral suspension at multiplicity of infection of 20 was added to the cells and incubated for 5 min ahead of plating. Sixteen hours after transduction, the culture medium was completely replaced with a fresh medium. To assess efficiency of viral transduction viruses coding GFP were used in parallel.

### **3.5 IMMUNOFLUORESCENCE**

The myogenic nature of the isolated cells was confirmed by immunocytochemical staining. Cells were fixed in 4% paraformaldehyde for 10 min at 4°C and then permeabilized with 0.05% Triton X-100 for 5 min. Nonspecific binding was blocked by incubation of permeabilized cells in 15% FCS for 30 min. Cells were incubated for one hour at room temperature with the following primary antibodies: anti-Desmin (D33, DAKO, Denmark), anti-Myosin heavy chain (MAB4470, R&D, USA), anti-Ryanodine receptor 1 (D4E1, Cell signaling, USA), anti-Mitofusin 2 (ab56889, Abcam, USA), anti-Lamin A/C (NCL-LAM-A/C, Novocastra, UK). The secondary antibodies conjugated with Alexa Fluor 488 or Alexa Fluor 546 (Molecular Probes, USA) were applied for 45 min at room temperature. Nuclei were counterstained with DAPI (Molecular Probes, USA).

### **3.6 MEASUREMENT OF CELLULAR CALCIUM FLUXES**

#### **3.6.1 Loading cells with calcium indicators**

Calcium indicators were used to measure changes in free calcium within the cell. Rhod-2 AM (Molecular Probes, USA) was used to monitor calcium in the mitochondrial matrix. Cytoplasmic calcium was monitored using either the ratiometric indicator, indo-1 AM (Molecular Probes, USA), or the non-ratiometric indicator, fluo-3 AM (Molecular Probes, USA). Cells were incubated for 30 min at room temperature with 5  $\mu$ M rhod-2 AM, 2  $\mu$ M fluo-3 AM, or 5  $\mu$ M indo-1 AM, then washed for 20 min with Tyrode buffer. Mitochondrial membrane potential was monitored with tetra-methyl rhodamine-ethyl ester (TMRE, Molecular Probes, USA).

#### **3.6.2 Stimulation of sarcoplasmic reticulum calcium release and laser confocal microscopy.**

Cells were stimulated chemically with 2 mM 2-chloro-m-cresol (CmC, Sigma, Germany) or continuously with 1 or 10 ms electrical individual pulses at various frequencies (1–100 Hz).

A BioRad MRC 1024 unit (BioRad Microscopy Division, Hertfordshire, England) with a dual Calypso laser (Cobolt, Solna, Sweden) attached to a Nikon Diaphot 200 inverted microscope was used. In the majority of experiments, a Nikon Plan Apo 20 $\times$  dry lens (N.A. 0.75) was used. The rhod-2 was excited with 531 nm light and the emitted light collected through a 585 nm long-pass filter, fluo-3 was excited with 491 nm light and emitted signal was collected at 515 nm. TMRE was excited with 531 nm and the emitted light was collected through a 585 nm filter. Confocal images were captured every 7 s and a total of 42 images were obtained for every experimental condition. Images were analyzed by means of ImageJ (National Institutes of Health [available at <http://rsb.info.nih.gov/ij/>]). The average pixel intensity in an area surrounding nuclei was defined as the rhod-2 mitochondrial intensity. Background fluorescence was measured in an area free of myotubes. Fluorescence intensity is

expressed as a ratio,  $F/F_0$ , where  $F$  is the fluorescence intensity after subtraction of background at each time point and  $F_0$  is the myotube fluorescence with background subtracted at the start of the experiment. Changes in  $F/F_0$  allowed comparison of mitochondrial and cytosolic calcium between different dishes of cells. The fluorescence emitted by indo-1 was measured with a system consisting of a xenon lamp, a mono-chromator, and two photomultiplier tubes (PTI, Photo Med GmbH, Wedel, Germany). Indo-1 was excited with 360 nm and the emitted light was measured at two wavelengths, 405 nm and 495 nm. The fluorescence of indo-1 was converted to  $[Ca^{2+}]_i$  using an intracellularly established calibration curve as described previously (173,174).

### **3.7 WHOLE-CELL PATCH-CLAMP**

Calcium current was recorded in muscle fibers and myotubes using the whole-cell patch-clamp technique. Current recordings were performed with an Axopatch 200B amplifier and Digidata 1440A AD/DA converter (Molecular Device, USA). Data collection and analysis were done using pClamp 10.2 (Molecular Device, USA). Patch pipettes (1.5–4 M $\Omega$ ) were pulled from borosilicate glass capillaries (World Precision Instruments, USA) by means of a micropipette puller P-1000 (Sutter Instruments, USA). The pipette solution had the following composition (mM): 120 CsCl, 5 MgATP, 10 EGTA, and 10 HEPES (pH adjusted to 7.4 using CsOH) and the bath solution contained the following (mM): 120 TEA-Cl, 10 CsCl, 1.8 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 HEPES, 0.001 TTX, and 10 glucose, (pH adjusted to 7.4 using TEA-OH). Calcium current was evoked with a series of 200 ms depolarizing steps from -30 to 40 mV with 10 mV increments. In order to compare currents in different cells, it was normalized to the membrane capacitance.

### **3.8 MEASUREMENT OF OXYGEN CONSUMPTION RATE**

The oxygen consumption rate (OCR) was measured using a XF96 Analyzer (Seahorse Bioscience, USA) that allows real-time determination of oxygen consumption rates. Freshly isolated satellite cells were seeded in 96-well FluxPaks microplate (Seahorse Bioscience Inc., USA) at the density of 5,000 cells/well and left to proliferate for seven days at 37°C. When cells confluence reached about 80%, differentiation was induced for another seven days. Before performing the Seahorse assay, cells were incubated for 30 min without CO<sub>2</sub> in DMEM (supplemented with 10 mM of pyruvate, 10 mM of glucose and 2 mM of L-glutamine, pH was adjusted to 7.3). The OCR measurements were taken for 3 min with 3 min mixing and 2 min waiting periods under basal conditions and after the addition of following inhibitors: ATP synthase inhibitor oligomycin (1  $\mu$ M); a mitochondrial uncoupler, fluoro-carbonyl cyanide phenylhydrazone (FCCP) (1  $\mu$ M); inhibitors of electron transport chain complexes I and III rotenone (1  $\mu$ M) and antimycin A (1  $\mu$ M) respectively. Five respiratory states were defined: (i) basal OCR, (ii) non-phosphorylating OCR, (iii) maximal OCR, (iv) non-mitochondrial OCR, and (v) spare respiratory capacity. After measurement of OCR, data were normalized to the level of basal respiration in each well.

## 4 RESULTS AND DISCUSSION. PAPERS OVERVIEW

### 4.1 PAPER I, PAPER II, AND PAPER III

The first three papers summarize data obtained after genetic studies of patients with cardiomyopathies.

#### 4.1.1 Paper I: Diagnostic challenge in desmin cardiomyopathy with transformation of clinical phenotypes

In this case-report we described the first case of desmin cardiomyopathy with transition from a hypertrophic to a restrictive and dilated phenotype and with the disease cause originally mimicking myocarditis due to the pericardial effusion, serum troponin elevation, and late gadolinium enhancement shown on MRI. Histological examination showed moderate myocardial disarray with cardiomyocyte hypertrophy, fragmentation and variation of nuclear morphology, and moderate perimyocyte and focal fibrosis. Staining with anti-desmin antibody showed local loss of regular cross striation and uneven desmin distribution, with areas of no desmin immunoreactivity. Sequencing of the desmin gene showed a splice-site mutation (IVS3+1G ->A), which was absent in 300 healthy control subjects.

#### 4.1.2 Paper II: Desmin A213V substitution represents a rare polymorphism but not a mutation and is more prevalent in patients with heart dilation of various origins

Recently A213V desmin gene substitution has been described in seven unrelated patients with three different phenotypes; distal skeletal myopathy, restrictive cardiomyopathy, and dilated cardiomyopathy (91,175). However, this substitution has been found also in a group of healthy donors with a frequency of approximately 1%, and has also been described in a familial case of dilated cardiomyopathy where it did not segregate with the disease phenotype (111). The framework of this paper was genetical study of patients with heart dilation due to ischemic heart disease, alcoholic cardiomyopathy, or viral myocarditis, and 300 healthy controls in order to determine the frequency of *DES* A213V substitution. In the analyzed patient group *DES* A213V substitution was found in 5 out of 108 cases, corresponding to approximately 4.6% ( $p < 0.035$ ). In the control group *DES* A213V substitution was identified in 3 out of 300 patients, representing a rare SNP with a frequency of approximately 1%, which corresponds to the earlier reported frequency. Therefore we conclude that *DES* A213V represents a disease-modifying conditional polymorphism, rather than disease-related mutation, and plays a role as a predisposing factor resulting in maladaptive heart remodelling in the presence of other pathological factors.

### 4.1.3 Paper III: Genetic spectrum of idiopathic restrictive cardiomyopathy uncovered by next-generation sequencing

The aim of this study was to identify the genetic background of idiopathic RCMP using high-throughput approach of NGS, and 24 patients with RCMP were included in the study. All described genetic modifications were referred to as disease-related (mutation) or disease-modifying (SNP) variants. Disease-related variants (n=39) were identified in 22 out of 108 studied genes and were found in 21 patients (88%). The highest number of variants was found in the *TTN* gene (n=10). Other identified genes with multiple variants were *MYH7* (n=4) and *MYBPC3* (n=3). Three genes, *TNNI3*, *MYOM1*, and *SYNE2*, contained two variants each, and the remaining 16 disease-related variants were evenly distributed across the rest 16 genes. Thus, among of 88% genotype-positive cases, single mutations were detected only in 38% of the cases, while 50% of the genotype-positive patients carried two or more combined mutations. The most frequent combination included mutations in genes of sarcomeric contractile and cytoskeletal proteins (21% of all cases). Disease-modifying variants were detected across 17 out of 108 studied genes (n=38). The highest number of disease-modifying variants was also found in the *TTN* gene (n=16). The spectra of genes displaying disease-related variants and disease-modifying variants in RCMP differed, sharing only 3 genes. Thus, no disease-modifying variants were detected in the group of sarcomeric contractile protein or mitochondrial protein genes. On the other hand, the number of disease-modifying polymorphisms in desmosomal and membrane-associated protein genes was significantly higher compared to the group of disease-related mutations. We compared the spectrum of all disease-associated genetic variants identified for RCMP patients and patients with early onset ventricular arrhythmias without diastolic dysfunction or structural cardiac abnormalities to analyze its overlapping. For the latter group only one disease-modifying variant with minor allele frequency of 0.017% was identified in the non-contractile cytoskeletal protein gene *MYOM1*, while no variants were found in sarcomeric contractile protein genes. Thus, the genetic spectrum of RCMP-associated variants does not represent a random combination and differs from that of other genetic cardiac disorders. To uncover the interaction of gene products affected in RCMP pathogenesis we created a protein interaction map with 36 proteins found in this study. Including a further 30 interlinking proteins the network consists of 66 proteins connected by 124 interactions. Out of the 124 physical and regulatory interactions described here 38 and 33 were supported by experiments performed in cardiac and other types of muscle cells, respectively. Most proteins of this network belong to one of four functional groups: (i) sarcomeric contractile proteins, (ii) mechanosensing Z-disc proteins, (iii) nuclear membrane, and (iv) outer mitochondrial membrane proteins. Thus, in this paper we underlined the role of cytoskeletal protein genes in RCMP development. We conclude that RCMP is often triggered by the deleterious combination of multiple mutations in sarcomeric contractile and cytoskeletal protein genes rather than by a single mutation. Mechanosensing and mechanotransduction proteins are key players in the development of restrictive cardiac pathology.

To sum up the data unveiled by papers discussed above, desmin-related myopathy diagnosis should be concurrently based on clinical phenotype, biopsy histological studies, and be genetically proven. When exploring *DES* modifications one cannot rely on mutations leading to severe protein rearrangements and aggregates formation only. Non-aggregate substitutions should also be taken into account, since they can act as provoking factor to DRM development. It is plausible that SNP adverse effect determined by co-existence of changes in multiple genes. These genes commonly belong to the families of sarcomeric contractile and cytoskeletal proteins, nuclear membrane, and outer mitochondria membrane-associated proteins.

#### **4.2 PAPER IV: PRIMARY MURINE MYOTUBES AS A MODEL FOR INVESTIGATING MUSCULAR DYSTROPHY**

The particular interest of this paper was establishment of a robust and relevant cellular model to perform functional studies of *DES* mutations, with emphasis on mitochondria. The model should meet the following requirements: availability, ease of cultivation, ability to undergo lentiviral genetic modifications, and demonstrate high level of similarity to mature muscles. We compared primary adult muscle fibers with myotubes formed upon satellite cells fusion. We assessed their morphological and physiological properties and analyzed the ability of cells to undergo LV genetic modification. We demonstrated that primary myotubes resembled primary adult muscle fibers in terms of morphology and physiology. Primary myotubes expressed myosin and desmin filaments with the typical cross-striated pattern found in adult muscle fibres. Mitofusin-2 was expressed throughout the cytoplasm of myotubes with no apparent cross-striation, indicating that the adult organization of mitochondria had not yet occurred. RyR staining in primary myotubes was found throughout the sarcoplasm indicating an extensive sarcoplasmic reticulum. Primary myotubes were able to contract and to release sarcoplasmic calcium in response to electrical and chemical stimulation indicating a functional excitation-contraction coupling pathway linking L-type channel activation and the RyR in the sarcoplasmic reticulum. Patch-clamp studies showed the presence of Ca<sup>2+</sup> currents in plasma membrane of primary myotubes, although the L-type Ca<sup>2+</sup> current density was less in myotubes than in adult muscle fibers. Furthermore, primary myotubes, in contrast to muscle fibers, successfully underwent genetic modification via LV transduction and expressed the encoded proteins in 72 hours after transduction for at least 14 days. We concluded that satellite cells constitute a promising cell model for further experiments aimed at exploring calcium pathways involved in muscle dystrophies caused by mutations in miscellaneous genes. Thus, this model was recruited for further functional studies of *DES* mutations.

#### **4.3 PAPER V: AGGREGATE-PRONE DESMIN MUTATIONS IMPAIR MITOCHONDRIAL CALCIUM UPTAKE IN PRIMARY MYOTUBES**

Desmin-mitochondria interactions were previously demonstrated in numerous studies. It has been explicitly shown that desmin filaments participate in mitochondrial anchoring within the cell, and localize them in areas of energy needs,

thus providing crosstalk between mechanochemical signaling and metabolism (For review see 109). The aim of the current paper was to examine the effect of two types of desmin point mutations (aggregate-prone versus non-aggregate-prone) on mitochondrial calcium uptake. Four mutations, all found in patients with myopathy/cardiomyopathy, resulting in different degree of desmin aggregate formation, were analyzed. Our results revealed that mitochondrial capacity to take up calcium depended on desmin conformation within the muscle cells and was altered by desmin structural changes, and the degree of this effect depended on the type of desmin mutation. Aggregate-prone mutations demonstrated a considerable decrease in mitochondrial calcium upon both electrical and chemical stimulation when compared to control cells. Cells carrying desmin gene WT did not differ from non-transduced cells in the extent of mitochondrial calcium uptake, as well as cells carrying non-aggregate desmin gene mutations. Resting  $[Ca^{2+}]_i$  as well as post-stimulation tetanic  $[Ca^{2+}]_i$  were significantly higher in cells carrying aggregate mutation than in WT cells, while measurement of  $[Ca^{2+}]_i$  did not reveal differences in peak concentrations at electrical stimulations between cells expressing WT or mutant desmin gene. Therefore, we concluded that mutations in desmin gene had no impact on evoked SR calcium release, and decreased  $[Ca^{2+}]_{mit}$  in mutant cells was due to the insufficient mitochondrial uptake in cells carrying aggregate desmin gene mutations, rather than to changes in SR release. The principal roles of mitochondrial calcium include regulation of ATP production, modulation of cytosolic calcium concentration in some cells such as cardiomyocytes, and initiating apoptosis upon highly increased  $[Ca^{2+}]_{mit}$ . Therefore, decreased mitochondrial calcium in desmin mutant cells could be expected to lead to a decline in ATP production with consequent effects on energy turnover and a shift in intracellular calcium homeostasis—key events in the development of cardiomyopathies and arrhythmias. Given the knowledge of desmin and mitochondria connections, we hypothesize that the observed effect of desmin mutations on mitochondrial calcium uptake might be the result of disruption of the organelle’s spatial orientation. Due to the altered desmin filamentous network, mitochondria could lose their normal positioning with respect to the SR (Fig. 4).

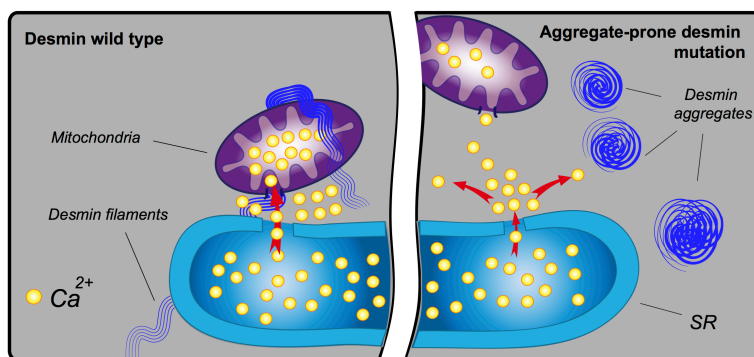


Figure 4. Proposed model of disturbed interaction between mitochondria and sarcoplasmic reticulum (SR) in the presence of desmin aggregates (adapted from Smolina et al., *Cell Calcium*. 2014; 56: 269-275, (176); republished with permission).

#### 4.4 UNPUBLISHED RESULTS: DESMIN MUTATIONS DEPRESS MITOCHONDRIAL METABOLISM

Our data have demonstrated decreased mitochondrial calcium levels in muscle cells carrying aggregate desmin gene mutations. It was speculated that decreased mitochondrial calcium might affect mitochondrial respiratory function, e.g. respiration parameters expressed as oxygen consumption rate (OCR). Mitochondrial respiration is the process of conversion of energy of taken up substrates into the production of ATP through enzymatically-mediated reactions. It takes place in mitochondrial matrix and mitochondrial inner membrane, and is important component of cellular metabolism.

To extend knowledge of the impact of desmin mutations on cellular metabolism Cell Mito Stress Test (Seahorse Bioscience, USA) experiments were performed. Seahorse equipment allows measuring oxygen consumption in living cells and estimate key parameters of mitochondrial respiration in real-time mode. Four key mitochondrial respiration parameters were measured according to the manufacturer's protocol—basal OCR, ATP-linked (non-phosphorylating) OCR, maximal OCR, and non-mitochondrial OCR. Each experiment encompassed six experimental groups corresponding to (i) non-transduced cells and cells transduced via LV encoded (ii) *Des* WT, (iii) *Des* L345P, (iv) *Des* A357P, (v) *Des* L370P, (vi) *Des* D399Y with 8–16 technical replicates per group. We normalized obtained data to the basal OCR level due to the obstacle of protein normalization. One-way ANOVA was employed to evaluate statistical significance, with  $p < 0.05$  considered significant. Tukey's post-hoc analysis was used to compare individual groups. We demonstrated that all cell types had similar bioenergetic profiles (Fig. 5(a)): decreasing OCR after oligomycin application, rapid OCR increase following FCCP application, and drop of OCR after rotenone/antimycin application. We showed that it was only maximal OCR that declined in the presence of desmin mutations; all other parameters did not display any significant difference between cells expressing endogenous *Des* or various forms of exogenous *Des*. Relative increase of OCR after FCCP application was  $1.95 \pm 0.09$  for non-transduced cells,  $2.36 \pm 0.09$  for *Des* WT,  $1.85 \pm 0.12$  for *Des* L345P,  $1.81 \pm 0.11$  for *Des* A357P,  $1.95 \pm 0.11$  for *Des* L370P and  $1.87 \pm 0.12$  for *Des* D399Y. Thus, *Des* L345P and A357P, being the most prominent aggregate-prone mutations, resulted in the most prominent decline in maximal OCR in comparison with *Des* WT, while other mutations also decreased maximal OCR but not as dramatically. Furthermore, it was found that only cells expressing mutant desmin had relative increase of maximal OCR less than one, implying lack of spare respiratory capacity in some of these cells (Fig. 5(c)).  $[Ca^{2+}]_{mit}$  activates several key enzymes in the mitochondrial matrix resulting in stimulation of ATP production (177–179), often through modulation of nitric oxide level and oxygen consumption rates (180), recent findings confirmed dose-dependent effect of calcium ions on muscle oxidative phosphorylation cascade (181). Therefore, we assumed that impaired mitochondrial calcium uptake might confine mitochondrial respiration parameters. We showed that in the presence of desmin mutations maximal OCR was decreased in comparison to cells harbouring *Des* WT.



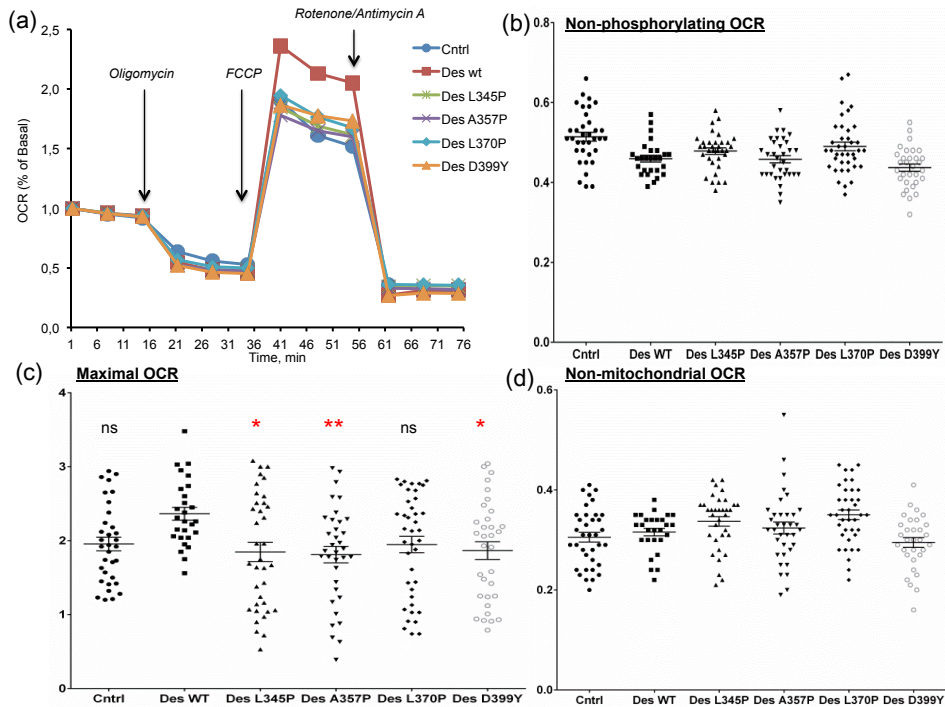


Figure 5. Mitochondrial respiration in primary myotubes harboring endogenous desmin (Cntrl) or various exogenous desmin variants (a) cells bioenergetic profiling obtained after application of mitochondrial inhibitors and reflecting four main parameters of mitochondrial respiration, three measurements for each parameter: [1–3] basal OCR, [4–6] non-phosphorylating OCR (after oligomycin to inhibit ATP synthase), [7–9] maximal OCR (after FCCP to uncouple the IMM), [10–12] non-mitochondrial OCR (after rotenone/antimycin A to inhibit complexes I/III). OCR data were normalized to the basal OCR level; Dot plots represent selected measurements for each parameter: (b) [6] non-phosphorylating OCR; (c) [7] maximal OCR; (d) [12] non-mitochondrial OCR. Data represent means  $\pm$  SEM of 3 independent experiments: \* $p < 0.05$  VS Des WT, \*\* $p < 0.01$  VS Des WT; ns—no significance difference.

We demonstrated spare respiratory capacity rate declining as well. These results are in agreement with previously established reduction of maximal rate of ADP-stimulated oxygen consumption in desmin-null cardiac and soleus muscles compared with controls measured in situ (59). It is of interest to note that measurement of oxygen consumption in isolated mitochondria from control and desmin-null mice did not show any variance in ADP-stimulated, ADP-limited, or uncoupled respiration rates (59), thus confirming the importance of surrounding environment for proper mitochondria functioning. On the other hand, our results contradict recently reported data obtained using mice lacking mitochondrial calcium uniporter-mediated calcium entry in mitochondria but exhibiting elevated or unaltered levels of oxygen consumption (182). This might be explained by the fact that measurements were performed on the whole hearts or isolated mitochondria, whereas in our experiments mitochondria were analysed in culture of myotubes. Moreover, it might be inferred that not only  $[Ca^{2+}]_{mit}$ , but also other mitochondrial regulating functions are impaired in conjunction with desmin gene mutations. It is plausible that the site of desmin-mitochondrial interplay lost its

correct spatial orientation, thus affecting other participants of these interactions as well. As discussed by Capetanaki et al. (109), desmin interacts with mitochondria at contact sites, where proteins responsible for mitochondria biogenesis, morphology, and functions are located, e.g. VDAC channels, ANT, and the MICOS complex. Spare respiratory capacity is a characteristic of cell bioenergetics reflecting the ability of cells to respond to rapid changes in energy demand, encompassing substrate supply and electron transport chain performance and resulting in the production of extra ATP molecules. Assessment of spare respiratory capacity allows defining the bioenergetic limits of the cell that is of a special importance for muscle cells undergoing high ATP consumption. The lack of spare respiratory capacity implies mitochondrial dysfunction that might be not obvious in normal conditions, however becomes overt under the load (183). Moreover, insufficiency of spare respiratory capacity was shown to be a hallmark of heart pathologies, e.g. heart failure (184,185). Several explanations were proposed for decreased maximal OCR and reserve respiration capacity: decreased substrate availability, decreased mitochondrial mass, poor electron transport chain integrity (186). Based on our and other's findings, we on the following explanations: declined functions of enzymes—participants of respiratory chain due to the impaired mitochondrial calcium uptake discussed above, and mitochondrial ADP consumption dependence on cytoskeletal components as was showed earlier by Kay et al. (187), Appaix et al. (188), and Varikmaa et al. (189).

#### **4.5 LIMITATIONS OF THE STUDY**

In this section limitations are specified that might impact on our findings and our competence in the approach to address the research questions raised. The main limitation presents in the majority of studies in this thesis is the application of models, for which the relevance may be debated. We recruited primary murine satellite cells isolated from the soleus muscle as the cell model, and the cell genome was modified via LV transduction. There are several principal limitations regarding this model. The first—differences between in vitro and in vivo studies. In vivo studies are more preferable over in vitro studies, since they provide better insights and allow investigation of complex interactions in disease pathology at organismal level. The following limitations concern differences between murine and human muscle physiology. Muscles, being a heterogenic tissue, display a high diversity in fiber types content resulting in variation of muscle physiological properties between species. Further, fiber size matters, which is much smaller in mouse compared to human. Last, TG mouse model carrying the mutation resulted in severe clinical manifestation in human, e.g. *DMD* gene mutations, might display relatively benign phenotype. The final limitation refers to the stochastic mode of LV genomic integration. LV belong to the retroviruses, and are able to the genome integration. However, the pattern of integration is unpredictable, and might lead to the randomic impairment of gene transcription. In order to overcome previously stated limitations, TG mouse model might be used for each examined mutation. However, this approach is costly and time-consuming. Satellite cells obtained from the patients might be applied to increase the relevance of the study, but biopsy material was difficult to access in our studies. Therefore, despite that limitations, we used murine primary satellite cells

due to the following reasons: ease of cell isolation, genome modification, cultivation, differentiation, and the high level of similarity between primary myotubes and adult myofibers (190); proven molecular resemblance of murine soleus muscle to human skeletal muscles (191); and absence of overt adverse effects of LV transduction on the muscle cell physiology (190). In the future studies we plan to utilize induced pluripotent stem cells (iPS), obtained from the patient carrying a mutation, as well as from the healthy donors as a control. These iPSc will be differentiated into cardiomyocytes and undergo further investigations. We assume this cell model will be relevant for studying the role of desmin mutations in development of the cardiomyopathy phenotype of DRM.

## 5 CONCLUSIONS AND FUTURE PERSPECTIVES

The principal goal of current thesis was to evaluate the role of intermediate filament desmin in the development of desmin-related myopathy (DRM), with an emphasis on development of cardiomyopathy. To address this goal we analyzed in different systems the effect of various types of *DES* mutations. First, we analyzed the clinical course of a patient carrying a *DES* splice-site mutation (IVS3+1G →A), resulting in DRM with transition of phenotypes: from a hypertrophic to a restrictive and dilated cardiomyopathy. Next, we continued with a population study in order to assess the prevalence of *DES* A213V, the significance of which was unknown. We found the *DES* A213V substitution to be a disease-modifying polymorphism with characteristics of a conditional mutation, rather than a primary disease-related mutation. Further, NGS approach was applied to unveil mutant genes underlying RCMP, one of cardiac DRM manifestation. By help of the high-throughput sequence technologies it became apparent that for most patients with RCMP it was not single disease-related mutation, but rather a combination of two or more disease-related–disease-modifying or diseases-related–disease-related variants that underlie the development of RCMP. To perform functional studies of *DES* mutations, we established a robust cell model—primary myotubes, demonstrating high levels of morphological and physiological similarity with mature muscle fibers and easily undergoing genome modification via lentiviral transduction. Therefore, we adopted it to conduct functional studies, aiming at comparing effects of aggregate-prone and non-aggregate-prone *DES* mutations on mitochondrial calcium uptake and respiration parameters. We found that aggregate-prone mutations resulted in decline of mitochondrial calcium uptake, as well as decreased maximal oxygen consumption rate (OCR) and spare respiratory capacity. However, non-aggregate-prone mutations did not disturb mitochondrial calcium, but still reduced maximal OCR and depressed spare respiratory capacity.

Mechanosensing and mechanotransduction functions of cytoskeletal proteins are of great interest today. Current findings strongly support the notion that diseases associated with certain mutations of genes encoding cytoskeletal proteins cannot be solely attributed to structural defects; but rather concern also wide range of other functions, organelle positioning, and signaling. Our data confirmed this perception for desmin. The finding that both aggregate-prone and non-aggregate-prone mutations influenced mitochondrial bioenergetics indicates that not only intermediate filament polymer formation contributes to the disease development.

Intriguing results were obtained from NGS analysis of patients with RCMP. Fifty percent of the genotype-positive patients carried two or more combined mutations. Most gene products of these combinations belonged to one of four functional groups: (i) sarcomeric contractile proteins, (ii) mechanosensing Z-disc proteins, (iii) nuclear membrane, or (iv) outer mitochondrial membrane proteins. This may imply that RCMP development is triggered by the combination of several mutations in mechanosensing and mechanotransduction proteins. The existence of multiple mutations, other than in the *DES*, may also explain the varying *DES* A213V effect on patients. Moreover, relatively mild effects of *DES* mutations in vitro studies in

comparison to those observed in patients could be linked to this as well. In line with this it may be speculated that studying cells obtained directly from a patient with DRM, harbouring the entire genetic background would reveal greater differences in mitochondrial functions between *DES* WT and *DES* mutants.

In conclusion:

1. Distortion of desmin mechanotransduction functions play an important role in DRM onset, affecting mitochondrial bioenergetics properties. The impact of aggregate-prone mutations was greater than of non-aggregate-prone, implying increased deleterious effect of desmin aggregates.
2. A combination of mutations in mechanosensing and mechanotransduction protein genes, rather than a single mutation predisposes to development of RCMP.

Further studies are necessary for detailed understanding of mitochondrial physiology in the presence of various desmin mutations. Such future analyses may include the following experiments. Estimation of the proximity between mitochondria and sarcoplasmic reticulum in order to validate the proposed model about mitochondrial spatial orientation within the cells harbouring desmin aggregate-prone mutations. Further, to estimate mitochondrial fusion-fission due to the known fact of mitochondrial dysfunction to result in increase in mitochondrial number. In addition, to estimate mitochondrial membrane potential and ADP/ATP ratio, thus evaluating the efficiency of mitochondrial respiratory activity. To increase the relevance of our studies we plan to use not only primary myotubes, but also iPS obtained from the patient carrying *DES* mutation and differentiated into cardiomyocytes.

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