Heterozygous $Lmna^{d e l K32}$ mice develop dilated cardiomyopathy through a combined pathomechanism of haploinsufficiency and peptide toxicity

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Dilated cardiomyopathy (DCM) associates left ventricular (LV) dilatation and systolic dysfunction and is a major cause of heart failure and cardiac transplantation. $LMNA$ gene encodes lamins A/C, proteins of the nuclear envelope. $LMNA$ mutations cause DCM with conduction and/or rhythm defects. The pathomechanisms linking mutations to DCM remain to be elucidated. We investigated the phenotype and associated pathomechanisms of heterozygous $Lmna^{d e l K32/1}$ (Het) knock-in mice, which carry a human mutation. Het mice developed a cardiac-specific phenotype. Two phases, with two different pathomechanisms, could be observed that lead to the development of cardiac dysfunction, DCM and death between 35 and 70 weeks of age. In young Het hearts, there was a clear reduction in lamin A/C level, mainly due to the degradation of toxic $\Delta K32$-lamin. As a side effect, lamin A/C haploinsufficiency probably triggers the cardiac remodelling. In older hearts, when DCM has developed, the lamin A/C level was normalized and associated with increased toxic $\Delta K32$-lamin expression. Crossing our mice with the Ub⁷⁶V-GFP ubiquitin-proteasome system (UPS) reporter mice revealed a heartspecific UPS impairment in Het. While UPS impairment itself has a clear deleterious effect on engineered heart tissue’s force of contraction, it also leads to the nuclear aggregation of viral-mediated expression of $\Delta K32$-lamin. In conclusion, Het mice are the first knock-in $Lmna$ model with cardiac-specific phenotype at the heterozygous state. Altogether, our data provide evidence that Het cardiomyocytes have to deal with major dilemma: mutant lamin A/C degradation or normalization of lamin level to fight the deleterious effect of lamin haploinsufficiency, both leading to DCM.

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INTRODUCTION

The main features of dilated cardiomyopathy (DCM) are left ventricular (LV) dilatation, systolic dysfunction, myocyte death and myocardial fibrosis. DCM represents a major cause of heart failure and cardiac transplantation. Analysis of relatives of affected patients reveals a familial disease in 20–35% of cases (1). Among all the mutated genes reported in familial DCM, LMNA gene represents one of the first known causes of non-syndromic DCM in patients, estimated at 6% of DCM (1). The LMNA-related cardiomyopathy associates DCM, conduction and/or rhythm defects (DCM-CD) (2,3) and is mainly dominantly inherited.

To date, more than 450 LMNA gene mutations have been reported (http://www.umd.be/LMNA/), which lead to a wide spectrum of diseases collectively referred to as laminopathies (4). These pathologies involve either specific tissues (striated muscles, adipose tissue, peripheral nerves) or several systems (progeroid syndromes) with overlapping phenotypes. Among the LMNA mutations identified so far, most of them lead to cardiac diseases (DCM-CD) isolated or associated with skeletal muscles involvement, e.g. LMNA-related congenital muscular dystrophy (5), Emery–Dreifuss muscular dystrophy (EDMD) (6) and limb-girdle muscular dystrophy (7). In those pathologies, DCM-CD constitutes the most serious and life-threatening manifestation of the disease.

LMNA encodes A-type lamins, mainly comprising lamins A and C (8). Lamins A/C are ubiquitous proteins of the type V intermediate filament family. They assemble in highly organized structures and, together with B-type lamins, form a meshwork beneath the inner nuclear membrane: the nuclear lamina (9). The physiological roles of lamin A/C are not yet fully understood. Their numerous interactions with proteins of the nuclear membrane support a role in maintaining the structure and stiffness of the nuclear envelope. They also largely interact with proteins of the nucleoplasm (transcription factors and histones) and with chromatin, which suggests physiological functions in the regulation of gene expression (9,10).

The mechanisms by which mutations in LMNA lead to tissue-specific diseases remain unclear. An important function of the nuclear lamina is to maintain the structural integrity of cells. Therefore, lamina defects might result in cellular ‘weakness’ and damages, critical for muscular cells subjected to constant mechanical stress. In addition, altered tissue-specific gene expression might also be involved in the diseases (9). Cumulative evidence indicates that the steady-state level of lamin A/C is crucial for normal cell morphology and function, especially in the heart. Overexpression of wild-type lamin A/C in cells and tissues leads to nuclear abnormalities and higher sensitivity to cell death (11,12). Lmna+/− mice develop severe DCM and die prematurely at 8 weeks of age (13), and heterozygous Lmna+/− mice that express only 50% of lamin A/C suffer from DCM-CD arising at adulthood (14,15).

We created a new Lmna mutant mouse model deleted for lysine 32 of lamin A/C (LmnaΔK32 mouse) (16). Homozygous LmnaΔK32ΔK32 mice exhibit maturation defects of skeletal and cardiac muscles, and severe metabolic disorders responsible for premature death at 2 weeks of age, suggesting that the ΔK32-lamin A/C protein itself and/or the overall reduction in the lamin A/C level (~80%) have deleterious effects (16). At this age, heterozygous LmnaΔK32+/ mice did not have an obvious pathological phenotype. In the present paper, we further investigated the phenotype of the heterozygous LmnaΔK32+/ adult mice (hereafter called Het mice).

RESULTS

Absence of skeletal muscle or metabolic defects in Het mice

As LmnaΔK32ΔK32 mice showed skeletal muscle abnormalities (16), we investigated the skeletal muscle phenotype of old Het mice (57 weeks of age). Spontaneous activity test, in vitro contractile function of diaphragm, soleus and extensor digitorum longus muscles, and histological analysis of gastrocnemius muscle were performed in Het and Wt mice (Supplementary Material, Table S1 and Fig. S1). All these parameters did not differ between Het and Wt mice, suggesting no skeletal muscle defect in Het mice. However, the lamin A/C protein level in gastrocnemius was >50% lower in Het than in Wt mice (Supplementary Material, Fig. S2). In addition, Het mice showed no evidence of organ abnormalities (normal weight and aspect of liver, kidney, spleen, skin and vasculature) or metabolic disorders (normal fat deposition and glycaemia, triglycerides and cholesterol levels in serum; data not shown).

Development of dilated cardiomyopathy in Het mice

Het mice displayed a shortened lifespan, dying between 35 and 70 weeks of age without gender-difference (Fig. 1A). To assess the cardiac phenotype, we performed echocardiographic and electrocardiographic (ECG) longitudinal studies of male and female Het and Wt mice from 12 weeks of age until death. These revealed a progressive development of cardiac dysfunction in Het mice, as shown by decreased fractional shortening (FS), which started a bit earlier in males than in females (Fig. 1B; Supplementary Material, Tables S1 and S2). This was followed by an increased LV end-diastolic diameter (LVEDD; Fig. 1C; Supplementary Material, Tables S2 and S3). These features matched with a DCM phenotype. Despite LV dilation, there was no major wall thinning (interventricular septal wall thickness in diastole, IVSd, and posterior wall thickness in diastole, PWd; Supplementary Material, Tables S2 and S3). Importantly, whatever the age of onset of cardiac dysfunction (from 32 to 70 weeks of age), the timeline of the disease evolution was very reproducible between mice, i.e. 15–20 weeks between reduced FS and death. Surface ECG recordings did not reveal any conduction or rhythm defect in Het mice. Some unspecific abnormalities (slight bradycardia, higher PR interval, QRS complex broadening) were observed at the very end stages of the DCM only (data not shown). The progression of DCM was associated with cardiac hypertrophy (Fig. 1D) and ventricular and atrial enlargement (Fig. 1G) in both males and females. At the end stage, the LV of Het mice presented slight fibrosis (Fig. 1H and I). Het mice eventually presented with congestive heart failure as demonstrated by marked LV hypokinesia and lung oedema reflected by an increase in the lung weight to tibia length (TL) ratio (Fig. 1E).

Analysis of mRNA levels of cardiac remodelling markers at 10 and 57 weeks revealed no change in Nppa mRNA level, whereas Nphp mRNA amounts were higher in Het than in Wt mice at both ages (Fig. 1F). The Mxyh7 mRNA level did not differ between 10-week-old Het and Wt, but was 5-fold higher.
in 57-week-old Het mice. These results suggest that cardiac remodelling has already started at 10 weeks of age in Het mice.

**Abnormal nuclear shape in Het hearts**

Immunostaining analysis of lamin A/C in the heart of 10, 30 and 57-week-old Het mice revealed nuclear rim localization similar to age-matched Wt (Fig. 2). However, a large proportion of nuclei were more elongated and thinner in Het than in Wt hearts at all ages (Fig. 2), even before the onset of DCM (Fig. 2A). Myocardial ultrastructure analysed by electron microscopy revealed a preserved sarcomeric architecture, but many elongated nuclei with enlarged nuclear intermembrane space in young asymptomatic Het mice compared with Wt.
In Het animals with mild cardiac dysfunction, severe alterations were found in many nuclei, such as extremely enlarged nuclear intermembrane space, accumulation of large perinuclear vacuoles (Fig. 2F and G) or envelope rupture with extravasations of chromatin into the cytoplasm (Fig. 2H). At the end stage, in addition to the pronounced alteration of a majority of nuclei, nonspecific degenerative changes were observed: disrupted sarcomeres, myofibrillar lysis, vesicular proliferation of sarcomeric reticulum, intracytoplasmic junctions, electron dense residual bodies and pericellular fibrosis (data not shown).

**Figure 2.** Lamin A/C localization and nuclear morphology in Wt and Het hearts. (A–C) Lamin A/C staining in Wt and Het hearts before (10 weeks, A), at early stage (30 weeks, B) and at end stage of DCM (57 weeks, C). Nuclei are counterstained with DAPI (bar = 10 μm). (D–H) Ultrastructural features of myocardial nuclei of 12-week-old Wt (D) and Het (E) mice and 26-week-old Het mice (F–H). Widened nuclear intermembrane space (inset 2E compared with 2D, and G, arrows), large vacuoles surrounding the nucleus (F, asterisks), nuclear membrane rupture and chromatin exit (H, arrows) (bar = 2 and 500 nm in insets).

**Modulation of lamin A/C levels with the progression of dilated cardiomyopathy**

We evaluated the amount of lamin A/C proteins in the heart of 10-, 30- and 57-week-old Het and Wt mice. Cardiac lamin A/C levels did not change with aging in Wt mice (data not shown). In contrast, whereas the cardiac lamin A/C level was 50% lower in 10- and 30-week-old Het than in age-matched Wt mice, it did not differ between 57-week-old Het and Wt mouse hearts (Fig. 3A and B). Furthermore, the level of lamin A was negatively correlated with the FS in Het hearts (Fig. 3C; $r = -0.581$; Pearson’s correlation test, $P < 0.01$). These data suggest that progression of DCM is associated with an increase in the total (wild-type and mutant) lamin A/C level in Het hearts. This normalization of the lamin A/C level was not due to an increase in the number of cells in Het hearts, as the level of emerin, another nuclear protein, did not differ in Het and Wt hearts at all tested ages (Fig. 3A). In order to determine the relative amount of ΔK32-lamin A/C in the heart, mass spectrometry (MS) was employed (Fig. 3D and Supplementary Material, Fig. S3–S4). Lamin A/C peptides were generated by trypsin digestion of protein extracts from heart samples of Het mice. Analysis of peptide signal intensities from the two unique Wt-peptides (33EDLQELNDR41 and 29LQEQLNDR29) and from the unique ΔK32-peptide (29LQEQLNDR29) showed that the ΔK32-peptide represented 8.4 and 8% of total lamin A/C proteins in the heart of 10- and 57-week-old Het mice, respectively (Fig. 3D). This indicates that the ratio of ΔK32-to-total lamin A/C was very low in Het hearts throughout
the evaluated lifespan. Furthermore, the MS quantification confirmed that the overall amount of total lamin A/C increased (1.5-fold change) with age in Het mice (Supplementary Material, Fig. S4E), as observed by western blot (Fig. 3A and B). This suggests that the absolute amount of ΔK32-lamin A/C increased in 57- compared with 10-week-old Het hearts.

We then evaluated whether modulation of total lamin A/C protein and reduced ΔK32-lamin A/C expression were due to modulation of Lmna gene expression. The total Lmna mRNA level did not differ between Het and Wt mice at 10, 30 and 57 weeks of age (Fig. 3E). Of note, Lmna mRNA was equally expressed in Wt hearts at all tested ages. Allele-specific mRNA level assessment by a RT-qPCR showed that both alleles were equally expressed in the heart of Het mice at the three stages (Fig. 3F). This suggests that the lower lamin A/C protein level in young Het hearts results from post-transcriptional regulations. We thus hypothesized that (i) in young Het hearts, lamin A/C haploinsufficiency results from specific degradation of ΔK32-lamin A/C and (ii) in old Het hearts, normalization of lamin A/C via the increase in

Figure 3. Evolution of mutant lamin A/C expression. (A–B) Lamin A and C, emerin protein levels in the heart of 10-, 30- and 57-week-old Het and Wt mice. Actin is used as a loading control. Representative blots (A) and quantification (B) of total lamin A (LaA) and C (LaC). (n = 6–9 per group). ** P < 0.01 compared with Wt. Mean ± SEM. (C) Correlation between the lamin A protein level and FS in Het mice at the three ages. (D) Ratio of mutant ΔK32-lamin A/C to total lamin A/C quantified by MS based label-free quantification in the heart of 10- and 57-week-old Het mice (n = 7–9 per group). Mean ± SEM. (E) Level of total Lmna mRNA in the heart of 10-, 30- and 57-week-old Het and Wt mice (n = 5–6 per group). Mean ± SEM. (F) Ratio of the specific allele level in the heart of 10-, 30- and 57-week-old Het mice. Wt allele = Lmna Wt, mutant allele = Lmna ΔK32.
ΔK32-lamin A/C results from an impairment/saturation of this degradation system.

Degradation of lamin A via the ubiquitin-proteasome system in cardiomyocytes

In mammalian cells, the ubiquitin-proteasome system (UPS) is the major non-lysosomal degradation system involved in protein homeostasis (17). To determine whether the UPS was involved in the degradation of Wt or ΔK32-lamin A/C, neonatal mouse cardiomyocytes (NMCMs) were transduced with adeno-associated virus serotype 6 (AAV6) encoding Flag-Wt- or Flag-ΔK32-prelamin A. The level of Flag-ΔK32-lamin A was lower than the level of Flag-Wt-lamin A (Fig. 4), suggesting lower protein stability of Flag-ΔK32-lamin A. Transduced NMCMs were then treated for 24 h with 500 nm of the UPS inhibitor epoxomicin. Epoxomicin treatment induced accumulation of both Flag-Wt- and Flag-ΔK32-lamin A, indicating that UPS degrades both Wt and ΔK32-lamin A in cardiomyocytes.

Impairment of the ubiquitin-proteasome system in Het hearts

We then hypothesized that the age-dependent increase in cardiac lamin A/C level is the result of heart-specific UPS impairment in Het mice. To assess the global function of the UPS, Het and Wt mice were crossed with UbG76V-GFP mice. UbG76V-GFP (hereafter called Ub-GFP) mice ubiquitously express a green fluorescent protein (GFP)-based proteasome substrate that allows monitoring of the UPS in vivo, the Ub-GFP protein level being inversely correlated with UPS function (18). The Ub-GFP level was very low in young Wt/Ub hearts and increased with age (Fig. 5A), indicating a general dysfunction of the UPS with aging. Interestingly, the Ub-GFP level was highly increased in 10-week-old Het/Ub-GFP hearts and persists thereafter although the difference was milder (Fig. 5A and B). UPS impairment appeared prior to normalization of lamin A/C level in Het/Ub-GFP mice, as lamin A/C level was 50% lower than in Wt/Ub-GFP mice at these ages (Fig. 5A and C). Of note, the UPS function was preserved in skeletal muscle (gastrocnemius) as reflected by similar Ub-GFP level in 30-week-old Het/Ub-GFP and Wt/Ub-GFP mice (Supplementary Material, Fig. S5) as well as in liver and kidney from both Het/Ub-GFP and Wt/Ub-GFP mice (data not shown). Altogether, these
results indicate that UPS impairment starts early and is strictly restricted to the heart in Het mice.

**Importance of a functional UPS for EHTs contractility and for ΔK32-lamin aggregates clearance**

We then hypothesized that the early UPS dysfunction is responsible for the first signs of cardiac dysfunction in young Het mice, which is worsened in old heart by the increase in ΔK32-lamin A/C. To test this hypothesis, we evaluated the contractile properties of engineered heart tissues (EHT; (19)) treated or not with an UPS inhibitor. Epoxomicin treatment (24 h) reduced force of contraction when compared with untreated EHTs (Fig. 6A), suggesting that an active UPS is required for efficient contractile function of cardiomyocytes.

We then evaluated the impact of AAV6-mediated gene transfer of Flag-Wt- and Flag-ΔK32-prelamin A on contractile properties of EHTs in the absence or presence of proteasome inhibitor. When the UPS is functional (DMSO-treated EHTs), overexpression of Flag-Wt- or Flag-ΔK32-lamin did not change the force of contraction of EHTs when compared with untransduced cardiomyocytes (Fig. 6A). Epoxomicin treatment leads to the increase in Flag-Wt- and Flag-ΔK32-lamin expression (Fig. 6B), demonstrating that UPS is involved in the regulation of Flag-lamin A expression level in EHTs. Despite the increase in Flag-ΔK32-lamin level after epoxomicin treatment, EHTs’ force of contraction did not differ from Flag-Wt-lamin and untransduced controls EHTs treated with epoxomicin (Fig. 6A). This result indicates that short time increased ΔK32-lamin expression is not sufficient to worsen contractile dysfunction due to UPS inhibition in EHTs.

Immunostaining revealed a normal nuclear rim localization of Flag-Wt-lamin in both DMSO- and epoxomicin-treated EHTs (Fig. 6C). However, Flag-ΔK32-lamin A was located in the nucleoplasm in DMSO-treated EHTs, as previously reported (16), and aggregated into large nucleoplasmic foci after UPS inhibition (Fig. 6C).

These results suggest that degradation of ΔK32-lamin A via the UPS in reconstituted heart tissue limits its aggregation and its putative negative effect on contractility.

**DISCUSSION**

We report here that a heterozygous in-frame amino acid deletion in the N-terminal domain of lamin A/C caused a cardiac-specific phenotype in mice. We show that (i) Het mice developed a progressive cardiac dysfunction and DCM evolving to death between 35 and 70 weeks of age; (ii) young Het hearts, which are still unaffected, exhibited lamin A/C haploinsufficiency, whereas old affected Het hearts had increased lamin A/C amount; (iii) lamin A/C haploinsufficiency is in part due to ΔK32-lamin A/C (alone and/or associated with Wt A-type lamin) degradation through the UPS; (iv) Het hearts exhibited UPS impairment before DCM development; (v) UPS inhibition had deleterious effects on contractile properties of EHTs; (vi) alteration of ΔK32-lamin degradation induced its nuclear aggregation in EHTs with inhibited UPS. These data provide evidence for a major role of the UPS in the regulation of cardiac function and of lamin A/C level in the heart of Het mice that appears to be a key pathophysiological component leading to DCM in Het mice.

**Haploinsufficiency of lamin A/C in Het mice**

Young Het mice presented with 50% less lamin A/C than age-matched Wt in heart and muscles (Fig. 3, Supplementary Material, Figure S2; (16)). With age, they developed cardiac disease but no skeletal muscle defects. This segmental phenotype affecting only cardiac muscle has already been observed in striated muscle-specific laminopathies (20). The heart seems to be more vulnerable than skeletal muscles to haploinsufficiency in patients. Indeed, genotype–phenotype correlations using LMNA Universal mutation database (http://www.umd.be/LMNA/) showed that 67% of nonsense and truncating mutations, which putatively lead to lower lamin A/C levels, cause...
cardiac diseases without muscle involvement (21–23). To date, no clear evidence explains the contrasting sensitivity to lamin A/C haploinsufficiency between the heart and skeletal muscles. It might result from structural and functional differences of these two tissues. The heart contracts in a ‘twist’ way, generating torsion, whereas skeletal muscles generate unidirectional shortening. In addition, distinct organizations of the cellular cytoarchitecture and nuclei positioning (central in cardiomyocytes versus subsarcolemmal in muscle) result in singular forces applied on cardiac nuclei compared with myofibres. This may explain, in part, the higher susceptibility of cardiac muscle to nuclear mechanical defects and deformations induced by a loss of lamin A/C function, as observed in Het mice.

Like in human, lamin A/C haploinsufficiency induces heart disease in mouse. Lmna+/− mice, presenting 50% of lamin A/C in tissues, exhibit apoptosis of atrio-ventricular nodal myocytes and progressive electrophysiologic disease starting at 10 weeks of age (14). They also develop mild dilated cardiomyopathy (15). Like in Lmna+/− mice, part of the cardiac phenotype of Het mice can be attributed to haploinsufficiency. Jahn et al. (24) have recently reported that, in addition to haploinsufficiency, Lmna+/− mice exhibited a truncated lamin A (lamin AΔ8–11) probably resulting in moderate toxic effect. Interestingly, our data show that Het mice developed a more severe phenotype than Lmna+/− mice and with several different features. Both the mouse strains displayed DCM, with a relatively earlier onset for Lmna+/− mice (15). However, only 20% of Lmna+/− mice suffered from life-threatening cardiac dysfunction, whereas all Het mice died from congestive heart failure.

No cardiac electrical abnormalities were observed in Het mice using surface ECG, whereas Lmna+/− mice showed rhythm and conduction defects, suggesting specific mechanisms related to the deletion of lysine 32. These results suggest a stronger ‘toxic’ effect of ΔK32-lamin A/C compared with lamin AΔ8–11. The presence of ΔK32-lamin and its relative increase with time might lead via a toxic peptide effect to the aggravation of defects due to lamin haploinsufficiency in Het hearts.

**UPS dysfunction in the heart of Het mice**

Whatever the primary cause (mechanical overload, ischaemia or mutant proteins), myocardial remodelling is a common feature of chronic heart failure and involves several pathways. Cumulative evidences suggest that alteration of proteasomal-mediated protein degradation contributes to the initiation and/or progression of cardiac diseases in humans and in experimental models (25). Especially, in familial cardiomyopathies, reports have demonstrated the role of defective UPS activity in the pathophysiological mechanisms. Indeed, mutations in genes encoding αB-crystallin (26) or cardiac myosin-binding protein C (27) involve cardiac UPS impairment in patients and mice (28–30).

In our study, we clearly observed major contractile dysfunction of EHT when treated with an UPS inhibitor.

As for cardiac and muscular laminopathies, Kandert et al. reported dysfunction of the UPS associated with impairment of proliferation and differentiation capacities of myoblasts from EDMD patients expressing p.R545C lamin A/C (31). In our study, we raised the hypothesis that UPS dysfunction could be an indirect consequence of the lamin haploinsufficiency. Additionally, we showed that the sole presence of ΔK32-lamin did not lead to accumulation of ubiquitinated proteins neither in NMCM (Fig. 4) nor in EHTs (Fig. 6B), suggesting that UPS impairment was not triggered directly by ΔK32-lamin. Although we cannot exclude that ΔK32-lamin A/C steady-state degradation might directly overwhelm the proteolytic capacity of the UPS in vivo, our data suggest that UPS impairment might rather occur as a consequence of high degradation workflow imposed by cardiac remodelling in Het mice. This notion is supported by the absence of UPS dysfunction in non-pathological tissues (skeletal muscle, liver and kidney) of Het mice, indicating a cardiac-specific impairment of this system. Of note, degradation of ΔK32-lamin and UPS impairment is one of the mechanisms leading to the modulation of lamin A/C expression in Het hearts, but some other processes might exist in parallel and need to be further investigated.

Even if the mechanisms leading to UPS impairment are not fully established in heart diseases, UPS is known to be essential for the balanced turnover of functionally important cardiac proteins such as contractile proteins (32), membrane receptors (33,34), signalling pathways like NF-κB (35), β-catenin (36) or p53 (37), regulating inflammation, cardiac remodelling and apoptosis. Hence, appropriate activity of UPS is essential to maintain normal cardiac function.

**Dominant-negative effect of ΔK32-lamin A/C**

We recently reported that homozygous LmnaΔK32/ΔK32 mice died at 2 weeks of age because of severe global maturation defects and metabolic disorders (16). They exhibited only ΔK32-lamin A/C in all tissues, albeit at a reduced level. Interestingly, LmnaΔK32/ΔK32 mice showed a more severe phenotype than Lmna+/− mice (which die at 8 weeks of age) (13), suggesting that ΔK32-lamin A/C is highly deleterious. Dominant-negative effect of ΔK32-lamin A/C is also supported by data from *Caenorhabditis elegans*. Indeed, Ce-ΔK46-lamin (homologous to ΔK32-lamin) filaments displayed defective lateral assembly leading to abnormal lamin network in vitro (38).

In the heterozygous context, we supposed that Wt and ΔK32-lamin form homodimers (Wt-Wt and ΔK32-ΔK32) as well as heterodimers (Wt-ΔK32) that further assemble into heterogeneous Wt/ΔK32 filaments. As in *C. elegans*, the absence of lysine 32 might impact lamin filament conformation in Het mice, leading to the degradation of filaments composed by a too high proportion of ΔK32-lamin. The results obtained by MS revealed that the ratio of ΔK32-lamin to Wt-lamin is maintained to 8% over time, suggesting that filaments composed of >8% of ΔK32-lamin are targeted for degradation. This hypothesis may explain the important reduction of the ΔK32-lamin but also the partially reduced pool of Wt-lamin, resulting in haploinsufficiency in the heart of 10- and 30-week-old Het mice.

The key and original finding of the present study is the altered proteasomal-mediated degradation system restricted to the heart with the course of DCM, which associates with the increase in the amount of lamin A/C in Het hearts. These results suggest that ΔK32-lamin A/C degradation via the UPS occurs as a protective mechanism to impede its aggregation into nuclei, as seen in EHTs.

Altogether, these observations reinforce the idea that the presence of ΔK32-lamin in the heart of Het mice may disturb correct filament assembly of Wt-lamin, leading to laminas...
defects. Our data further support the notion of ‘toxic’ effect of ∆K32-lamin A/C already observed in Lmna∆K32/∆K32 mice and C. elegans. Further studies are needed to elucidate how ∆K32-lamin A/C exerts its negative effect, either by increasing sensitivity to mechanical stress and/or by modifying interactions with partners essential for heart function.

CONCLUSION

Lmna∆K32/+ mice are the first knock-in Lmna mice associated with a cardiac-specific phenotype at the heterozygous state, and therefore recapitulate features of DCM associated with dominant A-type lamin mutations in patients. Lmna∆K32/+ mice constitute a reliable model for further evaluations of potential therapies of A-type lamin related-DCM. To recapitulate our findings, we propose the following sequence of events (Fig. 7): (i) ∆K32-lamin A/C disturb the assembly into protofilaments and are thus targeted for degradation through the UPS. (ii) The resulting lamin A/C haploinsufficiency contributes to heart tissue vulnerability. (iii) Remodelling mechanisms counteract the higher myocardial sensitivity but have global deleterious effects including UPS function. (iv) The latter causes the amount of ∆K32-lamin A/C to increase, which worsens its dominant-negative effect on Wt-lamin function. (v) This precipitates cardiac dysfunction and leads to congestive heart failure and death of Het mice. Overall, our findings suggest that the balance between degradation of mutant lamin to reduce its toxic effect on the one hand and global reduction in the lamin A/C level (haploinsufficiency) resulting from this elimination on the other hand constitutes the major pathomechanism leading to DCM in Het mice.

MATERIALS AND METHODS

Animals

Lmna∆K32 knock-in mice were generated as previously described (16). Mice were studied according to the protocols approved by the European legislation (L358–86/609/EEC). We explored the cardiac phenotype of Het mice at three different ages corresponding to different stages of heart function: 10-week-old mice without any cardiac dysfunction, 30-week-old mice starting heart dysfunction and 57-week-old mice at advanced stage of DCM (Supplementary Material, Tables S2 and S3). The UbG76V-GFP/1 mice were generated and characterized as previously described (18). They ubiquitously express a GFP-fused proteasome substrate.
Cardiac function measurement

Transthoracic echocardiography was performed at room temperature using an echocardiography-Doppler (General Electric Medical systems Co, Vivid 7 Dimension/Vivid 7 PRO) with a probe emitting ultrasounds with 9–14 MHz frequency. Mice were slightly anesthetized with 0.5–1% isoflurane in 100% O2. The two-dimensionally guided Time Motion mode recording of the LV provided the following measurements: IVSd, PWd, LVEDD and LV end-systolic (LVESD) diameters. Each set of measurements were obtained from the same cardiac cycle. At least three sets of measures were obtained from three different cardiac cycles. LV mass (LVM) and percentage of LV FS (%) were calculated as follows: [(IVSd + PWd + EDD)² – EDD²] × 1.055 (39) and (LVEDD – LVESD)/LVEDD × 100, respectively.

Electrocardiographs (ECGs) were performed in conscious mice. Mice were placed in cages in a way that each limb contacted a receiver for transmission of ECG signals. ECG traces were recorded during 20 min and analysed with ECG Auto software (EMKA). QRS duration, RR and PR intervals were measured.

Production of adeno-associated virus

FLAG-tag was inserted before the ATG codon of WT- and ΔK32-human prelamin A constructs by a PCR. They were cloned into the pSMD2-CMV vector for adeno-associated virus serotype 6 (AAV-6) production as described previously (40). Virus titres ranged from 1.92 × 10¹² to 1.03 × 10¹³ virus genomes/ml.

Cardiomyocytes culture and analysis

Neonatal mouse cardiac myocytes (NMCMs) were isolated from C57/BL6J mice as previously described (28). NMCMs were transduced with AAV-6 encoding Flag-Wt- or Flag-ΔK32-prelamin A at MOI (Multiplicity Of Infection) of 30 000. Cells were treated with 500 nM epoxomicin in 10% DMSO or with DMSO alone for 24 h. NMCMs were harvested 48 h after transduction.

Engineered heart tissue generation and analysis

Fibrin-based engineered heart tissues (EHTs) from neonatal rat heart cells were generated and cultured as previously described (19). Briefly, for each EHT, a 100 μl-reconstitution mix containing 4 × 10⁶ cells/EHT, bovine fibrinogen, aprotinin and DMEM was mixed with 3 μl thrombin and pipetted around two elastic silicone posts. EHTs were transduced with AAV-6 encoding Flag-Wt- or Flag-ΔK32-prelamin A at MOI of 1000 directly added in the reconstitution mix. At Day 17, EHTs were treated with 500 nm epoxomicin in 10% DMSO or with DMSO alone for 24 h. Contraction measurements were performed by video optical recording on Days 8, 11, 15, 16 and 17 as previously described (19). Average force and contraction and relaxation velocities were calculated from the recorded contractions by an algorithm that takes into account the elastic properties of the silicone posts.

Histology and immunohistochemical analysis

Fresh heart samples were snap frozen in liquid-nitrogen-cooled isopentane, and stored at −80°C until further processing. Frozen sections (8 μm) of transversal cardiac muscle were stained with Sirius red for fibrosis visualization. Sections were analysed by light microscopy.

For immunohistochemical analysis, tissue sections were fixed for 10 min in 100% acetone at −20°C and incubated for 30 min with blocking solution (5% bovine serum albumin IgG-free in PBS) at room temperature. For detection of lamin A/C and lamin B, a primary rabbit anti-lamin A/C polyclonal antibody (Ab) (1:100, Santa Cruz) and primary goat anti-lamin B polyclonal Ab (1:100, Santa Cruz) were diluted in 5% bovine serum albumin IgG-free in PBS and the sections were incubated for 90 min at room temperature. Sections were washed three times with PBS and incubated with secondary Ab (1:500, Alexa fluor 488 chicken anti-rabbit IgG and Alexa fluor 568 donkey anti-goat IgG) for 30 min at room temperature. Cardiac sections were mounted with mounting medium (Vectashield) with 40,6-diamidino-2-phenylindole dihydrochloride (DAPI) and images were collected using a Carl Zeiss Axioptilfluorescence microscope.

For immunofluorescence analysis of EHTs, the entire EHTs were analysed using confocal imaging. EHTs were rinsed with PBS and fixed with Histofix® (Roth) overnight at 4°C. The samples were then removed from the silicon post and treated 24 h with blocking solution (TBS 0.05 M, pH 7.4, 10% FCS, 1% BSA, 0.5% Triton X-100) at 4°C. Immunofluorescence was performed with a mouse anti-FLAG monoclonal Ab (1:250, Sigma Aldrich) and MF20 (1:500, DSHB) and Alexa-fluor 488 anti-mouse and Alexa-fluor 546 anti-rabbit secondary Ab (1:600, Invitrogen). Nuclei were stained with DAPI. The incubation period was 24 h for the primary and secondary antibodies, respectively. Finally, the EHTs were fixed between a cover slip and a glass slide, and the fluorescence signal was analysed using a Carl Zeiss confocal microscope (Zeiss LSM 510 META).

Electron microscopy

The ultrastructure of the myocardium of 12 mice was analysed: 6 Het and 6 WT siblings (4 at 12 weeks, 2 at 35 weeks, 2 at 51 weeks, and 4 at 67 weeks of age). Freshly harvested left ventricle apex was cut into small pieces and immediately fixed by immersion in 2.5% glutaraldehyde diluted in saline phosphate buffer (PBS) during 1 h at room temperature. After abundant washing in PBS, samples were post-fixed with 2% OsO₄, dehydrated in a graded series of acetone including 2% uranyl acetate in 70% acetone step and finally embedded in an epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate, examined using a CM120 Philips transmission electron microscope and photographed with a digital SIS Morada camera, using iTEM software.

Protein analysis

For western blot analyses, proteins were extracted from frozen total heart (ventricles and atria), gastrocnemius muscle, liver and kidney as previously described (16). Total proteins were
separated by SDS–PAGE and hybridized with a primary rabbit anti-lamin A/C polyclonal Ab (1:2000, Santa Cruz), primary rabbit anti-emerin polyclonal Ab (1:1000, kindly provided by G.E. Morris, NEWI, UK), primary rabbit anti-actin polyclonal Ab (1:1000, Sigma Aldrich), mouse anti-ubiquitinated monoclonal Ab (1:5000, Enzo LifeSciences), mouse anti-FLAG-M2 monoclonal Ab (1:1000, Sigma Aldrich), mouse anti-vinculin monoclonal Ab (1:1000, Sigma Aldrich) or rabbit anti-GFP polyclonal Ab (1:1000, AbCam) and with secondary rabbit anti-mouse (for monoclonal Ab) or goat anti-rabbit (for polyclonal Ab) IgG HRP-conjugated Ab (1:2000, Dako A/S). Recognized proteins were visualized by enhanced chemiluminescence (Pierce or Millipore). Hybridization signals were quantified using MultiGauge software and normalized to actin, vinculin or Ponceau staining.

**LC-MS/MS analysis**

Heart protein extracts for MS analysis were prepared as described in the western blotting procedure (Supplementary Material, methods) and separated using SDS–PAGE. The gel lanes containing the lamin A and C proteins were cut out and digested separately using in-gel tryptic digestion as previously described (41). Briefly, the gel bands were shrunk using acetonitrile, in-gel reduced and alkylated followed by saturation of the gel pieces with trypsin. Digestion was performed overnight at 37°C. The resulting peptides were desalted using in-house made StageTips as previously described (42) and analysed by a nanoLC-MS/MS system (LTQ-Orbitrap XL, Thermo Fisher Scientific). The peptides were eluted from an in-house packed fused-silica (length: 20 cm; i.d. 75 μm) reversed-phase column (3 μm C18, ReproSil-Pur C18 AQ, Dr Maisch, Germany) using an 80% acetonitrile (ACN), 0.5% acetic acid gradient, starting with 2% ACN and ending with 80% ACN. The MS full scans were detected in the Orbitrap, with MS/MS scans being detected in the LTQ iontrap. The peptides were identified by searching tandem MS spectra using the Mascot search engine (Matrix Science) against a custom-made mouse database, containing the sequence of the ΔK32 variant [Uniprot release 2011_01, restricted to UniProtKB/Swiss-Prot and Taxonomy: *Mus musculus* (mouse) (49954 sequences)]. The label-free quantification was performed using two different approaches: (i) MaxQuant’s intensity-based label-free quantification where total peptide signals were determined in the mass-to-charge, elution time and intensity space (43,44) and (ii) integration of peptide extracted ion-chromatograms using the ICIS integration algorithms in the Qual browser program (Thermo Fisher Scientific) (45). Details and validation of the label-free quantification approach are provided in Supplementary Material, Figure S4A and 4B.

**mRNA analysis**

Hearts were dissected and rapidly frozen in liquid nitrogen. Total RNA extraction and Q-PCR were performed as previously described (16). Individual expression values were normalized by comparison with Rplp0 mRNA, encoding ribosomal protein large P0. The sequences of oligonucleotides used for Q-PCR analysis are listed in Supplementary Material, Table S4. Lmna expression level was measured using oligonucleotides matching with a common region of Wt and mutant on the cDNA. Lmna Wt and ΔK32 expression levels, encoding Wt and mutant lamin A/C protein, respectively, were measured using a hydrolysis probe technique. Hydrolysis probes were designed to match specifically with a Wt or deleted nucleotide region of Lmna cDNA with LightCycler 480 Probes Master kit (Roche Diagnostic).

**Skeletal muscle analysis**

Spontaneous activity and immunostaining were performed as previously described (46). The morphology of the gastrocnemius muscle was analysed blindly by the histopathologists from the Myology Institute. For measurement of isometric contractile properties of muscles, after removal, muscles were soaked in an oxygenated Krebs solution (95% O2 and 5% CO2) containing 58.5 mM NaCl, 24 mM NaHCO3, 5.4 mM KCl, 1.2 mM KH2PO4, 1.8 mM CaCl2, 1 mM MgSO4 and 10 mM glucose, pH 7.4, and maintained at a temperature of 22°C. One of the muscle tendons was attached to a lever arm of a servomotor system (300B Dual-Mode Lever; Aurora Scientific). After equilibration (30 min), electrical stimulation was delivered through electrodes running parallel to the muscle. Pulses (1 ms) were generated by a high power stimulator (701B; Aurora Scientific). Po was measured at L0 during isometric contractions in response to electrical stimulation (frequency of 50–125 Hz; train of stimulation of 1500 ms). Time to peak and time to half relaxation were recorded. Muscles were weighted and the specific force IsoN was calculated by dividing Po by muscle weight.

**Statistical analysis**

Differences between groups were assessed using ANOVA and Student’s t-test with Sigmastat software. The values of P < 0.05 were considered statistically significant.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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Conflicts of Interest statement. None declared.
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