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COMMENTARY

Nebulin: Size matters for optimal muscle function

Katarina Pelin

In this issue of the *Journal of General Physiology*, Gohlke et al. (2021) report novel insights into the role of nebulin for skeletal muscle function. The nebulin gene, *NEB*, is one of the largest genes in the human genome and encodes one of the gigantic structural proteins in the skeletal muscle sarcomere (Labeit and Kolmerer, 1995). Mutated *NEB* causes nemaline myopathy and related inherited skeletal muscle disorders (Lehtokari et al., 2014). Nebulin is crucial for physiological force levels; it controls thin filament length and stiffness, promotes thin filament activation, and enhances cross-bridge recruitment (Kiss et al., 2018; Kiss et al., 2020). By analyzing and comparing nebulin gene and protein structures across 53 species, Gohlke and colleagues concluded that the length of the nebulin protein correlates with animal size (i.e., larger animals have longer nebulin proteins). Moreover, the size of nebulin correlated with thin filament length and longer sarcomeres in larger animals, allowing for optimal and energy-efficient force production. Gohlke et al. (2021) also found a positive correlation between the number of actin-binding super repeats and the number of Z-disk width–determining simple repeats allowing wider Z-disks in larger animals.

The main source of the nebulin length variation in different species is the number of actin- and tropomyosin-binding super-repeat domains. One super repeat consists of seven simple repeats and contains seven actin-binding sites and one tropomyosin-binding site (Labeit and Kolmerer, 1995; Fig. 1). Each super repeat lengthens the thin filament by ∼38.5 nm. The highest number of super repeats (i.e., 31) was found in chimpanzee and camel, and the lowest (i.e., 21) was found in some fish species and small birds (hummingbird and lance-tailed manakin; Gohlke et al., 2021). Human and chimpanzee nebulin are almost 100% identical, though human full-length nebulin has only 29 super repeats. However, due to recurrent copy-number variation in the centrally located triplicate region of *NEB* (exons 82–105), the number of super repeats in full-length human nebulin is 27–31 (Kiiski et al., 2016). The high sequence homology of the exons in the triplicate region of *NEB* increases the likelihood of misalignment of homologous chromosomes during meiosis and unequal recombination, resulting in deletions and duplications, which is thought to be the basis of the recurrent copy-number variation in this region. Due to the high sequence homology of the nebulin gene in primates (Gohlke et al., 2021), it is tempting to speculate that similar copy-number variation in the triplicate region exists in chimpanzee, gorilla, and orangutan.

Mutations in *NEB* are the most common cause of autosomal recessive nemaline myopathy, a congenital myopathy characterized by muscle weakness and nemaline rods consisting of Z-disk proteins in the muscle fibers. Nemaline myopathy varies from mild to severe disease (Sewry et al., 2019). Recessive mutations in *NEB* may also cause distal nebulin myopathy without nemaline rods, core-rod myopathy, distal forms of nemaline myopathy, and lethal multiple pterygium syndrome. More than 250 different recessive disease-causing mutations have been reported in *NEB* (Lehtokari et al., 2014). In addition, a dominant in-frame deletion encompassing *NEB* exons 14–89 causing distal nemaline/cap myopathy in a three-generation family has recently been described (Kiiski et al., 2019).

The susceptibility of the triplicate region to copy-number variation predisposes the region to disease-causing variation. When the total number of super repeats exceeds 33 it will result in nemaline myopathy, if both alleles are mutated (Kiiski et al., 2016). The recessive inheritance of *NEB* alleles with large in-frame duplications indicates a loss-of-function mechanism (i.e., too-large nebulin proteins are not incorporated into the sarcomere, or the mRNA is degraded). Yet, a variant with a large in-frame deletion that removes 17 super repeats is readily expressed and causes a dominant form of nemaline/cap myopathy (Kiiski et al., 2019). Interestingly, smaller in-frame deletions that remove one to five simple repeats in the super-repeat region are all recessive loss-of-function mutations causing disease (Lehtokari et al., 2014). These mutations disrupt the super-repeat structure of nebulin. Intact super-repeat structure with the correct spacing of actin- and tropomyosin-binding sites is crucial for nebulin function (Fig. 1). If the protein is significantly shorter or longer than normal, then function is impaired.

Molecular and Integrative Biosciences Research Programme, Faculty of Biological and Environmental Sciences, University of Helsinki, Helsinki, Finland.

Correspondence to Katarina Pelin: katarina.pelin@helsinki.fi

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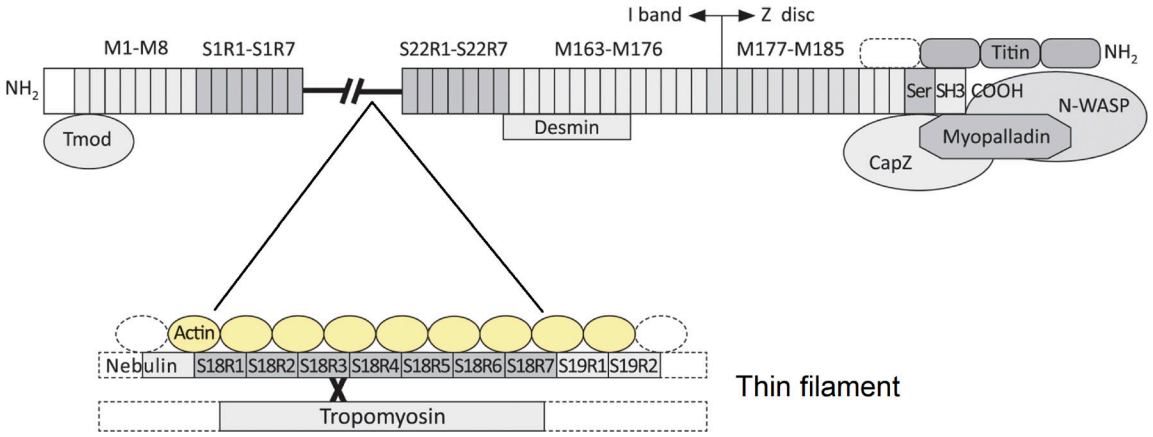

Figure 1. **A schematic presentation of the nebulin protein and some of its known protein interaction partners.** The upper part of the figure shows the domain structure of nebulin. M1-M8 are the N-terminal simple repeats that are preceded by a unique N-terminal domain. Tropomodulin (Tmod) binds to nebulin at the N terminus. S1R1–S1R7 is the first super repeat (S1) and S22R1–S22R7 is the last super repeat (S22), and each super repeat consists of seven simple repeats (R1–R7). M163–M176 are C-terminal simple repeats, and M177–M185 are Z-repeats. Desmin binds nebulin at the C-terminal simple repeats. The C terminus consists of a serine-rich domain (Ser) and an SH3 domain. Titin, myopalladin, CapZ, and N-WASP interact with nebulin inside the Z-disk. The lower part of the figure shows a detailed view of one super repeat (S18) and the binding sites for actin and tropomyosin. Each super repeat binds seven actin monomers and one tropomyosin dimer. The binding site for tropomyosin is in the third simple repeat in all the super repeats. The numbering of the super repeats and simple repeats is based on the data in Figs. 2 and 7 by Labeit and Kolmerer (1995).

The results by Gohlke et al. (2021) suggest that there are limits for optimal nebulin size within a species, but some variation is tolerated. Mouse models developed by Kiss et al. (2020) support this notion. Homozygous mice with a deletion of super repeats 9–11 or a duplication of the same super repeats developed normally and were indistinguishable from wild-type mice. Furthermore, mice compound heterozygous for the deletion as well as the duplication expressed both length variants of nebulin and were phenotypically normal (Kiss et al., 2020). However, it may be important which of the super repeats that are deleted or duplicated. Laitila et al. (2019) reported significantly stronger actin-binding capacity of the super repeats located in the beginning or at the end of the super-repeat region. Gohlke et al. (2021) show that the super-repeat expansion during evolution has preferentially occurred in the central part of nebulin. Therefore, copy-number variation involving centrally located super repeats may be better tolerated than deletion or duplications of super repeats at the ends.

NEB exons 63–66, encoding super repeat 12 in human, are either included in or excluded from *NEB* transcripts (Donner et al., 2004). Both transcript variants seem to be equally expressed in different muscle types, suggesting the presence of nebulin proteins that differ in length by one super repeat within one muscle type in human (Laitila et al., 2012). This was also the case with the compound heterozygous mice described by Kiss et al. (2020); both length variants of nebulin were readily incorporated into the sarcomeres.

Donner et al. (2004) reported alternative splicing of *NEB* exons 82–105 (i.e., exon 81 spliced to exon 106), but Laitila et al. (2012) could not confirm the results in a more extensive study. Exons 82–105 encode six super repeats, which would be missing from an alternatively spliced isoform. The results reported by Gohlke et al. (2021) underscore the importance of nebulin length within a species. Therefore, it seems unlikely that removing six super repeats is compatible with a fully functional nebulin.

Extensive alternative splicing occurs in the 3′ end of *NEB* that encodes the C-terminal simple repeat domains that anchor nebulin into the Z-disk (Donner et al., 2004; Laitila et al., 2012; Millevoi et al., 1998). In humans, *NEB* exons 167–177 are spliced independently of each other, resulting in a large number of nebulin isoforms with different combinations and numbers of Z-repeats within one muscle type (Donner et al., 2004; Laitila et al., 2012). Similar size variation in this nebulin region has also been observed in rabbit and mouse (Kiss et al., 2020; Donner et al., 2004; Millevoi et al., 1998).

Z-disk width varies in different fiber types. Slow muscle fibers have wider Z-disks and express more nebulin Z-repeats than fast muscle fibers (Kiss et al., 2020; Millevoi et al., 1998). Gohlke et al. (2021) propose that having a large number of nebulin Z-repeats with more interaction sites for nebulin and Z-disk components in wide Z-disks allow the stable anchoring of the thin filament into the Z-disks during the long-lasting contractions of slow muscle fibers. Loss-of-function mutations in the Z-repeat encoding exons are common in patients with all forms of nemaline myopathy, ranging from mild to severe phenotypes. Loss-of function mutations in alternatively spliced exons are expected to reduce the nebulin isoform diversity in skeletal muscle (Lehtokari et al., 2014).

An interesting result that emerged from Gohlke and colleagues' analysis, namely the extraordinary conservation of the last three simple repeat domains encoded by nebulin exons 178–180 across species. No specific function has been ascribed to these domains, but they should be the focus of future research. No disease-causing mutations have been reported in *NEB* exons 178 and 179, but frameshift and nonsense mutations in exon 180 cause severe or intermediate forms of nemaline myopathy (Lehtokari et al., 2014).

In conclusion, by comparing nebulin structure in 53 species, Gohlke et al. (2021) have revealed the importance of the super repeat and Z-repeat regions of nebulin for optimal muscle function. Knowing the number of super repeats within closely related species can help us to assess the limits for normal and disease-causing variation. On the basis of previously published results, deletion or duplication of three super repeats in the central part of nebulin is tolerated (Kiss et al., 2020), but it is not known if this variation is the limit for normal muscle function. Whether the location of the deleted/duplicated super repeats has an impact on thin filament function remains to be determined. The results by Gohlke and colleagues confirm the role of nebulin Z-repeats in the determination of Z-disk width and the importance of wide Z-disks for withstanding long-lasting force in slow muscle.

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