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## Nebulette Mutations are Associated with Dilated Cardiomyopathy and Endocardial Fibroelastosis

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### Abstract

**Objectives**—Four variants (K60N, Q128R, G202R and A592E) in the nebulette gene (*NEBL*) were identified in patients with dilated cardiomyopathy (DCM) and endocardial fibroelastosis (EFE). We sought to determine if these mutations cause cardiomyopathy.

**Background**—Nebulette aligns thin filaments and connects them with the myocardial Z-disk.

**Methods**—We produced transgenic mice with cardiac-restricted over-expression of human wild-type (WT) or mutant nebulette. Chimera and transgenic mice were examined at 4, 6 and 12 months of age by echocardiography and cardiac MRI. The hearts from embryos and adult mice were assessed by histopathologic, immunohistochemical, ultrastructural and protein analyses. Rat H9C2 cardiomyoblasts with transient expression of nebulette underwent cyclic mechanical strain.

**Results**—We identified lethal cardiac structural abnormalities in mutant embryonic hearts (K60N and Q128R). Founders of the mutant mice lines developed DCM with severe heart failure. An irregular localization pattern for nebulette and impaired desmin expression was noted in the proband and chimera Q128R mice. Mutant G202R and A592E mice exhibited left ventricular dilation and impaired cardiac function accompanied with the specific changes in I-band or Z-disk proteins by 6 months of age, respectively. The mutations modulated distribution of nebulette in the sarcomere and the Z-disks during stretch of H9C2 cells.

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No conflict of interest

**Conclusions**—*NEBL* is a new susceptibility gene for EFE and DCM. Different mutations in nebullette trigger specific mechanisms converging to a common pathological cascade leading to EFE and DCM.

### Keywords

nebullette; dilated cardiomyopathy; endocardial fibroelastosis; Z-disk

## INTRODUCTION

Dilated cardiomyopathy (DCM) is a myocardial disease characterized by left ventricular (LV) dilation and dysfunction and is believed to be of genetic origin in approximately 35% of all DCM cases.<sup>1</sup> Endocardial fibroelastosis (EFE), characterized by diffuse endocardial thickening due to proliferation of fibrous and elastic tissue, is a rare cardiac disorder with poor prognosis that occurs in infants and young children.<sup>2</sup> Although virus-induced fetal myocarditis, or X-linked Barth or autosomal recessive inherited genes have been reported to be causative for EFE, most cases are of unknown etiology.<sup>3</sup> Various molecular mechanisms are involved in the development of these disorders due to the overlapping functions of cytoskeletal proteins beginning from their involvement in sarcomeric structure, to their functions in cardiac differentiation, myofibrillogenesis and transcriptional regulation.<sup>4,5</sup>

The nebulin protein family is a group of actin-binding proteins whose universal feature is the presence of “nebulin-repeats” which bind a single actin subunit and is involved in tropomyosin-troponin assembly.<sup>6</sup> Moreover, expression of the specific nebulin isoforms with specific numbers of “nebulin-repeats” correlate thin filament length in a striated muscle-specific manner.<sup>7</sup> The only cardiac-specific member in the nebulin family is nebullette, a 107kDa protein, that appears to be involved in early events of myogenesis and myofibrillar organization in cardiac muscle.<sup>8,9</sup> The “nebulin-repeat”- domain of nebullette tethers it in the I-band, while the C-terminus is embedded inside the Z-line lattice, interacting with  $\alpha$ -actinin2,<sup>9</sup> the PEVK-domain of titin,<sup>7</sup> myopalladin,<sup>10</sup> desmin,<sup>11</sup> and filamin C.<sup>12</sup> The significant increase in the affinity of tropomyosin-troponin for F-actin by nebullette was observed previously.<sup>13</sup> Involvement in all these protein-protein interactions suggests the functional importance of nebullette.

Nebullette polymorphism (Asn654Lys) in the actin-binding motif was associated with non-familial DCM.<sup>14</sup> To determine if the nebullette gene (*NEBL*) plays a role in the genetic and cellular basis of cardiac disorders, we screened the nebullette gene in patients with cardiomyopathies. Transgenic (Tg) mice with cardiac-specific over-expression of nebullette were created and functional analysis was pursued to explore the molecular mechanisms of the development and regulation of the clinical phenotypes seen in humans. We investigated the effect of the mutations by applying cyclic mechanical strain in transient nebullette-expressing H9C2 cardiomyoblasts.

## METHODS

### Mutational Analysis

We assessed genomic DNA from 260 patients with DCM recruited after informed consent in the NIH-funded Pediatric Cardiomyopathy Specimen Registry (PCMR) repository (PCSR; R01 HL087000-01A1, JAT). Cytoskeletal and sarcomeric genes were investigated for mutations as previously described.<sup>15</sup> We screened more than 20 candidate genes that met criteria for disease-causation. For each variant identified, available family members, as well as 300 unrelated ethnic matched individuals, were screened.

## Human Heart Immunohistochemistry

Paraffin-embedded sample of explanted heart from patient S84-5162 carrying the Q128R variant was available. As control, heart tissue was obtained from normal donor subject after informed consent. The samples were dewaxed and stained with nebullette, desmin, myopalladin and  $\alpha$ -actinin2 antibodies using standard techniques.<sup>15</sup>

## Generation and Characterization of $\alpha$ -MyHC-Nebulette Transgenic Embryos and Mice

Full-length 3.1 kb human nebullette wild-type (WT) cDNA (#NM\_006393) was amplified by reverse transcription-polymerase chain reaction (RT-PCR) from human heart RNA (Ambion) and ligated into vector containing murine  $\alpha$ -myosin heavy chain ( $\alpha$ -MyHC) gene promoter with downstream human growth hormone (hGH) gene (Dr. Robbins, University of Cincinnati, OH). Four mutants, K60N, Q128R, G202R, and A592E were created using the QuikChange Site-directed Mutagenesis Kits (Stratagene, La Jolla, CA). The Tg mice were created according to the Baylor College of Medicine animal regulations. Offspring were genotyped using PCR of genomic DNA. Transcription and protein expression levels were confirmed by real-time quantitative RT-PCR and Western blot analysis. Embryos were studied at E7.5, 9.5, 12.5 and 16.5 stages (N>3).

To find the latent intolerance to exercise, Tg and age- and gender-matched non-transgenic (non-Tg) mice underwent graded treadmill testing at four and six months of age (N $\geq$ 5). The treadmill was set (Exer-6M, Columbus Instruments, OH) at a slope of 15° with a starting speed of 16 m/min. Speed was increased by 2 m/min every 5 min until the mouse exhibited signs of exhaustion, and the total running distance was recorded. Echocardiography and cardiac magnetic resonance imaging (cMRI) were performed as described previously (N=5).<sup>15</sup>

## Assessment of Transgenic Mouse Hearts

The mouse hearts were studied by histology, immunohistochemistry, Masson trichrome or Transmission Electron Microscope (TEM) as described previously.<sup>15</sup> Antibodies against nebullette and myopalladin, 11  $\alpha$ -actinin (Upstate, Chicago, IL), desmin (Abcam, Cambridge, MA), Z-band alternatively spliced PDZ-motif protein (ZASP; Dr. Georgine Faulkner, ICGEB, Trieste, Italy), tropomyosin, cardiac troponin I (cTnI), cardiac troponin T (cTnT) and filamin C (Santa Cruz Biotechnology) were diluted as recommended by the manufacturer. Multiple sections from at least 5 mice per group were analyzed.

The level of protein expression was quantified using Western blotting and relative intensity (RI) was carried out using a Kodak Image system. Beta-actin was chosen as a control, since nebullette only interacts with the muscle elements ( $\alpha$ - and F-actin) and not the nonmuscle elements ( $\beta$ -actin) in cardiac background.<sup>16</sup>

## Nebulette Expression in H9C2 Cells and Differentiation Under Cyclic Mechanical Stretch

Rat H9C2 cardiomyoblasts were used to study the effects of nebullette mutations on myofibrillar assembly during differentiation under mechanical stress. The human WT and mutant nebullette cDNA ligated into a pcDNA3.1NT-GFP-TOPO vector (Invitrogen, Carlsbad, CA) were prepared and transfected into H9C2 cells plated on BioFlex plates using an Effectine (Qiagen, Valencia, CA). After 48 hours, cells were cultured in DMEM containing 2% of heat-inactivated horse serum, and subjected to 10% one-hour/day cyclic mechanical stretch for 3 days, as previously described.<sup>17</sup> The H9C2 cells were fixed, then stained with anti-GFP followed by labeling with Alexa Fluor Phalloidin-594 (Invitrogen, Carlsbad, CA) and visualized using an Olympus confocal microscopy.

## Statistical Analysis

Statistical analysis was performed using the Student's *t* and ANOVA tests. Data were reported as the mean with standard error mean (SEM). Values of  $P < 0.05$  were considered significant.

## RESULTS

### Mutations and Clinical Correlation

We identified four variants in *NEBL*: c.180G>C (exon 3; p.K60N), c.383A>G (exon 5; p.Q128R), c.604G>A (exon 7; p.G202R) and c.1775C>A (exon 17; p.A592E) (Fig. 1A). K60N has previously been reported in the dbSNP database (rs41277374), but neither the population frequency nor effect on function has been reported. Variants K60N, Q128R and G202R are located in the “nebulin-repeat”-domain, which binds to F-actin and the tropomyosin-troponin complex, while A592E is located in the Z-disk binding region. None of these variants were identified in 300 race- and ethnicity- matched control subjects (600 alleles). Family analysis was available only for K60N variant, which confirmed *de-novo* origin. The clinical features of the affected subjects hosting these variants were heterogeneous. The newborn patient (S84-5162) carrying the Q128R variant had DCM and EFE with a clinical phenotype demonstrating endocardial thickening, deposition of elastic tissue and collagen (Fig. 3A) along with a severely dilated LV and severely depressed systolic function by echocardiography based on fractional shortening (FS) of 10%. The K60N and G202R variants, however, were identified in patients who developed clinical manifestations of DCM in adulthood. The patient carrying the A592E variant displayed the clinical features of DCM as a newborn; this patient also carried a digenic mutation in the  $\alpha$ -actinin2 (*ACTN2*) gene (Fig. 1B).

### Mutations, K60N and Q128R, Cause Embryonic Lethality and DCM in Chimera Mice

WT, G202R, and A592E mice showed 45%, 40% and 35% transmission frequencies, respectively, however, we were unable to identify positive livable offspring from K60N and Q128R founders. No mutant embryos were harvested from the K60N line at E7.5 or later, while mutant Q128R embryos were harvested up to age E12.5. Since K60N Tg embryos numbers were 50–70% less than expected, the possibility of embryonic lethality at an earlier stage of development could not be excluded. Histology of the Q128R embryonic hearts revealed severe cardiac changes including biventricular dilation (Fig. 2B-b, X2) and wall thinning (X40). Such cardiac structural changes are potential causes of embryonic death.

Founders of the K60N and Q128R lines died at 1 year of age with severe heart failure. Enlargement of the heart was evident (Fig. 2C). cMRI of hearts revealed mild increase in LV mass but remarkable dilation in mutants. Ejection fraction (EF) was decreased ( $p < 0.05$ ) in compared to control mice (Fig. 2D). TEM of the K60N and Q128R hearts revealed enlarged and deformed mitochondria (Fig. 2E, asterisks) as well as abnormal lysosomes and mitochondrial remnants (arrowheads). Localized intercalated disc disruption and accumulation of lipids in cardiomyocytes were noted in Q128R (arrows).

Comparative immunohistochemistry of human (control and S84-5162) and transgenic mouse hearts (non-Tg and Q128R) revealed that nebulin was seen disassociated with Z-lines, probably, depositing as aggregates throughout the sarcoplasm in the S84-5162 heart (Fig. 3A-e, arrowheads); the Q128R mutant mice exhibited punctate foci and more diffuse fluorescence for nebulin than the controls (Fig. 3B-e, arrows); myopalladin (f) and  $\alpha$ -actinin2 (g) were smeared in S84-5162 (asterisks), whereas loss of desmin association with Z-disks in both S84-5162 and Q128R-Tg hearts was notable (h, double arrows). In Q128R-Tg mouse hearts, (Fig. 3B-e), myopalladin (f), and  $\alpha$ -actinin2 (g) were not altered.

## Transgenic G202R and A592E Mice Exhibit Signs of DCM

WT, G202R and A592E Tg mice were born and initially developed normally. By 6 months of age, marked reduction in the tolerance against acute stress was identified in G202R and A592E mice ( $p < 0.01$ ). This was evidenced by decreased running distances ( $270.7 \pm 89.0$  m and  $273.4 \pm 55.77$  m, respectively) compared to non-Tg ( $398.2 \pm 21.54$ ) and WT-Tg mice ( $358.0 \pm 13.00$ ) (Fig. 4A, upper panel). Decreased FS in mutant mice was noted:  $50.37 \pm 0.7\%$  and  $55.24 \pm 1.5\%$  in non-Tg and WT-Tg, respectively, *versus*  $43.36 \pm 3.1\%$  in G202R-Tg and  $43.14 \pm 3.1\%$  in A592E-Tg ( $p < 0.05$ ). Significant dilation of the LV, as evidenced by increased LV end-systolic dimensions (LVESD) in G202R-Tg ( $1.66 \pm 0.04$  mm) and A592E-Tg ( $1.67 \pm 0.02$  mm) compared to non-Tg ( $1.47 \pm 0.04$  mm) and WT-Tg ( $1.51 \pm 0.07$  mm) mice without changes in LV end-diastolic dimensions (LVEDD) were detected (Fig. 4, lower panels). The LV mass-body weight ratio was unchanged compared to normal (data not shown).

Cardiac enlargement due to LV dilation (Fig. 4B, arrows) along with myocyte disarray and interstitial cell infiltration was evident in the hearts of the G202R and A592E mice (Fig. 4C, c and d, respectively). TEM revealed focal separation of intercalated disks (Fig. 4D, arrow), increased residual body liposome and mild variation in mitochondrial shape in the G202R mutants (asterisks), while the A592E-Tg hearts had mild accumulation of lipids (arrowheads) and abnormalities of mitochondrial shape and size. Disorganization and disarray of over-expressed nebulin in G202R-Tg and A592E-Tg hearts were detected by immunochemical analysis (Fig. 5A, asterisks). Protein analysis (Fig. 5B) revealed over-expressed nebulin in Tg mouse hearts (RI=1.9 to 3.4) compared to non-Tg animals (RI=1.0).

## Effects of Nebulette Mutations on I-band and Z-disk Composition

Proteins expressed mainly in the I-band, such as cTnI (RI=0.5), cTnT (RI=0.3), and tropomyosin (RI=0.2) were significantly downregulated (Fig. 6B, arrows) and disrupted in G202R-Tg mutants (Fig. 6A, i-l, asterisks). The mobility of filamin C by Western blot analysis was abnormal suggesting cleavage (Fig. 6B, arrowhead). In contrast, Z-disk-associated proteins, such as  $\alpha$ -actinin-associated LIM protein (ALP) (RI=0.2), ZASP (RI=0.7),  $\alpha$ -actinin2 (RI=0.4), and myopalladin (RI=0.3) appeared to be downregulated (Fig. 7B, arrows) and more disrupted (Fig. 7A, m-p, asterisks) in the A592E-Tg hearts. Localization of myopalladin in Z-disks in the G202R- and A592E-Tg mouse hearts appeared normal; however, Western blotting revealed a second lower molecular weight band in the G202R mice, suggesting cleavage (Fig. 7B, double arrows). We also noted that ALP expression was downregulated in WT (RI=0.2, arrowhead) mouse hearts compared to non-Tg (RI=1.0) and G202R-Tg mice.

## Abnormal Distribution of Mutated Nebulette Under Cyclic Mechanical Stretch

We have chosen rat H9C2 embryonic cardiomyoblasts (ATCC, Manassas, VA) as a model of cardiomyocytes to investigate nebulin expression pattern during differentiation. Transfection efficiency of WT and mutant nebulin-GFP in H9C2 cells was approximately 10%. Perinuclear cytoplasmic localization of nebulin-GFP was observed in differentiating WT and mutant cells (data not shown).

Further, we hypothesized whether nebulin can act as a stretch-induced mechanosensor in cardiomyoblasts.<sup>16</sup> To test this hypothesis as well as to investigate expression of mutated nebulin in differentiating cardiomyoblasts during mechanical stretch we performed cyclic mechanical strain of H9C2 cardiomyoblasts. On day 1 of cyclic mechanical stretch, both WT and mutant nebulin-GFP were localized uniformly to the perinuclear region in differentiating cells (Fig. 8A, asterisks). By day 3, WT nebulin was distributed throughout the cytoplasm along with F-actin filaments reaching the periphery of cells (Fig. 8B, d-f, arrows) and tended to form lines, presumably, assembling into the maturing Z-lines (higher resolution, right corner). In contrast, despite of normally distributed F-actin filaments extended to periphery of

sarcomere (Fig.8B, g–l, arrowheads), sustained perinuclear nebullette-GFP localization in all mutants was evident (asterisks, Q128R and A592E mutants representing I band or Z-disk mutations, respectively, are shown).

## DISCUSSION

Over the past decade, variants in a many genes encoding structural proteins have been reported in patients with cardiomyopathies.<sup>1</sup> In particular, genes encoding cytoskeletal and sarcomeric proteins, including Z-disk proteins, have been found to be the “final common pathway” of DCM.<sup>18</sup> The events leading to EFE are not well understood, but could result from an environmental insult, such as a virus or chemical exposure, in a genetically susceptible individual.<sup>2,3</sup> The goal of this study was to uncover the molecular basis of cardiac dysfunction in subjects with DCM. We focused in the sarcomere and Z-disk and identified *NEBL* gene variants (K60N, Q128R, G202R and A592E) in patients with DCM and EFE. We demonstrated that the mutations lead to a variety of cardiac phenotypes and levels of severity *in vivo*, from embryonic lethality due to a lethal cardiac abnormality (K60N and Q128R), to DCM with clinical signs of impairment in cardiac function (G202R and A592E) as seen in humans recapitulating the human disease phenotype, with or without EFE. Interestingly, G202R and A592E mutations were found to result in cardiac dysfunction despite causing different ultrastructural changes. I-band proteins were significantly disrupted in G202R mouse hearts, whereas Z-disk associated proteins appeared to be more disrupted in the A592E hearts. These facts led us to hypothesize that *NEBL* mutations have different effects on protein function depending on the location of the mutation in specific portions of the “nebulin-repeats”.

Nebulette is a component of the early dense body structures involved in myofibrillogenesis of developing cardiac muscle.<sup>8,9</sup> We demonstrated that cyclic mechanical strain appeared to be the factor which initiated the distribution of nebullette through the sarcomere and, probably, could be important determinant in stimulating formation of Z-lines in differentiating H9C2 cardiomyoblasts. To support this, we found that nebullette mutations did not affect nebullette distribution and differentiation of cardiomyoblasts. When H9C2 cells subjected to cyclic strain, we demonstrated that nebullette mutations perturbed the co-distribution of nebullette-GFP throughout the sarcomere with the actin filaments and delayed expression of nebullette-GFP in the maturing Z-lines under cyclic mechanical strain. Taken together, these data support our hypothesis that nebullette may function as a mechanosensor and may have signaling properties in stretch-induced activation of specific mechanotransduction pathways in cardiomyoblasts during myogenesis. These data also support our *in vivo* results that demonstrate that expression of mutant Q128R nebullette leads to embryonic lethality due to severe structural abnormalities in the embryonic heart. Moreover, an abnormal expression pattern for nebullette, myopalladin and desmin was discovered in the heart of patient carrying the Q128R mutation. It is possible that interruption of these proteins could cause the specific phenotype of EFE in humans. We speculate that nebullette-desmin chain transmits information from the Z-disks to the intermediate filaments maintaining the structural and functional integrity of myocytes.<sup>12</sup> Based on its timing during embryogenesis, as well as its functional effects on key binding partners, it appears likely that Q128R mutation alters cardiac development, specifically affecting myofibrillogenesis through the early nebullette-actin interactions, later nebullette-desmin association. We assume that disruption in nebullette-actin co-distribution and/or delay in nebullette expression in the maturing Z-disks at the earliest stages of myogenesis may be present in K60N mice.

In contrast to K60N and Q128R, the effect of the G202R mutation, which resulted in downregulated tropomyosin and troponins in the mutant mice, suggests that this mutation may cause changes in force generation in mature cardiomyocytes.<sup>19</sup> Our data was consistent with Bonzo *et al* reported that overexpression of nebullette fragments was associated with loss of

endogenous nebulin tropomyosin and troponins accompanied with decrease in thin filament length and concomitant loss of F-actin, but not overall actin staining.<sup>7</sup> In addition, “nebulin-repeats” was found to contribute to the assembly and the allosteric property of the striated muscle thin filament.<sup>14</sup> Although, we did not measure actin-filaments length in G202R mice, it is likelihood dysregulated tropomyosin, troponins and filamin C may cause development of LV dilation.

On the other hand, the A592E mutation led to cytoarchitectural changes with different patterns in expression of Z-disk proteins. It is believed that myofibrillar functional integrity is regulated by the Z-disk, network linking the sarcomere, cytoskeleton, sarcoplasmic reticulum and sarcolemma.<sup>4</sup> Dysregulation of Z-disk proteins in A592E-Tg hearts suggests that A592E may disturb force transmission. Mature cardiomyocytes respond to mechanical stretch with an immediate increase in contractility as well as long-term changes in gene expression resulting in myocyte hypertrophy.<sup>5</sup> Orchestrated cross-talking between multiple independent and mechanosensitive signaling pathways such as MAPK, PI3K/Akt, Ras, JAK/STAT and Ca<sup>2+</sup> signaling determines the final phenotype of the cardiomyopathies. The desmin-based unit is one of the major contributors in mechanotransduction<sup>12,16</sup> and it appears that I-band-nebulin-Z-disk connection may play a crucial role in transmitting information from the Z-disks to the intermediate filaments.

We identified novel nebulin mutations (K60N, Q128R, G202R and A592E) in patients with DCM, EFE and cardiac failure. We propose that *NEBL* is a new susceptibility gene for EFE and DCM and these mutations lead to cardiac remodeling and dysfunction. In summary, we suggest that disruption of the proposed signaling pathways leads to unfavorable cardiac remodeling and consequently cardiac dysfunction. Furthermore, mutations in certain domains of nebulin will alter the function of its interacting proteins in distinct, domain-defined manner and ultimately will result in activation and/or disruption of signaling cascades, culminating in structural remodeling of cardiomyocytes.

## Abbreviations

<b>EFE</b>	endocardial fibroelastosis
<b>DCM</b>	dilated cardiomyopathy
<b>PCMR</b>	Pediatric Cardiomyopathy Registry

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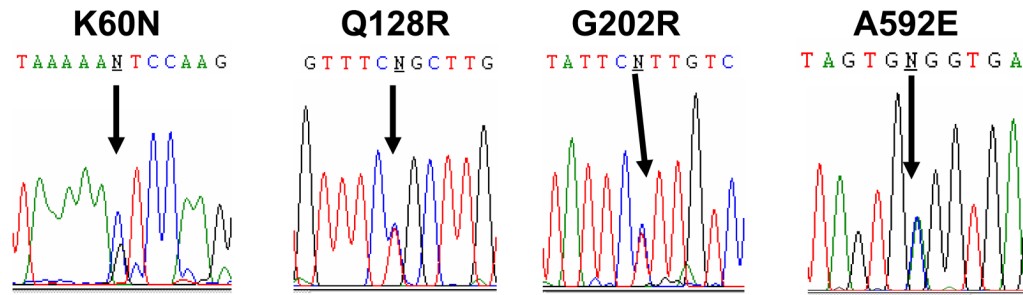
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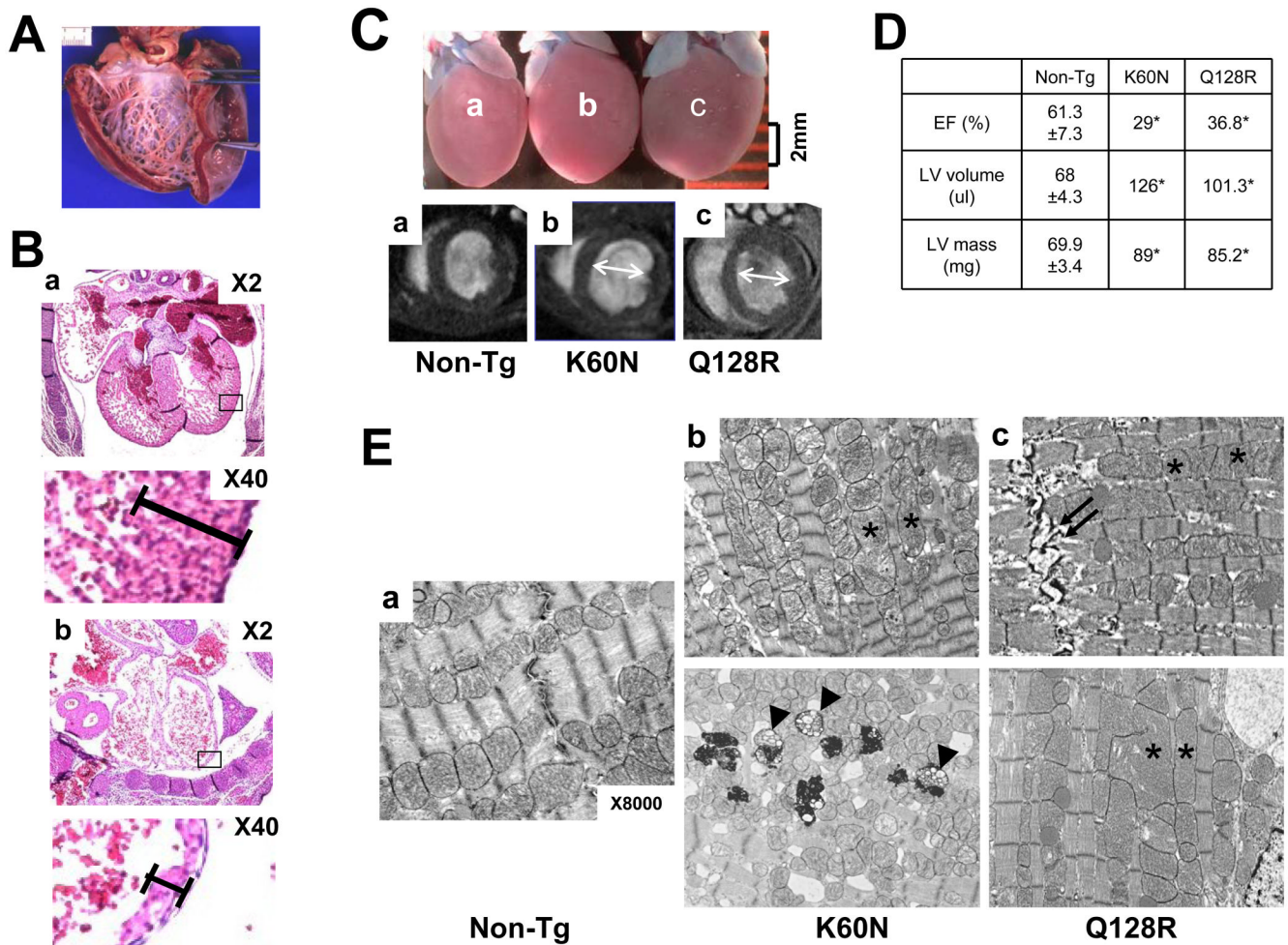


ID	Mutation	Localiza- tion	Gen- der	Age at onset	Clinical diagnosis	FH of CM	NYHA	LAD (mm)	LVDd (mm)	%FS	Other remarks
H424214	K60N	Actin- binding domain	M	55 y	DCM	No	II	64	84	23	ACE inhibitor, diuretics
S84-5162	Q128R	Actin- binding domain	F	0 y	EFE	N/A	II			10	8 mo, heart transplantation
H693839	G202R	Nebulin repeats (I band)	F	41	DCM	No	II		65	18	ACE inhibitor, diuretics, ameodarone
73-2	A592E+ M374V in $\alpha$ -actinin2	Nebulin repeats (Z-disk)	F	0 y	DCM	No	I	N/A	N/A	20	ACE inhibitor

**Figure 1. Nebulette mutations in cardiomyopathy**

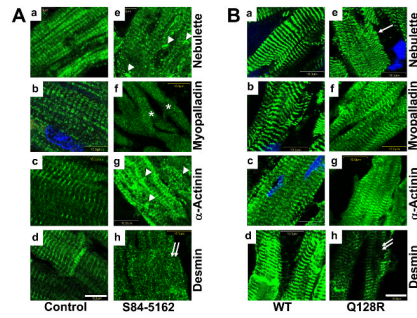
**Upper Panel.** Sequence electropherograms of *NEBL* showing the nucleotide substitutions.

**Lower Panel.** Genetic and clinical features of patients carrying mutations in *NEBL*.



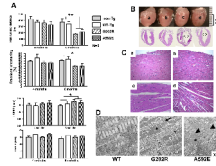
**Figure 2. Morphohistologic, functional and ultrastructural features of Q128R hearts of the proband, Tg embryos and chimera mice**

**Panel A.** Gross morphology of the Q128R proband heart. **Panel B.** Representative H&E sections of the hearts from (a) WT-Tg and (b) Q128R-Tg embryos at embryonic stage E12.5. Upper panel represents X2; lower panel represents X40 magnification. **Panel C.** Representative gross morphology and short axis LV end-diastolic cMRI images of mouse hearts. Heart enlargement and LV dilation (arrows) in K60N-Tg (b), and Q128R-Tg (c) chimera mouse hearts compared to non-Tg (a). of non-Tg (a), Bar-0.2cm. Panel C. cMRI analysis of mouse hearts. (\*-p<0.05). **Panel E.** TEM analysis of WT-Tg (a), K60N (b) and Q128RTg (c) mouse hearts (X8000).



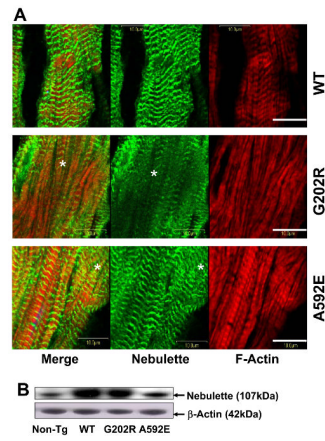
**Figure 3. Comparative immunohistochemical analysis of human or mouse hearts**

Panels a–d are sections from human (A) or WT-Tg mouse (B) control hearts. Panels e–h are sections from the S84-5162 proband's heart (A) or Q128R-Tg chimera heart (B), respectively. Nebulette (a, e); myopalladin (b, f);  $\alpha$ -actinin2 in (c, g); and desmin (d, h) are shown in green, nuclei are blue (DAPI). Bar=10  $\mu$ m.



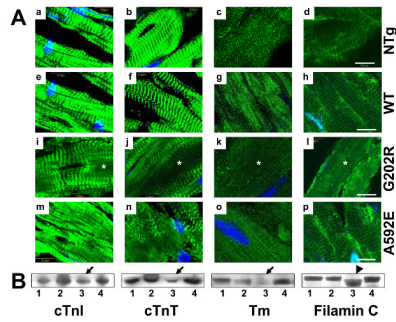
**Figure 4. Cardiac function and morphology in transgenic mice**

**Panel A.** Graded treadmill testing and echocardiography in 4- and 6-month-old mice (N=5). Mutant G202R (stripe) and A592E (dots) mice exhibited a marked reduction in exercise tolerance ( $p < 0.01^{**}$ ) compared to non-Tg (grey) and WT-Tg (white) mice. Decrease of FS ( $p < 0.05^{*}$ ) and increase of LVESD ( $p < 0.05^{*}$ ) was evident in mutants. **Panel B.** Morphology and long-axis four-chamber images of mouse hearts. Bar=0.5 cm. **Panel C.** Cardiac histology with H&E (X40). Representative sections from non-Tg (a), WT-Tg (b), G202R (c), and A592E (d) are shown. **Panel D.** Ultrastructural analysis of mouse hearts at X8000.



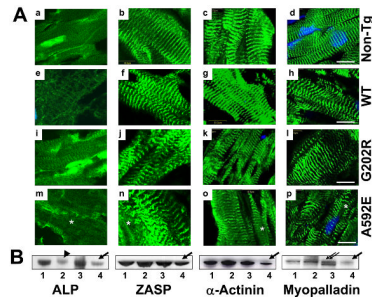
**Figure 5. Immunohistochemical analysis of nebullette in mouse hearts**

**Panel A.** Representative cardiac sections stained for nebullette (green), F-actin (red), and DAPI (blue). Bar=10 $\mu$ m. **Panel B:** Level of expression of nebullette in mouse hearts. Loading control is beta-Actin.



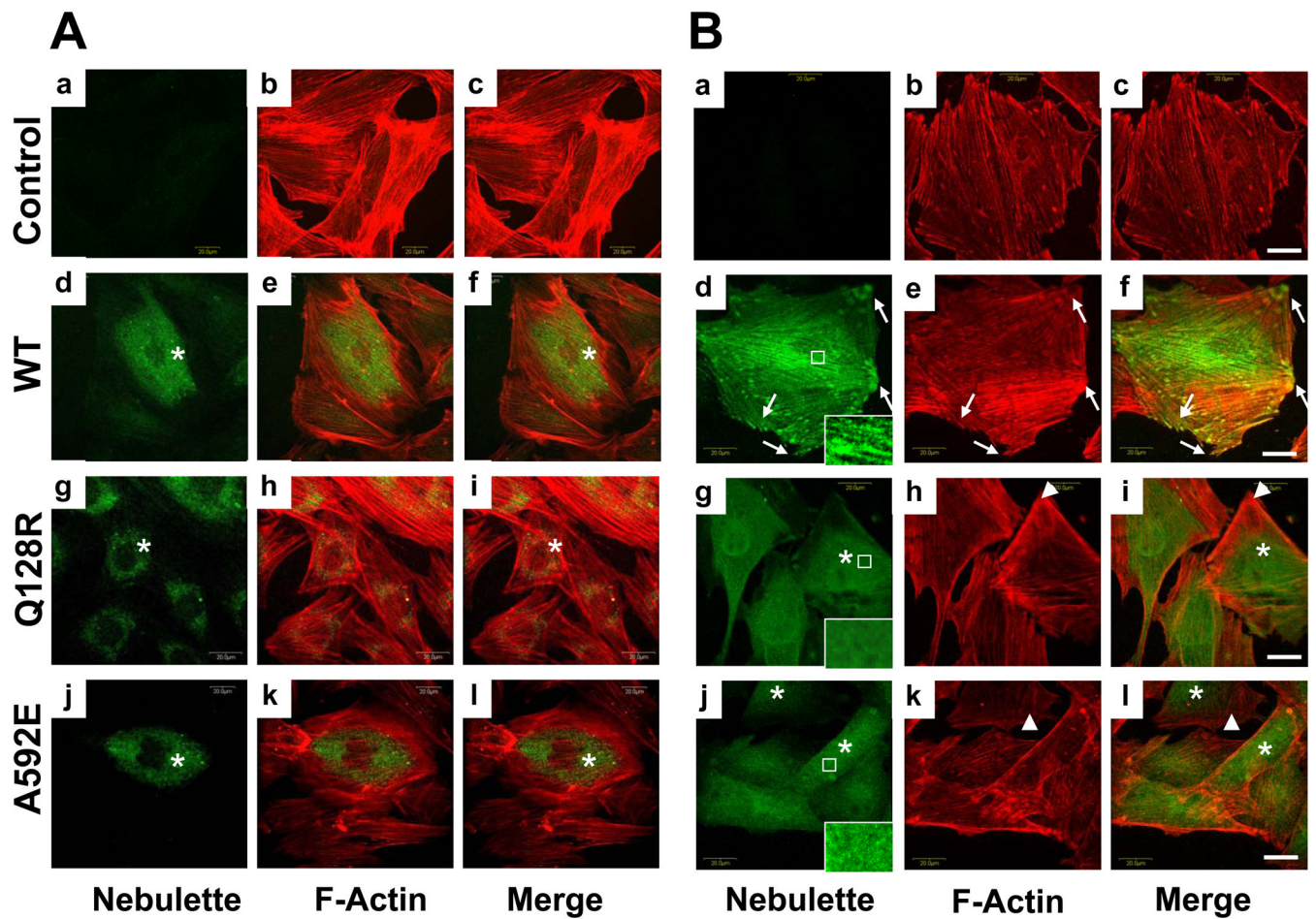
**Figure 6. Comparative analysis of I-band proteins in mouse hearts**

**Panel A.** Representative cardiac sections stained for cTnI (a, e, i and m), cTnT (b, f, j and n), Tm (c, g, k and o), and filamin C (d, h, l and p) from non-Tg (a–d), WT- (e–h), G202R (i–l) and A592E (m–p) mice. Proteins are green and nuclei are blue (DAPI). Bar=10 $\mu$ m. **Panel B.** Western blot analysis of I-band proteins in non-Tg (1), WT- (2), G202R- (3) and A592E-Tg (4) hearts. Beta-Actin is used as a loading control (Figure 5B). Downregulation is indicated by arrows and cleavage is indicated by arrowhead.



**Figure 7. Comparative analysis of Z-disk proteins in mouse hearts**

**Panel A.** Representative cardiac sections stained for ALP (a, e, i and m), ZASP (b, f, j and n),  $\alpha$ -actinin-2 (c, g, k and o), and myopalladin (d, h, l and p) from a non-Tg (a–d), WT (e–h), G202R (i–l) and A592E (m–p) mouse. Proteins are green and nuclei are blue (DAPI). Bar=10 $\mu$ m. **Panel B.** Western blotting of Z-disk proteins in non-Tg (1), WT (2), G202R (3) and A592E (4) hearts. Beta-Actin is used as a loading control (Figure 5B). Arrows and arrowhead indicate downregulation, asterisk indicates cleavage.



**Figure 8. Effects of cyclic mechanical stretch of H9C2 cells**

**Panel A.** Cells after cyclic strain without differentiation. **Panel B.** Differentiating H9C2 cells after cyclic mechanical strain. Expression of nebullette (green) and/or actin (red) are shown in control rat H9C2 cardiomyoblasts (a–b), H9C2 cells expressing WT-nebullette-GFP (c–e), Q128R (g–i) and A592E (j–l) representative cells with transient expression of each mutant nebullette-GFP, respectively. Bar=20  $\mu$ m. Experiments were repeated three times to confirm stability of results.