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## **Nemaline myopathy: pathophysiology and therapeutic targets**

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2017

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de Winter, J. M. (2017). *Nemaline myopathy: pathophysiology and therapeutic targets*.

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**DISCUSSION, SUMMARY  
AND FUTURE PERSPECTIVES**



## DISCUSSION AND SUMMARY

Nemaline myopathy is a debilitating muscle disease, for which currently no treatment exists as the mechanisms underlying muscle weakness are only partly understood. Therefore, the aim of this thesis was to gain more insight into the pathophysiology of muscle weakness in nemaline myopathy and to investigate genotype-functional phenotype correlations to direct targeted therapeutic interventions (Chapter 2 – 5), and to test potential therapeutic targets to restore muscle strength (Chapter 6 – 8). Here, first the outcomes of the studies are described and discussed, followed by a discussion on future directions.

### ***Pathophysiology of nemaline myopathy***

#### *Chapter 2*

Nemaline myopathy is a disease that affects the skeletal muscle thin filament. As the overlap between the thick and the thin filament determines the number of cross-bridges that can be formed, we investigated whether mutations implicated in nemaline myopathy contribute to force loss by affecting thin filament length. The force-sarcomere length dependency of force was studied in fifty-one biopsies covering the majority of genes implicated in nemaline myopathy (*NEB*, *ACTA1*, *TPM2*, *TPM3*, *TNNT1*, *KBTBD13*, *KLHL40* and *KLHL41*). This study thought us that all mutations resulted in muscle weakness at the myofilament level, but that the contribution of shorter thin filament length to muscle weakness was specific for mutations in *ACTA1* and *NEB*. Next, findings in a nebulin-deficient mouse model for nemaline myopathy revealed that skeletal muscle compensates for shorter thin filament length by adding more sarcomeres in series.

This study was designed to answer a key question in the nemaline myopathy field: are there genotype-functional phenotype correlations in nemaline myopathy? Clear correlations will guide targeted treatment of muscle weakness. The present study reveals that only specific mutations in the *NEB* and *ACTA1* gene result in changes in thin filament length. Note that from two gene cohorts - *KLHL40* and *KLHL41* – only one patient sample was studied. Both *KLHL40* and *KLHL41* are known to interact with either nebulin and leiomodin-3 and/or actin (Garg et al., 2014; Gupta et al., 2013). Mutations in leiomodin-3 have recently been reported to affect thin filament length

regulation (Yuen et al., 2014). Hence, next to *NEB*, *ACTA1* and *LMOD3*, mutations in *KLHL40* and *KLHL41* are potential players in thin filament length regulation.

It is presumed that mutations in *TPM2*, *TPM3* and *KBTBD13* do not compromise thin filament length regulation. The finding in the *TPM3* gene cohort is supported by previous structural studies on biopsies from nemaline myopathy patients with mutation in *TPM3* (Ottenheijm et al., 2011; Yuen et al., 2015). Of interest, another important parameter for force generation –the calcium-sensitivity of force generation - was affected bidirectionally in tropomyosin-based nemaline myopathy. The effect on the calcium-sensitivity of force generation was mutation-dependent: either a hypertonic or a hypotonic functional phenotype was observed (Donkervoort et al., 2015; Mokbel et al., 2013; Ottenheijm et al., 2011; Yuen et al., 2015). In line with these findings in tropomyosin-based nemaline myopathy, also in nebulin-based nemaline myopathy mutation-dependent changes in thin filament length regulation and activation are found. For example, a deletion of exon-55 compromised both thin filament length regulation and thin filament activation (lower calcium-sensitivity of force generations and lower cross-bridge cycling kinetics (Ottenheijm et al., 2009, 2010, 2013; de Winter et al., 2013), but compound heterozygous splice site mutations (in exons 3 and exon 22, respectively), revealed normal thin filament lengths and normal calcium-sensitivity of force generation but impaired cross-bridge cycling kinetics (Ochala et al., 2011). Thus, combining the present data with data that was reported previously, the concept arises that in nemaline myopathy the functional phenotype is mutation-dependent. Hence, we should revise our research question into: are there *mutation*-functional phenotype correlations in nemaline myopathy? High-throughput studies in flies or fish might aid in elucidating mutation-functional phenotype correlations in nemaline myopathy (Berger et al., 2014; Sztal et al., 2015; Telfer et al., 2012).

In part two of Chapter 2 we investigated whether muscle responds to shorter thin filament lengths by using the recently developed conditional nebulin knockout mouse. This model recapitulates nemaline myopathy: muscle fibers generate lower maximal active tension, have shorter thin filaments and as a consequence, the optimal sarcomere length for force production is shorter (Li et al., 2015). However, conditional nebulin knock-out mice increased the number of sarcomeres in series, and as a result the optimal muscle length

for force generation in intact muscle was comparable to wild-type mice. Hence, the addition of sarcomeres in series allowed the muscles to operate at a shorter sarcomere length, a length closer to their optimal sarcomere length. Adapting the number of sarcomeres in series to compensate for changes in thin filament length constitutes a novel control mechanism in muscle. Whether this control mechanism is also present in human diseased muscle is unknown. Simultaneous measurement of total fiber and sarcomere length in humans is challenging. In this respect, knowledge on the *in vivo* sarcomere length range in thin filament myopathy patients might provide valuable information: shorter sarcomeres would indicate more sarcomeres in series. The elucidation of this control mechanism might have implications for treatment strategies. In rodents, active stretching of muscle stimulates the addition of sarcomeres in series (Riley and Van Dyke, 2012). Thus, active stretching of muscles of patients with shorter thin filament lengths might be an interesting direction to explore to stimulate the addition of sarcomeres in series and alleviate muscle weakness.

### Chapter 3

The aim of Chapter 3 was to investigate whether there are correlations between the ultrastructure of muscle fibers from nemaline myopathy patients with mutations in the nebulin gene and the functional phenotype and clinical phenotype of muscle fibers from these patients. We found that the clinical severity was associated with the functional phenotype and the sarcomeric ultrastructure. The latter is defined here as the alignment of myofibrils. Interestingly, related to this was the finding that the subsarcolemmal position of rods appeared to inversely correlate with the clinical and functional phenotype. Thus, when the nemaline bodies are located at the subsarcolemmal region, myofibrillar alignment is preserved which favors contractile performance. The question remains what mechanisms orchestrate the organization of nemaline bodies and whether this is correlated to nebulin protein levels. These are interesting questions for future research. Although the ultrastructure of the muscle fibers of mildly affected nemaline myopathy patients is relatively preserved, contractile weakness is observed compared to the contractile performance of control fibers. This phenomenon has been reported in literature previously (Donkervoort et al., 2015; Ottenheijm et al., 2011) de Winter et al., 2016, and suggests that intrinsic dysfunction of the contractile proteins accounts for the contractile weakness observed

at the single fiber level. An elegant way to investigate the contribution of purely contractile protein function to muscle fiber weakness is to study the contractile performance of myofibrils (Stehle et al., 2009). A muscle fiber (diameter ~ 100  $\mu\text{m}$ ) consists of a large collection of myofibrils (diameter ~ 1  $\mu\text{m}$ ), hence changes in the ultrastructure of the muscle fiber – e.g. the connectivity of adjacent myofibrils – influences the contractile performance of this interconnected collection of myofibrils. In a recent study, it was shown that the contractile deficit in myofibrils isolated from nebulin-deficient mouse muscle resembles the contractile deficit that was observed in single fibers from that muscle (Ottenheijm et al., 2013). These data suggest that dysfunctional contractile proteins are a main cause of weakness in nebulin-based nemaline myopathy.

### *Chapter 4*

In Chapter 4 we characterized the *in vivo* and *in vitro* contractile performance of heterozygous nebulin-knockout mice, and investigated whether changes in sarcomeric gene and protein levels were correlated to the contractile phenotype of intact soleus muscle. This study revealed that heterozygous nebulin-knockout mice have normal nebulin protein levels. However, the mRNA levels encoding slow-twitch regulatory proteins troponin C, T and I and slow-twitch myosin heavy chain were 5-7 fold upregulated. In line with the upregulation of slow-twitch-myosin heavy chain mRNA, a shift in the myosin heavy chain composition was observed at the protein level: muscle of nebulin-knockout mice display a higher slow-twitch over fast-twitch myosin heavy chain ratio. The switch towards a slow-twitch proteomic phenotype was accompanied by mild muscle weakness at maximal stimulation frequencies at the *in vitro* intact muscle level. It's striking that the absence of one nebulin allele has no effect on nebulin protein levels, but does affect the levels of slow-twitch regulatory proteins. Also in other mouse models for nemaline myopathy (Corbett, 2001; Nguyen et al., 2011; Ravenscroft et al., 2011; Tian et al., 2015), muscle biopsies of nemaline myopathy patients and in other neuromuscular conditions (D'Antona et al., 2007) this switch is observed. An important question that remains to be addressed is what triggers this induction of a slow-twitch gene program. And why muscle responds this way: is it a preventive strategy, as a slow-twitch muscle is more fatigue resistant? These are interesting questions for future research. Especially, as a recent study on a novel nebulin-deficient mouse model revealed that the soleus

muscle – a typical example of an oxidative muscle in mice - was relatively spared compared to the glycolytic muscles (Li et al., 2015). In addition to that, nemaline myopathy patients with mutations in *KBTBD13* display mild muscle weakness and hypertrophy of slow-twitch muscle fibers (Gommans et al., 2002; Olivé et al., 2010; Sambuughin et al., 2010). Thus, when muscle possesses the potential of inducing a slow-twitch gene program, this might be a favorable strategy in nemaline myopathy.

### Chapter 5

The aim of Chapter 5 was to investigate the role of KBTBD13 in the pathogenesis of muscle weakness. Contractile studies on single fibers isolated from biopsies of NEM6 patients with mutations in the *KBTBD13* gene revealed that at least part of the muscle weakness and muscle slowness is sarcomere-based. In addition, muscle characteristics of the *Kbtbd13*-KO mouse model partly phenocopy the muscle weakness and slowness of NEM6 patients. Hence, we developed a novel nemaline myopathy mouse model that allows us to further unravel the role of KBTBD13 in health and disease and to test therapeutic strategies for NEM6 patients. Important aspects to focus on in the near future are to elucidate the interaction partner(s) of KBTBD13 and the localization of the protein. The only identified interaction partner thus far is Cullin E3 (Sambuughin et al., 2012). For KLHL40 and KLHL41, interactions with both Cullin E3 and specific thin filament proteins are reported in literature (Cenik et al., 2015; Garg et al., 2014; Gupta et al., 2013). Therefore it is hypothesized that KBTBD13 plays a role in the turnover of (a) thin filament protein(s). Hence, kelch proteins are a very exciting new class of proteins that stabilize thin filament proteins. Therefore, they can be of interest to stabilize specific thin filament proteins that are implicated in nemaline myopathy. As *KBTBD13* is expressed in both skeletal and cardiac muscle tissue, the availability of a *Kbtbd13*-deficient mouse provided us with the possibility to investigate cardiac function under various conditions. Cardiac muscle of *Kbtbd13*-KO had a lower contractile reserve upon stress. Thus far, compromised cardiac function has not been reported in mouse models for nemaline myopathy. To date, some case studies reported cardiac complications in patients with nemaline myopathy (Finsterer and Stöllberger, 2015), but for the NEM6 phenotype this observation is new. As the lower contractile reserve was evident only during stress conditions, the possibility exists that also in other types of nemaline myopathy caused by mutations in

genes that are expressed in the heart, cardiac dysfunction develops during stress conditions. Thus, future studies should take into account the potential of cardiac involvement in nemaline myopathy.

### ***Therapeutic targets in nemaline myopathy***

#### *Chapter 6-8*

In Chapters 6-8 we investigated the potential of slow- and fast troponin activation to augment muscle strength in muscle fibers from a nebulin-deficient nemaline myopathy mouse model and from nemaline myopathy patients with mutations in the nebulin gene. Troponin activators increase the calcium-sensitivity of force generation. As we found that nebulin-deficient muscle has a lower calcium-sensitivity of force generation, these drugs could have a great potential to restore muscle strength. First, the effect of a slow troponin activator – Levosimendan – was tested. Unfortunately, no gain of function was observed in muscle fibers of controls and patients upon administration of Levosimendan. Besides Levosimendan, other slow troponin activators are available for pre-clinical and clinical use (Schlecht et al., 2016). Therefore, it would be of interest to investigate whether these slow troponin activators have the potential to augment muscle force in nemaline myopathy.

However, note that a potential drawback of slow troponin activators is that they might exert their effect on slow troponin isoforms that are present in the heart muscle. Thus, to specifically target skeletal muscle without risking potential cardiac complications, we moved our attention to a fast troponin activator – CK-2066260. First, we tested the ability of this compound to augment muscle strength in fibers from a nebulin-deficient mouse model that phenocopies nemaline myopathy. Here, a gigantic increase in muscle force was observed at submaximal calcium levels. In addition to the increase in the calcium-sensitivity of force generation, also the cross-bridge cycling kinetics were increased at submaximal calcium levels. Then, we tested the effect of the fast troponin activator on muscle fibers isolated from biopsies of nemaline myopathy patients with mutations in the nebulin gene. To our enthusiasm, also in patient fibers a great increase in muscle force at submaximal calcium levels was observed. This can be of clinical relevance, as these submaximal calcium levels reflect the physiological activity levels: i.e. activity levels that we use in daily life activities as breathing and walking. It is therefore hypothesized that administration of a fast troponin activator will lower fatigue

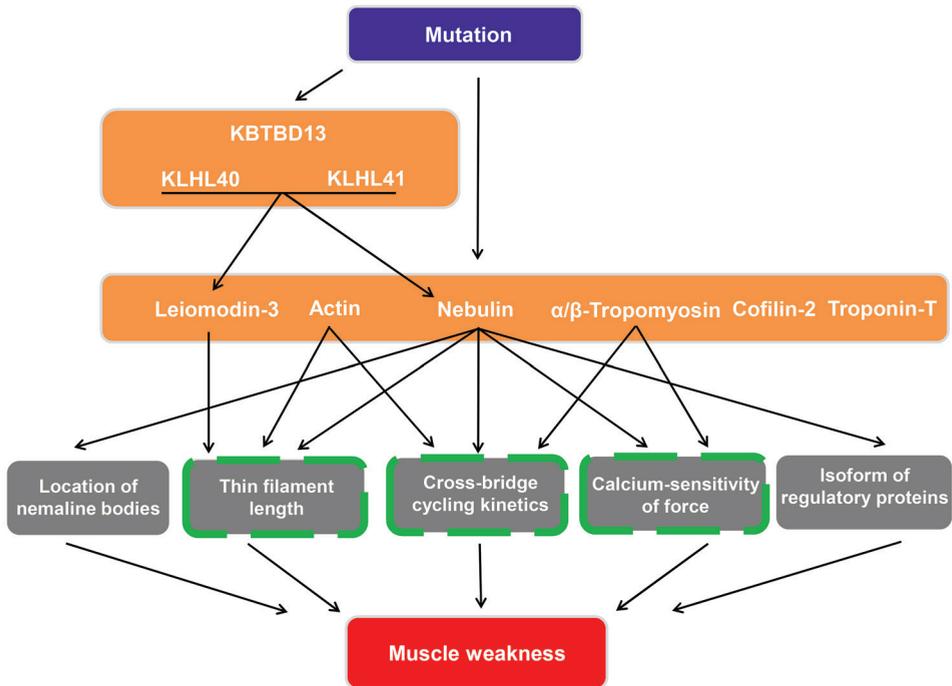
in daily life activities and thus contribute to the quality of life in nemaline myopathy patients with nebulin mutations. Recent studies in healthy controls reveal that a fast skeletal troponin activator could also amplify the response of the sarcomere *in vivo* (Hansen et al., 2014). In addition to that, positive effects on respiratory function are reported in patients with amyotrophic lateral sclerosis and myasthenia gravis upon administration with a fast troponin activator (Sanders et al., 2015; Shefner et al., 2012). Thus, it would be of great interest to investigate the ability of this fast troponin activator to alleviate muscle weakness in patients with nemaline myopathy. The first crucial steps that we will undertake are to study the effect of the compound on *in vivo* force production in various mouse models for nemaline myopathy.

## FUTURE PERSPECTIVES

Besides the promising concept of fast troponin activation, also other strategies are of highly interest to further explore in the quest for directions to alleviate muscle weakness in nemaline myopathy. First, as disturbed calcium-handling can contribute to muscle weakness (Ottenheijm et al., 2008), compounds that target either the release of calcium or the re-uptake of calcium by the sarcoplasmic reticulum will aid in the alleviation of muscle weakness. In other neuromuscular conditions such as mitochondrial myopathy and duchenne muscular dystrophy, small molecules that target calcium-handling result in improved contractile performance (Cheng et al., 2015; Gineste et al., 2015; Mázala et al., 2015). Another exciting field to further explore is the role of mitochondria in nemaline myopathy. Hitherto, mitochondrial function has not been investigated in nemaline myopathic muscle. In addition to that, also the role of oxidative stress in the pathogenesis of muscle weakness in nemaline myopathy is obscure. As the primary contractile deficits caused by the gene mutations that are implicated in nemaline myopathy can put a high stress on the mitochondria, one hypothesis is that nemaline myopathic muscle can benefit from redox-modulators that reduce the overload of oxidative stress that results from disturbed mitochondrial function. Recent studies in the neuromuscular field reveal that redox-modulators can ameliorate muscle weakness in various mouse models (Cheng et al., 2016; Kang et al., 2015; Lanner et al., 2012; Paolini et al., 2015). A final interesting approach to counteract muscle weakness is to target the trophicity of skeletal muscle cells by inhibiting specific ubiquitin-proteasome pathway enzymes that target skeletal muscle proteins, such as MuRF1 (Bodine et al., 2001; Eddins et al., 2011). That way, loss of contractile proteins can be inhibited to alleviate muscle weakness.

In conclusion, this thesis focused on various mechanisms underlying the pathophysiology in nemaline myopathy, on elucidating genotype-functional phenotype correlations in nemaline myopathy and on the potential of therapeutic strategies to augment muscle force. By setting up a bank for nemaline myopathy biopsies and by the engineering of various mouse models for nemaline myopathy, unique muscle tissue was available to perform translational studies. Mechanisms that can contribute to muscle weakness are changes in (1) thin filament length regulation, (2) the calcium-sensitivity of force generation, (3) the cross-bridge cycling kinetics, (4) the

location of nemaline bodies and (4) the isoform composition of regulatory proteins. These changes revealed to be mutation-dependent. A promising therapeutic target to augment muscle strength in nemaline myopathy is fast skeletal troponin activation.



**Figure 1 Thesis summary**

Schematic overview of the mechanisms by which mutations in genes implicated in nemaline myopathy cause muscle weakness. Mechanisms that can contribute to muscle weakness are changes in (1) the location of nemaline bodies, (2) thin filament length regulation, (3) the cross-bridge cycling kinetics, (4) the calcium-sensitivity of force generation, and (5) the isoform composition of regulatory proteins. These changes revealed to be mutation-dependent. Green dashed lines indicate potential therapeutic targets. The addition of sarcomeres in series is an interesting approach to improve thick-thin filament overlap, and fast troponin activation is a promising target to modulate cross-bridge cycling kinetics and calcium-sensitivity of force.

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