Roles of Nebulin Family Members in the Heart

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The members of the nebulin protein family, including nebulin, nebulinette, LASP-1, LASP-2, and N-RAP, contain various numbers of nebulin repeats and bind to actin, but are otherwise heterogeneous with regard to size, expression pattern, and function. This review focuses on the roles of nebulin family members in the heart. Nebulin is the largest member predominantly expressed in skeletal muscle, where it stretches along the thin filament. In heart, nebulin is detectable only at low levels and its absence has no apparent effects. Nebulette is similar in structure to the nebulin C-terminal Z-line region and specifically expressed in heart. Nebulette gene mutations have been identified in dilated cardiomyopathy patients and transgenic mice overexpressing nebulin mutants partially recapitulate the human pathology. In contrast, nebulin knockout mice show no functional phenotype, but exhibit Z-line widening. LASP-2 is an isoform of nebulin expressed in multiple tissues, including the heart. It is present in the Z-line and intercalated disc and able to bind and cross-link filamentous actin. LASP-1 is similar in structure to LASP-2, but expressed only in non-muscle tissue. N-RAP is present in myofibril precursors during myofibrillogenesis and thought to be involved in myofibril assembly, while it is localized at the intercalated disc in adult heart. Additional *in vivo* models are required to provide further insights into the functions of nebulin family members in the heart. (*Circ J* 2015; **79**: 2081–2087)

Key Words: Dilated cardiomyopathy; N-RAP; Nebulette; Nebulin family

The nebulin family of actin-binding cytoskeletal proteins comprises nebulin, nebulinette, N-RAP (Nebulin-related anchoring protein), LASP-1 (LIM and Src homology 3 (SH3) Protein-1), and LASP-2 (LIM and SH3 Protein-2/LIM-Nebulette), a splice variant of nebulinette.¹ The members of the family contain various numbers of 35-residue nebulin repeats, containing a central conserved SDXXYK consensus motif^{2,3} and named after the founding member nebulin, which includes up to 185 copies of the repeat (Figure).^{4,5} Nebulin is the largest member of the nebulin family, with a molecular weight ranging from 600 to 900 kDa and expressed predominantly in skeletal muscle, where it stretches along the actin thin filament with its C-terminal region anchored in the sarcomeric Z-line and its N-terminal region extending towards the pointed end of the thin filament.^{4,6} For reviews on the sarcomere and the Z-line, see Frank et al⁷ and Sheikh et al.⁸ Within the central part of nebulin (repeats 9–162), the nebulin repeats are organized into “super repeats” of 7 repeats, containing a conserved WLKGIGW motif at the end of the third repeat of each super repeat.⁴ Each nebulin repeat binds to an actin monomer,^{9–12} while the nebulin super repeats correspond to the architecture of the thin filament with 1 tropomyosin/troponin complex for every 7 actin subunits, and interact with troponin T and tropomyosin along the length of the thin fila-

ment.^{4,10,11,13–15} N-RAP is the second largest member of the nebulin family (193–196 kDa in humans) containing 46 nebulin repeats of which 35 are organized into 5 super repeats,^{16,17} while nebulinette (109 kDa),^{3,18} LASP-1 (37 kDa),^{19,20} and LASP-2 (34 kDa)^{21–23} contain only simple nebulin repeats. Nebulette contains up to 23 repeats,³ while LASP-1 and LASP-2 contain only 2 and 3 repeats, respectively. Except for N-RAP the family members also share an SH3 domain at their C-terminus, and N-RAP, LASP-1, and LASP-2 contain an N-terminal LIM domain not present in nebulin or nebulinette. Apart from their similar domain structure and linkage to actin, the members of the nebulin family are quite heterogeneous both with respect to molecular size (34–900 kDa) and expression pattern as well as function, which includes roles in stabilization and scaffolding of cytoskeletal structures, cell migration, and organization of the actin cytoskeleton. In the present review, we will focus on the roles of nebulin family members in the heart.

Nebulin

Mutations in the *nebulin* gene are causative for nemaline myopathy^{24,25} and the function of nebulin in skeletal muscle has been extensively studied both *in vitro* and *in vivo*, revealing its multifunctional role in various processes required for efficient myofibrillar force generation, including (1) regulation

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of thin filament length through prevention of depolymerization and stabilization of actin filaments, allowing filaments to grow beyond the length of nebulin;²⁶⁻³³ (2) promotion of strong actomyosin interactions;^{27,34-36} (3) calcium handling;^{33,37} (4) Z-line alignment and integrity;^{26,32,33,38} and (5) maintenance of sarcomeric structure during muscle use.^{26,39} On the other hand, the potential role of nebulin in the heart is less clear. Nebulin was originally thought to be absent from cardiac muscle,^{4,40,41} where instead the smaller homolog, nebullette, is expressed, providing a possible explanation for the more variable thin filament lengths in cardiac muscle compared with skeletal muscle.^{18,42} However, in subsequent studies nebulin was found to be detectable in the heart at low levels with the same molecular layout as in skeletal muscle.⁴³⁻⁴⁶ A functional role of nebulin in the heart was suggested by an RNA interference (RNAi) study in rat fetal cardiomyocytes where nebulin-deficient cardiomyocytes were found to have dramatically elongated thin filaments, and depolymerization resulted in reassembly of thin filaments to unrestricted lengths, suggesting the hypothesis that nebulin is required to restrict the length of thin filaments.⁴⁷ However, two different nebulin knockout mouse models did not exhibit any cardiac phenotype,^{26,33} and lineage analyses by cross-breeding of heterozygous nebulin knockout mice in which Cre was under the control of the endogenous *nebulin* promoter with Rosa26 reporter mice showed expression of nebulin only in approximately 50% of atrial cardiomyocytes and a small percentage of ventricular cardiomyocytes in the inner layer of the myocardium.²⁶ Consistently, nemaline myopathy caused by nebulin mutations is not commonly associated with cardiac effects.⁴⁸ Thus, it is difficult to interpret the results of the study in rat cardiomyocytes.⁴⁷ Also, the observed increase in thin filament lengths in cardiomyocytes⁴⁷ is in contrast to observations in nebulin knockout mice and skeletal myoblast cultures, where nebulin ablation was found to result in shorter thin filaments.^{26,32,33,49}

Nebulette

Nebulette is specifically expressed in the heart and similar in structure to the C-terminal region of nebulin, containing 23 simple nebulin repeats, a serine-rich linker region, and an SH3 domain (Figure).³ Like the nebulin C-terminus, nebullette is positioned at the sarcomeric Z-line and only extends a short distance along the actin filament, consistent with its smaller size compared with nebulin. Based on this, nebullette has been proposed to have overlapping functions with nebulin in the Z-line, which was recently tested through both in vitro and in vivo studies. Based on immunoelectron microscopy, nebulin and nebullette were originally proposed to extend only partially into the Z-line with their N-termini projecting out of the Z-line along the I-band.^{3,50} However, this model does not fit with the location of binding sites for the Z-line associated proteins CapZ⁴⁹ and desmin⁵¹ within nebulin repeats M160–164, and an alternative model was therefore proposed in which nebulin from adjacent sarcomeres overlap within the Z-line and bind to CapZ at the barbed end of thin filaments from the opposing thin filament, thereby cross-linking neighboring sarcomeres.^{49,52} It seems pragmatic to assume that nebullette is inserted into the Z-line in a similar way, although yeast two-hybrid assays do not suggest its binding to desmin⁵¹ and it is unknown whether nebullette interacts with CapZ. On the other hand, nebullette, but not nebulin, has been found to bind to the actin cross-linking protein filamin C both through its N-terminal acidic region and the nebulin repeats.¹⁴ Both nebulin and nebullette contain a C-terminal SH3 domain preceded by a serine-rich linker region containing phosphorylation sites.⁵³ The SH3 domains of both nebulin and nebullette bind to proline-rich regions within various proteins, including (1) myopalladin, a striated muscle-specific protein associated with the actin cross-linking protein α -actinin in the Z-line;^{54,55} (2) palladin, an ubiquitously expressed homolog of myopalladin associated with α -actinin and filamentous actin (F-actin), playing an important role in the organization of the actin cyto-

skeleton;^{54,56} (3) the Zis1 and PEVK regions of titin, a giant protein stretching half a sarcomere from the Z-line to the M-line;^{33,55,57} (4) zyxin, associated with focal adhesions and actin based structures, involved in cytoskeletal organization;²² (5) neuronal Wiscott-Aldrich syndrome protein (N-WASP/WASL), regulating actin polymerization;⁵³ and (6) the Xin actin-binding repeat-containing (XIRP) family members Xin and XIRP2 with which they interact transiently during development and remodeling.⁵⁸ Furthermore, both the SH3 domain and the nebulin repeats bind with high affinity to α -actinin in the Z-line.¹⁰

Similar to observations in nebulin-deficient skeletal muscle and myoblasts,^{26,27,29,30,32,33} reduced endogenous nebulin levels in chick embryonic cardiomyocytes as a consequence of overexpression of the nebulin serine-rich linker region or SH3 domain, resulted in shorter thin filaments.⁵⁹ Thus, based on these results nebulin, like nebulin, appears to play a role in the stabilization of thin filaments despite its smaller size and localization in the Z-line. Furthermore, reduction of endogenous nebulin resulted in impaired beating of cultured cardiomyocytes as well as loss of tropomyosin and troponin T from the thin filament.^{13,59} Surprisingly, nebulin knockout mice exhibited no functional cardiac phenotype and the localization of tropomyosin, troponin T, and other nebulin binding partners was unaffected.⁶⁰ On the other hand, ultrastructural studies of nebulin knockout mouse hearts showed moderate Z-line widening, including localized regions with extremely widened Z-lines. This is somewhat similar to observations in nebulin knockout mouse skeletal muscle, which exhibits Z-line widening and misalignment reminiscent of nemaline myopathy.^{26,33,38} Thus, the two proteins may play similar roles in determining Z-line width and maintaining Z-line integrity in cardiac and skeletal muscle, respectively. On the other hand, nebulin knockout mice do not show Z-line misalignment,⁶⁰ which in nebulin knockout mice is thought to be caused by the loss of the connection of desmin to the Z-line through its binding to the nebulin M160–164 repeats.^{26,38} This is consistent with the absent binding of nebulin to desmin in yeast two-hybrid studies.⁵¹ Nebulin single repeats in the C-terminal Z-line region are differentially expressed during development and in different muscles, and the number of repeats has been shown to correlate with Z-line width in skeletal muscle.^{3,61} Likewise, the corresponding repeats in nebulin have been found to be absent from the fetal nebulin isoform.³ Thus, the increased Z-line width in cardiac and skeletal muscle of nebulin and nebulin knockout mice, respectively, is likely to be related to the role of their nebulin repeats in determining Z-line width. On the other hand, the SH3 domain does not appear to influence Z-line width, because our recent studies of a mouse model in which the nebulin SH3 domain has been deleted (Neb Δ SH3 mice) showed no Z-line widening or ultrastructural abnormalities in skeletal muscle.³⁹ Also, we found that the nebulin SH3 domain is dispensable for skeletal muscle development and structure and not involved in force generation. Instead, the nebulin SH3 domain appears to be important for the maintenance of Z-line integrity during load, as Neb Δ SH3 mice were more susceptible to eccentric contraction-induced skeletal muscle injury following downhill running.³⁹ Surprisingly, neither knockout of nebulin or deletion of the nebulin SH3 domain affected the localization of nebulin/nebulin SH3 domain binding partners in the Z-line.^{39,60} In particular, the absent effect on the Z-line localization of N-WASP in skeletal muscle was unexpected because N-WASP was previously reported to be targeted to the Z-line through binding to the nebulin/nebulin SH3 domain in response to

insulin-like growth factor 1 (IGF-1)-induced protein kinase B (PKB/Akt) activation [inactivating glycogen synthase kinase 3 β (GSK3 β), which was found to ablate the binding by phosphorylation of nebulin at two sites within the serine-rich linker region], promoting actin nucleation and elongation.⁵³ This finding was supported by in vivo results showing that knockdown of N-WASP in skeletal muscle prevents IGF1-induced actin incorporation into the sarcomere, resulting in reduced fiber cross-sectional area. As neither nebulin knockout mice nor Neb Δ SH3 mice showed any changes in cell size or signs of actin cytoskeletal abnormalities, these findings are likely unrelated to the binding of N-WASP to the nebulin SH3 domain and may instead be related to the role of N-WASP in myoblast cell fusion.⁶² Furthermore, N-WASP is likely to have other interaction partner(s) in the Z-line responsible for its targeting to the Z-line in the absence of nebulin or the nebulin SH3 domain in cardiac and skeletal muscle, respectively.

The first indication that nebulin may be linked to cardiac disease was the association of the N654K polymorphism in the actin-binding motif of nebulin repeat 18 in human *nebulin* (*nebl*) in the homozygous state to idiopathic dilated cardiomyopathy (IDCM) in the Japanese population (7.54% in IDCM patients (n=106) vs. 1.21% in healthy control subjects (n=331); P=0.002).⁶³ More recently, 4 heterozygous missense mutations (K60N, Q128R, G202R, A592E) with different locations within the *nebl* gene were identified in patients with DCM and absent from 300 ethnically matched control subjects.⁶⁴ The 4 variants are all localized in the nebulin repeat region with the K60N, Q128R, G202R, and A592E variants residing in nebulin repeats 1, 3, 5, and 16, respectively (Figure). The Q128R and A592E variants were identified in newborn patients, whereas the patients carrying the K60N and G202R variants developed DCM in adulthood. In particular, a patient carrying the Q128R variant exhibited both DCM and endocardial fibroelastosis associated with severe systolic dysfunction, and underwent heart transplantation at 8 months of age. Immunohistochemistry of the explanted heart showed partial dissociation of nebulin from the Z-line, diffuse myopalladin and α -actinin localization as well as loss of desmin association with the Z-line.⁶⁴ The patient with the A592E variant also carried the M374V missense mutation in α -actinin. Overexpression of GFP-tagged wild-type (WT) and mutant nebulin in H9C2 cardiac cells showed targeting of both WT and mutant nebulin to the perinuclear region. However, while cyclic mechanical stretch resulted in colocalization of WT nebulin with F-actin filaments and assembly into maturing Z-lines throughout the cytoplasm, the localization of the Q128R and A592E nebulin mutants remained diffuse and mainly perinuclear,⁶⁴ suggesting a role of nebulin in mechanosensing. The effect of the *nebl* mutations was also assessed in vivo through analysis of transgenic mice.⁶⁴ Transgenic overexpression of the K60N and Q128R mutations in the mice resulted in embryonic lethality associated with severe cardiac abnormalities, and the founders exhibited cardiac dilation and abnormal lysosomes and mitochondria, leading to death at 1 year of age. Additionally, lipid accumulation and localized disruption of intercalated discs were observed in the Q128R founder. G202R and A592E transgenic mice survived to adulthood, but developed cardiac dilation, systolic dysfunction, and mitochondrial abnormalities at 6 months of age.⁶⁴ Cardiac magnetic resonance imaging at 3 months of age before the onset of the DCM phenotype showed geometric remodeling and reduced torsion in G202R transgenic mice, while A592E transgenic mice exhibited enhanced twist and untwisting rate.⁶⁵ Furthermore, ex vivo experiments on isolated adult cardio-

myocytes from the same mice showed an increased baseline calcium decay rate in the G202R transgenic mice, suggesting reduced myofilament calcium sensitivity, whereas cardiomyocytes from A592E transgenic mice were shorter and exhibited blunted calcium decay in response to isoproterenol-mediated stress.⁶⁵ Ultrastructural analyses of G202R transgenic hearts compared with controls showed increased sarcomere length and I-band width, enlarged t-tubules as well as desmosomal separation at the intercalated disc.⁶⁵ Similar intercalated disc abnormalities, although not as severe, were observed in hearts from A592E transgenic mice. At the molecular level, downregulation and disruption of troponin I, cardiac troponin T, and tropomyosin as well as cleavage of filamin C and myopalladin were found in the hearts of 6-month-old G202R transgenic mice compared with control mice.⁶⁴ This was preceded by downregulation of α -actinin and connexin 43, lateralization of connexin 43 as well as increased phosphorylation of cardiac troponin I at 3 months of age, possibly explaining the enhanced ex vivo calcium decay.⁶⁵ Transgenic overexpression of the A592E mutation resulted in downregulation of the Z-line proteins ALP, Cypher/ZASP, α -actinin, myopalladin at 6 months of age, corresponding to the localization of the mutation within the nebulin repeats in the C-terminal Z-line portion of nebulin.⁶⁴ In addition, downregulation of desmin and lateralization of connexin 43 were found at 3 months of age.⁶⁵ Altogether, the findings in transgenic mice overexpressing different nebulin variants are consistent with nebulin mutations being causative for DCM.

The observed phenotypes of the transgenic mice suggest a role of nebulin in various processes in the heart, although it is unclear which of the observed abnormalities are directly caused by the mutations and which are indirect secondary changes as a consequence of the resulting cardiomyopathy. Also, one should be cautious with the interpretation of results obtained from transgenic mice as the overexpression of nebulin mutants may have possible nonspecific effects. It would be interesting to study knock-in mice carrying the mutations to more faithfully mimic the situation in human patients. The weak phenotype of nebulin knockout mice is somewhat surprising, given the results obtained both from in vitro studies and analyses of transgenic mice. A possible explanation could be that alternative mechanisms can compensate for the loss of nebulin, while human nebulin mutations have dominant gain-of-function effects as has been described in other cases.^{66,67} This would also explain why no DCM patients have been reported with nebulin loss-of-function mutations resulting in absent nebulin expression. In conclusion, although recent in vivo studies have provided new insights into the role of nebulin in the heart, conflicting results make it difficult to interpret the data, and studies of more physiological models such as knock-in mice will be required to further dissect the molecular mechanisms leading from *nebl* mutations to cardiomyopathy.

LASP-2 (LIM and SH3 Protein-2)

LASP-2, also called LIM-nebulin, is a shorter splice variant of nebulin transcribed from a distinct promoter upstream of the *nebl* promoter.^{21,22} *Lasp2* contains 7 exons, of which 4 are upstream of *nebl* and 3 are shared with *nebl*, corresponding to exons 24, 27, and 28 at the 3' end of *nebl*. This gives rise to a protein containing a LIM domain, 3 nebulin repeats, a linker region, and an SH3 domain (Figure). The region identical to nebulin starts from the middle of the second nebulin repeat, but half of the nebulin linker region, corresponding to *nebl* exons 25 and 26, is absent from LASP-2.^{22,68} Unlike nebulin,

which is expressed exclusively in the heart, LASP-2 is expressed in multiple tissues, in particular brain, lung, and kidney.^{22,23} Furthermore, LASP-2 is expressed at lower levels in the heart and skeletal muscle, where it is present in nascent Z-lines of premyofibrils as well as in Z-lines, intercalated discs, and focal adhesions of mature cardiomyocytes.^{22,69,70} Overexpression of GFP-tagged LASP-2 deletion constructs in chick cardiomyocytes revealed that the C-terminal region, containing the linker and SH3 domain, is required for the targeting of LASP-2 to the Z-line.⁷⁰ Like nebulin, LASP-2 binds to α -actinin⁷⁰ and zyxin²² and based on the identify of its C-terminal region to nebulin, likely also the other interaction partners of the nebulin SH3 domain. Furthermore, LASP-2 was found to directly interact with F-actin,²³ as well as to bundle F-actin, in a dose-dependent manner,⁷⁰ suggesting its role in organizing the actin cytoskeleton. It remains to be determined whether LASP-2 cross-links actin through dimerization or whether it contains two F-actin binding sites. In fibroblasts, overexpression of LASP-2 was found to increase the rates of attachment and spreading.⁷¹ Based on these findings, LASP-2 has been suggested to play a role as a molecular scaffold involved in myofilament assembly and stabilization.^{70,72} However, this remains to be determined in vivo. In nebulin knockout mice, which did not exhibit a functional phenotype, we found a 23-fold downregulation of *lasp2* transcript levels, suggesting that LASP2 is not critical for normal cardiac function.⁶⁰ However, this would have to be verified in a mouse model with complete absence of LASP-2.

LASP-1 (LIM and SH3 Protein-1)

LASP-1 has a similar domain structure as LASP-2, except that it contains only 2 nebulin repeats and a distinct linker region (Figure).²³ LASP-1 is expressed in various non-muscle tissues and localized at sites of actin assembly, such as focal adhesions, lamellipodia, and filopodia,^{19,20,72-74} where it is involved in cell signaling, proliferation, migration, adhesion, and survival (reviewed by Grunewald and Butt⁷²). However, because LASP-1 is absent from the heart, it will not be further discussed.

N-RAP (Nebulin-Related Anchoring Protein)

N-RAP is specifically expressed in heart and skeletal muscle and composed of a LIM domain followed by 11 simple repeats and 5 super repeats, thus containing a total of 46 nebulin repeats (Figure).^{16,17,75} In heart, the N-RAP-c isoform is expressed, while in skeletal muscle 2 different isoforms are expressed (N-RAP-s and in lower amounts N-RAP-c).^{17,76} The N-RAP-c isoform is missing nebulin simple repeat 9 compared with the N-RAP-s isoform. N-RAP is expressed from embryonic day 10.5, initially in myofibril precursors and subsequently within the Z-line and M-line of maturing myofibrils.^{77,78} However, it is absent from mature myofibrils and is expressed exclusively at the intercalated disc in the adult heart and myotendinous junctions of skeletal muscle. N-RAP has been reported to interact with several proteins through its different regions. Its N-terminal LIM domain was found to interact with α -actinin,⁷⁹ as well as with talin,⁸⁰ which links integrin to the actin cytoskeleton at the cell surface.⁸¹ The nebulin simple repeats can bind to actin,⁸⁰ α -actinin,⁸² the Z-line protein muscle LIM protein (MLP),⁸³ as well as Kelch-like family member 41 (KLHL41/Krp1),⁸² a scaffolding protein thought to promote the lateral fusion of nascent myofibrils into mature myofibrils.⁸⁴ The super repeats within the C-terminal part of N-RAP interact with filamin C,⁸² as well as with vinculin,⁸⁰ which binds to talin at adherens junctions and focal adhesions.⁸⁵

Overexpression of GFP-tagged N-RAP truncation constructs in cultured chick embryonic cardiomyocytes showed different targeting of the individual regions.⁸⁶ The N-RAP LIM domain targeted the cell membrane, whereas the N-RAP simple repeat region and super repeat region targeted premyofibrils. Furthermore, all 3 regions of N-RAP could inhibit the formation of mature myofibrils, consistent with an essential role of N-RAP in myofibril assembly. Additional studies based on deletion mutants revealed that the simple repeat region is required for actin incorporation into the Z-line, while the super repeat region is critical for actin organization during myofibril assembly.⁸⁷ These findings were further supported by N-RAP sRNAi studies in mouse embryonic cardiomyocytes, where knockdown of N-RAP was found to be associated with inhibition of α -actinin assembly into Z-lines and myofibril assembly.⁸⁸ Subsequent time-lapse confocal microscopy studies of cultured embryonic chick cardiomyocytes showed that during myofibrillogenesis, N-RAP is incorporated into premyofibrillar actin filament structures, whereafter α -actinin is recruited and assembled into I-Z-I bodies.⁸⁹ As the myofibril matures, the dynamic exchange of N-RAP increases, leading to the complete removal of N-RAP from mature sarcomeres. Thus, several lines of evidence suggest a transient role of N-RAP as a scaffold for myofibril assembly during cardiomyocyte development. On the other hand, in adult heart N-RAP is localized at the intercalated disc, where it is thought to link terminal actin filaments to membrane complexes, potentially playing a role in force transmission from the sarcomere to the extracellular matrix.^{16,78,80} As yet, although cell cultures studies suggest a role of N-RAP in various important processes both during cardiac development and in the adult, the role of N-RAP in vivo remains unknown and there is no known link between N-RAP mutations and human cardiomyopathies. On the other hand, N-RAP was found to be upregulated in different mouse models of DCM before the development of the pathology and the appearance of other alterations.^{83,90} This is thought to be a compensatory mechanism to reinforce the connection between the myofibrils and the membrane at the intercalated disc, although more studies are required to determine this. Future studies are required to elucidate the precise role of N-RAP in vivo.

Future Perspectives

Despite the structural similarities among the nebulin family members and their connection to actin, each of the members has different expression patterns and cellular localizations and appears to have rather distinct functional roles. The recent generation and analysis of knockout, knock-in, and/or transgenic mice for nebulin and nebulette has greatly improved the understanding of their functional role in vivo. However, in the case of nebulette, the absent functional phenotype of nebulette knockout mice as opposed to the effect of nebulette knockdown in cardiomyocytes and severe phenotypes observed in transgenic mice overexpressing nebulette mutants, makes it difficult to interpret the findings. In knockout mice, alternative mechanisms may compensate for the absence of nebulette, while in transgenic mice the nonphysiologic overexpression of nebulette may have nonspecific effects. Therefore, more studies are required to better understand the mechanisms leading from *nebl* mutations to DCM,^{63,64} which is essential for the future development of targeted therapies. The ideal would be to generate nebulette knock-in mice carrying mutations corresponding to the *nebl* mutations identified in human DCM patients, which would most reliably mimic the human condi-

tion and could be used both for the elucidation of disease pathways and for the testing of potential therapies. A complementary approach would be to generate and analyze human-based cardiomyocyte models of the nebulette mutants using patient-derived induced pluripotent stem cells or gene-edited human embryonic stem cells.⁹¹

Cell cultures studies have suggested important roles of N-RAP and LASP-2 in the heart. However, studies in mouse models would be required to determine whether the in vitro findings correspond to functional phenotypes in vivo. Also, as of now, nebulette is the only member with a known link to cardiac disease. Therefore, based on their putative functions in the heart, it would be relevant to take into consideration also N-RAP and LASP-2 as candidate genes when screening cardiomyopathy patients for gene mutations. Thus, although significant progress has been made in understanding the roles of the nebulin family members in the heart, many unanswered questions remain and additional in vivo models are required to provide further insights into their function in the heart.

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Disclosures

The authors declare no conflicts of interest.

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