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Phosphorylation of connexin43 on S279/282 may contribute to laminopathy-associated conduction defects

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Abstract

An understanding of the molecular mechanism behind the arrhythmic phenotype associated with laminopathies has yet to emerge. A-type lamins have been shown to interact and sequester activated phospho-ERK1/2(pERK1/2) at the nucleus. The gap junction protein connexin43 (Cx43) can be phosphorylated by pERK1/2 on S279/282 (pS279/282), inhibiting intercellular communication. We hypothesized that without A-type lamins, pS279/282Cx43 will increase due to inappropriate phosphorylation by pERK1/2, resulting in decreased gap junction function. We observed a 1.6-fold increase in pS279/282 Cx43 levels in *Lmna*^{-/-} mouse embryonic fibroblasts (MEFs) compared to *Lmna*^{+/+}, and 1.8-fold more pERK1/2 co-precipitated from *Lmna*^{-/-} MEFs with Cx43 antibodies. We found a 3-fold increase in the fraction of non-nuclear pERK1/2 and a concomitant 2-fold increase in the fraction of pS279/282 Cx43 in *Lmna*^{-/-} MEFs by immunofluorescence. In an assay of gap junctional function, *Lmna*^{-/-} MEFs transferred dye to 60% fewer partners compared to *Lmna*^{+/+} controls. These results are mirrored in 5–6 week-old *Lmna*^{-/-} mice compared to their *Lmna*^{+/+} littermates as we detect increased pS279/282 Cx43 in gap junctions by immunofluorescence and 1.7-fold increased levels by immunoblot. We conclude that increased pS279/282 Cx43 in the *Lmna*^{-/-} background results in decreased cell communication and may contribute to the arrhythmic pathology *in vivo*.

Keywords

nuclear lamins; pERK1/2; connexin43; gap junction; cardiac conduction

Introduction

Nuclear lamins are intermediate filament proteins associated with the inner nuclear membrane and the primary component of the nuclear lamina. The nuclear lamins perform a variety of functions such as providing structure to the nucleus [1–3], binding chromatin [4,

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Disclosures

None

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5], acting as a scaffold for other nuclear proteins (reviewed in [6–8]) and positioning the nucleus (reviewed in [9]). Likely due to their diverse roles, point mutations in the A-type lamin (*LMNA*) gene can cause a spectrum of diseases collectively termed laminopathies which include muscular dystrophies, lipodystrophies, dilated cardiomyopathy, restrictive dermopathy, peripheral neuropathy, and progeroid syndromes (reviewed in [10]). One pathology of particular interest in laminopathies is dilated cardiomyopathy with conduction system disease (DCM-CD), which can develop independently from any other skeletal muscle pathology or in conjunction with muscular dystrophies associated with A-type lamins, such as Emery-Dreifuss muscular dystrophy (EDMD) and limb-girdle muscular dystrophy (LGMD).

The association of DCM-CD with *LMNA* mutations is well established, as A-type lamin mutations are the most prevalent genetic cause of DCM-CD [11], and it has been found that many patients with DCM-CD ultimately have an undiagnosed laminopathy [12, 13]. Ultimately, most laminopathy patients that develop DCM-CD die of sudden cardiac death, expiring due to ventricular fibrillation, which in certain cases can be rescued by implantable cardiac defibrillators [14–17]. It is not yet clear how mutation of nuclear A-type lamin proteins cause defects in heart conduction. We hypothesize that loss of A-type lamin function could affect gap junction mediated conduction in the heart.

Gap junctions are specialized channels that serve as the intercellular conduits allowing ions to flow freely from the cytoplasm of one cell to another (reviewed in [18]). They play an especially important role in the heart, because they conduct the electrical signal from one cardiomyocyte to the other, thus allowing for the coordinated and regular contractions that we interpret as heartbeats. Connexin43 (Cx43) is the predominant gap junction protein in the heart, expressed in the working ventricular myocardium [19] and in a multitude of other tissues and cells throughout the body. Mice lacking Cx43 die shortly after birth due to cardiac hyperplasia obstructing the right ventricular outflow tract [20], while a heart-specific conditional knockout exhibited arrhythmia and premature death [21, 22]. There are at least 14 serines and 2 tyrosines in the cytoplasmic C-terminal region of Cx43 that are phosphorylated by a variety of kinases [23]. Of particular interest are three phosphorylation sites that have been identified as mitogen-activated protein kinase (MAPK) consensus recognition sites: S255, S279 and S282 [24]. It has been shown that phosphorylation of these residues can result in decreased conductivity of the gap junction and that the MAP kinase ERK1/2 is required for these effects [25, 26].

Mice lacking A-type lamins (*Lmna*^{-/-}) suffer from severe muscular dystrophy and dilated cardiomyopathy with conduction defect, dying prematurely at 5–7 weeks of age [27, 28]. Recent studies have found a link between the lamin A protein and ERK1/2, where the lamin A protein serves as a scaffold for activated ERK1/2, anchoring it to the nucleus and presumably keeping it in close proximity with the transcription factors it normally activates [29, 30].

As both *Lmna*^{-/-} mice and human laminopathy patients appear to suffer from dilated cardiomyopathy with conduction defect, we have investigated the regulation of the primary gap junction protein, Cx43, in the *Lmna*^{-/-} context. Previously, we have shown that *Lmna*^{-/-} mice exhibit decreased Cx43 levels at the gap junction, potentially contributing to conduction block and arrhythmias [31]. In the present study, we hypothesize that loss of lamin A impairs the normal sequestration of pERK1/2, resulting in a shift in its localization to the cytoplasm and subsequently inappropriate phosphorylation of Cx43. Here we show that loss of *Lmna* both *in vitro* and *in vivo* results in inappropriate ERK1/2 signaling, affecting phosphorylation of Cx43 on S279/282, which reduces intercellular communication.

Thus, inappropriate Cx43 phosphorylation may contribute to the conduction defects observed in laminopathies.

Results

Using immortalized MEFs isolated from *Lmna*^{+/+} and *Lmna*^{-/-} mice, we studied whether the regulation and function of Cx43 was perturbed by ERK1/2 activation due to loss of normal lamin A activity. *Lmna*^{-/-} cells exhibit a significant 1.6-fold increase in levels of Cx43 phosphorylated on serines 279 and 282 (pS279/282) compared to *Lmna*^{+/+} when normalized to total Cx43 (n=7, p < 0.01) (Figure 1A). Since phosphorylation of Cx43 at S279/282 has been associated with increased degradation, we examined the total levels of Cx43 using an antibody against the N-terminus of Cx43 (NT1), which recognizes both phosphorylated and unphosphorylated isoforms, and found a non-significant decrease (p = 0.19). Treatment of these samples with alkaline phosphatase collapses most of the Cx43-associated bands to its fastest migrating isoform (equivalent to non-phosphorylated) as shown with the NT1 antibody and dramatically reduces the pS279/282 Cx43 signal, indicating a reduction in the level of phosphorylation of these residues and confirming that the bands detected with pS279/282 are indeed phospho-Cx43 (Figure 1B). As S279/282 have been identified as consensus ERK1/2 phosphorylation sites [24], we expected to find an increase in the active, phosphorylated form of ERK1/2 (pERK1/2) concomitant with increased phosphorylation of Cx43. Surprisingly, we observe no significant increase in the total levels of pERK1/2 normalized to total ERK1/2 by Western blot in whole cell lysates. Therefore, we hypothesized that lack of pERK1/2 sequestration to the nucleus could increase Cx43 and pERK1/2 interaction. When we immunoprecipitated total Cx43 and blotted for pERK1/2, we found a 1.8-fold enrichment in pERK1/2 association with Cx43 in *Lmna*^{-/-} cells compared to *Lmna*^{+/+} (Figure 1C).

Activated ERK1/2 has been previously shown to associate with the nuclear envelope via lamin A [29]. Therefore, we tested whether the lack of normal lamin A activity in *Lmna*^{-/-} cells would result in a loss of the normal sequestration of pERK1/2 to the nuclear envelope and abnormal ERK1/2 activity in the cytoplasm of mouse embryonic fibroblasts (MEFs) with and without lamin A. This could explain why total levels of pERK1/2 were unchanged by immunoblot, while pS279/282 Cx43 was increased. By immunofluorescence, we observe an increased presence of punctate pERK1/2 foci in the cytoplasm and potentially at the plasma membrane of *Lmna*^{-/-} cells (Figure 2A). We then measured the fraction of pERK1/2 signal that lay outside the nucleus as defined by DAPI staining. After quantitation, we see a 3-fold increase in the levels of cytoplasmic pERK1/2 in *Lmna*^{-/-} cells (p < 0.001) (Figure 2B). When the pS279/282 Cx43 antibody is used to immunostain MEFs, we can see an increase in the amount of immunofluorescence in *Lmna*^{-/-} cells (Figure 2C). Note that there is some prominent non-Cx43 nuclear staining with the pS279/282 antibody that does not overlay with the IF1 antibody which labels total Cx43, so an unidentified phosphorylated nuclear protein that exists in these fibroblasts apparently reacts with the pS279/282 Cx43 antibody. We have observed similar staining in some other cell lines and tissues, while others do not show this nuclear staining (data not shown). Therefore, to determine the fraction of total Cx43 signal (IF1) that was phosphorylated on S279/282, we excluded the nuclear pS279/282 Cx43 signal (as defined by DAPI staining) and quantitated the colocalization of the remaining pS279/282 Cx43 signal with total Cx43 as recognized by IF1. This analysis shows a 2-fold increase in the levels of pS279/282 Cx43 in *Lmna*^{-/-} cells (p < 0.001) over the control, which agrees with our 1.6-fold increase as measured by Western (Figure 2D).

After establishing that Cx43 phosphorylation was affected by the loss of lamin A, we sought to determine whether gap junction function and intercellular communication were affected.

In order to measure gap junction assembly, we performed the well-established calcein/DiI transfer assay with *Lmna*^{+/+} and *Lmna*^{-/-} MEFs. For this assay, the “donor” population of cells is labeled with calcein, a fluorescent dye that can pass between cells via gap junctions after cleavage by intracellular esterases. A second population of cells is labeled with DiI, a lipophilic cyanine dye that marks the recipient cells. Dye transfer is then calculated as the number of cells that are both calcein- and DiI-positive (acceptor) and are adjacent to a calcein-positive, DiI-negative (donor) cell over the total interfaces between donors and all DiI-positive cells (Figure 3A). We observe that levels of dye transfer are reduced by 60% in *Lmna*^{-/-} cells compared to *Lmna*^{+/+} cells ($p < 0.001$) (Figure 3B). To determine whether the defect had any directionality, i.e., whether *Lmna*^{-/-} cells were defective when serving as either donor or acceptor, we mixed *Lmna*^{-/-} cells as either donor or acceptor with the respective *Lmna*^{+/+} acceptor or donor. We observed no difference in these mixed calcein/DiI experiments compared to results with *Lmna*^{-/-} alone and saw no improvement towards the levels exhibited by *Lmna*^{+/+} cells alone (data not shown). This shows that gap junctions in *Lmna*^{-/-} cells are deficient and that otherwise functional Cx43 in adjacent *Lmna*^{+/+} cells is not sufficient to rescue gap junctional communication.

Using the *Lmna*^{-/-} mouse strain, we performed Western blotting for pS279/282 Cx43 on whole heart lysates from *Lmna*^{-/-} mice and wild-type littermate controls. Consistent with our results in MEFs, we observe a 1.7-fold increase in levels of pS279/282 Cx43 normalized to total Cx43 in 6-week old *Lmna*^{-/-} mice compared to their wild-type littermates (*Lmna*^{+/+} $n = 5$, *Lmna*^{-/-} $n = 7$, $p < 0.01$) (Figure 4A and 4B). We also detect increased pERK1/2 in *Lmna*^{-/-} mice, as we have previously described [31]. Immunofluorescence staining of heart cryosections reveals the presence of pS279/282 Cx43 at the gap junction as demarcated with the IF1 Cx43 antibody in *Lmna*^{-/-} mice but not in their *Lmna*^{+/+} littermates (Figure 4C).

Discussion

In the course of these studies, we have established a pathway that may explain how loss of A-type lamin activity leads to conduction defects in the heart. We have demonstrated that in *Lmna*^{-/-} cells, pERK1/2 is not properly anchored and sequestered to the nuclear envelope, allowing a significant increase in the fraction of cytoplasmic pERK1/2 when compared to *Lmna*^{+/+} cells. This results in increased phosphorylation on S279/282 of Cx43 and contributes to decreased cell communication. We also observe a similar result *in vivo* as we see increased pERK1/2 levels and pS279/282 Cx43 in *Lmna*^{-/-} mice. S279/282 Cx43 phosphorylation has been previously identified to cause decreased conductance and channel open time [32], as well as increased degradation of Cx43 [33], so we assert that increased S279/282 phosphorylation likely contributes to the decreased conduction velocity previously measured by ECG in *Lmna*^{-/-} mice [28, 31] and potentially other mouse models for laminopathies that show increased ERK1/2 activation [34, 35].

Very recently, it was reported that there may be residual lamin A protein in the *Lmna*^{-/-} mouse [36], drawing into question whether *Lmna*^{-/-} mice really lack all A-type lamins. In contrast with their report, we did not detect the presence of lamin A in *Lmna*^{-/-} mice or cells in our experiments. While the data presented by Jahn, et al. are very interesting and potentially insightful, it remains to be seen whether the form of lamin A that is purportedly expressed has functional activity, as the *Lmna*^{-/-} mouse clearly displays phenotypes of cardiomyopathy, and thus remains a useful model. There are many other mouse models for laminopathies, such as the *Lmna*^{N195/N195K} and *Lmna*^{H222P/H222P} knock-in mice, which both exhibit dilated cardiomyopathy with conduction defect [34, 37], as well as the transgenic expression of a dominant negative M371K mutation in mice which resulted in cardiac defects as measured by pathology and death *in utero* [38]. Because of this, we feel that, whether or not the current *Lmna*^{-/-} model completely lacks all A-type lamin, it shares

many of the phenotypes with other disease-causing mutation models and remains a valuable tool for the study of laminopathies. Nonetheless, this recent study does highlight the importance of developing a new model that lacks all A-type lamins.

One of the remaining unanswered questions is how loss or mutation in A-type lamins could result in inappropriate ERK1/2 signaling. It has already been shown that lamins and ERK1/2 interact at the nuclear envelope [29, 30], and ERK1/2 activity is perturbed as a result of different EDMD-causing lamin mutations in mice, myoblasts and patient-derived fibroblasts [39–41]. Recently a crystal structure of the most C-terminal of the four α -helical segments in the central rod domain of lamin A, encoding either the R335W or E347K EDMD mutation, has been described [42]. The authors find no difference in the ability of these mutant alleles to form the parallel coiled-coil dimer, indicating that these disease causing residues do not significantly interfere with structure or polymerization. Instead, these particular residues associated with laminopathy are exposed at the surface of the coiled-coil dimer, leading to the hypothesis that mutation of these highly conserved residues affects the binding of A-type lamins to interacting proteins and/or nuclear lamina components.

Mutations in other genes such as emerin (*Emd*), another nuclear membrane protein that causes X-linked EDMD, and desmin (*Des*), a type III intermediate filament, are also associated with the development of cardiomyopathy. Increased ERK1/2 activity has been shown in both emerin-null *Emd*^{-/-} mice as well as in cells treated with *Emd*siRNA [43, 44]. Increased ERK1/2 activity has also been described in airway smooth muscle cells of *Des*^{-/-} mice resulting in increased *miR-26a* and hypertrophy [45]. Desmin aggregation in *Lmna*^{-/-} cardiac tissue has been previously described [28, 31] and a similar phenotype has been reported in mice with cardiac-specific expression of a 7-amino acid deletion of desmin (D7-des) [46]. D7-des mice exhibit desmin aggregation, loss of Cx43 from the intercalated disk, and decreased conduction velocity in a fashion very similar to *Lmna*^{-/-} mice. However, analysis of desmin in human patients with EDMD did not reveal any change in desmin localization or expression, implying that although desmin aggregation appears to be a downstream effect due to loss of A-type lamin function in mice, its role in human laminopathies may be more subtle [47]. Ultimately, the results from mice deficient in emerin or desmin illustrate the need for analysis of ERK1/2 activity in human patients to clarify its potential role in the development of laminopathies.

In conclusion, these data demonstrate a pathway by which loss of A-type lamins at the nuclear envelope result in decreased cell communication through phosphorylation of S279/282 on Cx43 by pERK1/2. We propose a mechanism in which the loss of normal A-type lamin decreases the interaction of pERK1/2 with the nuclear envelope and affects cellular function through Cx43 by increasing the rate of channel closure and possibly protein degradation. As ERK1/2 signaling has been demonstrated to be increased in two separate mouse models of laminopathy, it provides a promising target for further investigation of the development of laminopathies in humans.

Methods

Cell culture

Mouse embryonic fibroblasts from littermate-matched control and *Lmna*^{-/-} were derived and immortalized as previously described [27]. Cells were cultured and maintained in Dulbecco's Modified Eagle Medium (Cellgro) supplemented with 10% fetal bovine serum (SAFC Biosciences), 2 mM L-glutamine (Gibco) and penicillin/streptomycin (Gibco). The MEK1 inhibitor PD98059 (EMD) was diluted in DMSO and used at a 50 μ M concentration.

Animal husbandry and care

Lmna^{+/-} mice were originally generated and obtained from Dr. Colin Stewart [27] and were backcrossed on C57BL/6 (Jackson Laboratory) for 20 additional generations. The resulting *Lmna*^{+/-} progeny were interbred to produce *Lmna*^{+/+} and *Lmna*^{-/-} progeny.

All mice were bred and maintained under specific pathogen-free conditions. All experiments were performed in compliance with either the University of Washington Institutional Animal Care and Use Committee or the Fred Hutchinson Cancer Research Center Institutional Animal Care and Use Committee. Euthanasia of mice for analysis was accomplished by isoflurane overdose followed by cervical dislocation.

Genotyping on ear clip samples was performed using the Extract-N-Amp Tissue PCR Kit (Sigma Aldrich). Primers for the detection of *Lmna* and its knockout allele [27]: 5'-wild-type – TGCTGATGCCATGGATACTC; 5'-knockout – GCACGAGACTAGTGAGACGTG; 3'-common – GAGAAGGCAGAGGTGTGAGCAGC yielding ~1 kb and 700 bp fragments respectively.

SDS-PAGE and Western blotting

Mouse hearts were lysed by sonication on ice in 500 μ L of lysis buffer consisting of 3X Laemmli buffer supplemented with 5% beta-mercaptoethanol, 10 mM NaF, 0.5 mM NaVO₃, 2 mM PMSF and cOmplete mini protease inhibitor cocktail (Roche). MEF lysates were collected in the same lysis buffer by using a cell scraper followed by sonication on ice.

Lysates were separated via SDS-PAGE using 10% Tris-Glycine PAGER Gold precast gels (Lonza). Proteins were transferred to nitrocellulose and blocked in 1% milk supplemented with 10 mM NaF.

Nitrocellulose membranes were incubated with one or more of the following antibodies overnight at 4°C: lamin A/C (Cell Signaling 2032; 1:500), pERK1/2 (Cell Signaling 9101; 1:500), ERK1/2 (Santa Cruz sc-94; 1:5000), α -tubulin (Cell Signaling 2125; 1:1000), N-terminus connexin43 (NT1; FHCRC; 1:1000), and pS279/282 Cx43 (FHCRC; 1:1000). Appropriate secondary antibodies were then applied for 60 minutes at room temperature at 1:10000 dilution: AlexaFluor680 goat anti-rabbit (Invitrogen) or AlexaFluor800 donkey anti-mouse (Invitrogen). Blots were scanned using an Odyssey infrared imager (Li-Cor, Omaha, NE). Quantitation was performed with Odyssey software.

Co-immunoprecipitation

Recombinant protein Agarose (Pierce) was incubated with PBS containing 10% SDS and 1% BSA then rinsed thoroughly in PBS to remove any unbound protein A. The rinsed beads were then mixed with either pERK1/2 (Cell Signaling) or connexin43 IF1 and CT1 (FHCRC). Cells were lysed in PBS supplemented with 0.5% Triton X-100, 0.25% deoxycholate, 10 mM NaF, 0.5 mM NaVO₃, 2 mM PMSF and cOmplete mini protease inhibitor cocktail. Cell lysates were added to beads and rotated end over end at 4°C for 90 minutes then thoroughly rinsed with PBS. Before loading for SDS-PAGE, 3X Laemmli sample buffer supplemented with 5% beta-mercaptoethanol was added and boiled briefly.

Immunofluorescence

MEFs were grown in a 24-well dish on round coverslips and fixed in 2% formalin in PBS for 15 minutes. Cells were then permeabilized with PBS supplemented with 0.5% TritonX-100 for 10 minutes. Blocking was performed by incubating with PBS containing 0.2% Tween-20 (PBST), 5% goat serum and 0.2% fish skin gelatin (referred to as “blocking buffer”) at 37°C for 15 minutes. Primary and secondary antibodies were diluted in blocking

buffer and incubated with coverslips at 37°C for 60 minutes. DAPI staining was performed and coverslips were mounted in Fluoromount-G. Images were collected using an Nikon TE-400 microscope and a NIS-Elements (Nikon) camera and then analyzed using Metamorphsoftware (Molecular Devices).

Heart tissue was frozen in Tissue-Tek O.C.T compound, cryosectioned at 8µm in thickness on a Leica CM3050S and mounted on Superfrost Plus glass slides (Fisher). Sections were fixed in -20°C acetone for 20 minutes and air dried. Blocking was performed in PBS with 1% BSA (PBSB) supplemented with 0.3% TritonX-100 for 15 minutes. Primary antibodies were diluted in PBSB and applied to slides for 60 minutes at room temperature. Appropriate secondary antibodies were then applied for 60 minutes at 1:200 in PBSB. Following secondary antibody exposure, DAPI staining was performed with 5 µg/mL DAPI in PBS. Sections were then mounted in Fluoromount-G (Southern Biotech).

Primary antibodies included: lamin A/C (Cell Signaling 4777; 1:500), pERK1/2 (Cell Signaling 4370; 1:200), connexin43 IF1 (FHCRC; 1:1000), connexin43 CT1 (FHCRC; 1:2000), and pS279/282 connexin43 (FHCRC; 1:500). Secondary antibodies used include: AlexaFluor488 goat anti-mouse, AlexaFluor594 goat anti-rabbit, AlexaFluor488 goat anti-rabbit and AlexaFluor594 goat anti-mouse (Invitrogen).

Calcein/Dil transfer assay

For each calcein/Dil transfer assay, one 60-mm dish of cells were incubated with 2.5 ng/uL calcein-AM (Invitrogen) in PBS for 10 minutes and one 10-cm dish of cells were incubated with 5 nM Dil (Sigma) in PBS for 5 minutes. After thorough rinsing, the two dishes of cells were then trypsinized and plated together in a single well of a 6-well plate. The cells were then allowed to recover and were incubated at 37°C for 2 hours. Images were captured using a digital camera on a Nikon TE-400 inverted microscope. The captured images were manually scored using ImageJ to determine the total number of donor cells (calcein-labeled only) to adjacent Dil-labeled acceptor cells (i.e., total interfaces) that were either calcein-positive indicating dye transfer or negative, and then the ratio of positive transfer over total interfaces between was taken as a measure of cell communication.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 5. All analyses utilized a two-tailed unpaired t-test.

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Abbreviations

DCM-CD	Dilated cardiomyopathy with conduction system disease
EDMD	Emery-Dreifuss muscular dystrophy
LGMD	Limb-girdle muscular dystrophy
Cx43	Connexin43
MAPK	Mitogen-activated protein kinase

ERK	Extracellular signal-regulated kinase
MEF	Mouse embryonic fibroblast
pERK	phospho-ERK
pS279/282	Cx43 phosphorylated on S279/282

References

1. Broers JL, Kuijpers HJ, Ostlund C, Worman HJ, Endert J, Ramaekers FC. Both lamin A and lamin C mutations cause lamina instability as well as loss of internal nuclear lamin organization. *Exp Cell Res.* 2005; 304:582–592. [PubMed: 15748902]
2. Lammerding J, Fong LG, Ji JY, Reue K, Stewart CL, Young SG, Lee RT. Lamins A and C but not lamin B1 regulate nuclear mechanics. *J Biol Chem.* 2006; 281:25768–25780. [PubMed: 16825190]
3. Stuurman N, Heins S, Aeby U. Nuclear lamins: their structure, assembly, and interactions. *J Struct Biol.* 1998; 122:42–66. [PubMed: 9724605]
4. Stierle V, Couprie J, Ostlund C, Krimm I, Zinn-Justin S, Hossenlopp P, Worman HJ, Courvalin JC, Duband-Goulet I. The carboxyl-terminal region common to lamins A and C contains a DNA binding domain. *Biochemistry.* 2003; 42:4819–4828. [PubMed: 12718522]
5. Worman HJ, Courvalin JC. The inner nuclear membrane. *J Membr Biol.* 2000; 177:1–11. [PubMed: 10960149]
6. Stewart CL, Roux KJ, Burke B. Blurring the boundary: the nuclear envelope extends its reach. *Science.* 2007; 318:1408–1412. [PubMed: 18048680]
7. Goldman RD, Gruenbaum Y, Moir RD, Shumaker DK, Spann TP. Nuclear lamins: building blocks of nuclear architecture. *Genes Dev.* 2002; 16:533–547. [PubMed: 11877373]
8. Cohen TV, Hernandez L, Stewart CL. Functions of the nuclear envelope and lamina in development and disease. *Biochem Soc Trans.* 2008; 36:1329–1334. [PubMed: 19021550]
9. Dahl KN, Ribeiro AJ, Lammerding J. Nuclear shape, mechanics, and mechanotransduction. *Circ Res.* 2008; 102:1307–1318. [PubMed: 18535268]
10. Worman HJ. Nuclear lamins and laminopathies. *J Pathol.* 2012; 226:316–325. [PubMed: 21953297]
11. Malhotra R, Mason PK. Lamin A/C deficiency as a cause of familial dilated cardiomyopathy. *Curr Opin Cardiol.* 2009; 24:203–208.
12. van Tintelen JP, Hofstra RM, Katerberg H, Rossenbacker T, Wiesfeld AC, du Marchie Sarvaas GJ, Wilde AA, van Langen IM, Nannenberg EA, van der Kooij AJ, Kraak M, van Gelder IC, van Veldhuisen DJ, Vos Y, van den Berg MP. High yield of LMNA mutations in patients with dilated cardiomyopathy and/or conduction disease referred to cardiogenetics outpatient clinics. *Am Heart J.* 2007; 154:1130–1139. [PubMed: 18035086]
13. Parks SB, Kushner JD, Nauman D, Burgess D, Ludwigsen S, Peterson A, Li D, Jakobs P, Litt M, Porter CB, Rahko PS, Hershberger RE. Lamin A/C mutation analysis in a cohort of 324 unrelated patients with idiopathic or familial dilated cardiomyopathy. *Am Heart J.* 2008; 156:161–169. [PubMed: 18585512]
14. van Berlo JH, de Voogt WG, van der Kooij AJ, van Tintelen JP, Bonne G, Yaou RB, Duboc D, Rossenbacker T, Heidbuchel H, de Visser M, Crijns HJ, Pinto YM. Meta-analysis of clinical characteristics of 299 carriers of LMNA gene mutations: do lamin A/C mutations portend a high risk of sudden death? *J Mol Med.* 2005; 83:79–83. [PubMed: 15551023]
15. van Rijsingen IA, Arbustini E, Elliott PM, Mogensen J, Hermans-van Ast JF, van der Kooij AJ, van Tintelen JP, van den Berg MP, Pilotto A, Pasotti M, Jenkins S, Rowland C, Aslam U, Wilde AA, Perrot A, Pankuweit S, Zwinderman AH, Charron P, Pinto YM. Risk factors for malignant ventricular arrhythmias in lamin a/c mutation carriers a European cohort study. *J Am Coll Cardiol.* 2012; 59:493–500. [PubMed: 22281253]
16. Lu JT, Muchir A, Nagy PL, Worman HJ. LMNA cardiomyopathy: cell biology and genetics meet clinical medicine. *Dis Model Mech.* 2011; 4:562–568. [PubMed: 21810905]

17. Becane HM, Bonne G, Varnous S, Muchir A, Ortega V, Hammouda EH, Urtizberea JA, Lavergne T, Fardeau M, Eymard B, Weber S, Schwartz K, Duboc D. High incidence of sudden death with conduction system and myocardial disease due to lamins A and C gene mutation. *Pacing Clin Electrophysiol.* 2000; 23:1661–1666. [PubMed: 11138304]
18. Saez JC, Berthoud VM, Branes MC, Martinez AD, Beyer EC. Plasma membrane channels formed by connexins: their regulation and functions. *Physiol Rev.* 2003; 83:1359–1400. [PubMed: 14506308]
19. Davis LM, Rodefeld ME, Green K, Beyer EC, Saffitz JE. Gap junction protein phenotypes of the human heart and conduction system. *J Cardiovasc Electrophysiol.* 1995; 6:813–822. [PubMed: 8542077]
20. Reaume AG, de Sousa PA, Kulkarni S, Langille BL, Zhu D, Davies TC, Juneja SC, Kidder GM, Rossant J. Cardiac malformation in neonatal mice lacking connexin43. *Science.* 1995; 267:1831–1834. [PubMed: 7892609]
21. Gutstein DE, Morley GE, Tamaddon H, Vaidya D, Schneider MD, Chen J, Chien KR, Stuhlmann H, Fishman GI. Conduction slowing and sudden arrhythmic death in mice with cardiac-restricted inactivation of connexin43. *Circ Res.* 2001; 88:333–339. [PubMed: 11179202]
22. Gutstein DE, Morley GE, Fishman GI. Conditional gene targeting of connexin43: exploring the consequences of gap junction remodeling in the heart. *Cell Commun Adhes.* 2001; 8:345–348. [PubMed: 12064616]
23. Marquez-Rosado L, Solan JL, Dunn CA, Norris RP, Lampe PD. Connexin43 phosphorylation in brain, cardiac, endothelial and epithelial tissues. *Biochim Biophys Acta.* 2011
24. Warn-Cramer BJ, Lampe PD, Kurata WE, Kanemitsu MY, Loo LW, Eckhart W, Lau AF. Characterization of the mitogen-activated protein kinase phosphorylation sites on the connexin-43 gap junction protein. *J Biol Chem.* 1996; 271:3779–3786. [PubMed: 8631994]
25. Warn-Cramer BJ, Cottrell GT, Burt JM, Lau AF. Regulation of connexin-43 gap junctional intercellular communication by mitogen-activated protein kinase. *J Biol Chem.* 1998; 273:9188–9196. [PubMed: 9535909]
26. Rivedal E, Opsahl H. Role of PKC and MAP kinase in EGF- and TPA-induced connexin43 phosphorylation and inhibition of gap junction intercellular communication in rat liver epithelial cells. *Carcinogenesis.* 2001; 22:1543–1550. [PubMed: 11532878]
27. Sullivan T, Escalante-Alcalde D, Bhatt H, Anver M, Bhat N, Nagashima K, Stewart CL, Burke B. Loss of A-type lamin expression compromises nuclear envelope integrity leading to muscular dystrophy. *J Cell Biol.* 1999; 147:913–920. [PubMed: 10579712]
28. Nikolova V, Leimena C, McMahon AC, Tan JC, Chandar S, Jogia D, Kesteven SH, Michalick J, Otway R, Verheyen F, Rainer S, Stewart CL, Martin D, Feneley MP, Fatkin D. Defects in nuclear structure and function promote dilated cardiomyopathy in lamin A/C- deficient mice. *J Clin Invest.* 2004; 113:357–369. [PubMed: 14755333]
29. Rodriguez J, Calvo F, Gonzalez JM, Casar B, Andres V, Crespo P. ERK1/2 MAP kinases promote cell cycle entry by rapid, kinase-independent disruption of retinoblastoma-lamin A complexes. *J Cell Biol.* 2010; 191:967–979. [PubMed: 21115804]
30. Gonzalez JM, Navarro-Puche A, Casar B, Crespo P, Andres V. Fast regulation of AP-1 activity through interaction of lamin A/C, ERK1/2, and c-Fos at the nuclear envelope. *J Cell Biol.* 2008; 183:653–666. [PubMed: 19015316]
31. Frock RL, Chen SC, Da DF, Frett E, Lau C, Brown C, Pak DN, Wang Y, Muchir A, Worman HJ, Santana LF, Ladiges WC, Rabinovitch PS, Kennedy BK. Cardiomyocyte-Specific Expression of Lamin A Improves Cardiac Function in *Lmna*^{-/-} Mice. *PLoS One.* 2012; 7:e42918. [PubMed: 22905185]
32. Cottrell GT, Lin R, Warn-Cramer BJ, Lau AF, Burt JM. Mechanism of v-Src- and mitogen-activated protein kinase-induced reduction of gap junction communication. *Am J Physiol Cell Physiol.* 2003; 284:C511–520. [PubMed: 12388103]
33. Leykauf K, Salek M, Bomke J, Freeh M, Lehmann WD, Durst M, Alonso A. Ubiquitin protein ligase Nedd4 binds to connexin43 by a phosphorylation-modulated process. *J Cell Sci.* 2006; 119:3634–3642. [PubMed: 16931598]

34. Arimura T, Helbling-Leclerc A, Massart C, Varnous S, Niel F, Lacene E, Fromes Y, Toussaint M, Mura AM, Keller DI, Amthor H, Isnard R, Malissen M, Schwartz K, Bonne G. Mouse model carrying H222P-Lmna mutation develops muscular dystrophy and dilated cardiomyopathy similar to human striated muscle laminopathies. *Hum Mol Genet.* 2005; 14:155–169. [PubMed: 15548545]
35. Muchir A, Shan J, Bonne G, Lehnart SE, Worman HJ. Inhibition of extracellular signal-regulated kinase signaling to prevent cardiomyopathy caused by mutation in the gene encoding A-type lamins. *Hum Mol Genet.* 2009; 18:241–247. [PubMed: 18927124]
36. Jahn D, Schramm S, Schnolzer M, Heilmann CJ, de Koster CG, Schutz W, Benavente R, Alsheimer M. A truncated lamin A in the Lmna (–/–) mouse line: Implications for the understanding of laminopathies. *Nucleus.* 2012; 3:463–474. [PubMed: 22895093]
37. Mounkes LC, Kozlov SV, Rottman JN, Stewart CL. Expression of an LMNA-N195K variant of A-type lamins results in cardiac conduction defects and death in mice. *Hum Mol Genet.* 2005; 14:2167–2180. [PubMed: 15972724]
38. Wang Y, Herron AJ, Worman HJ. Pathology and nuclear abnormalities in hearts of transgenic mice expressing M371K lamin A encoded by an LMNA mutation causing Emery-Dreifuss muscular dystrophy. *Hum Mol Genet.* 2006; 15:2479–2489. [PubMed: 16825283]
39. Muchir A, Pavlidis P, Decostre V, Herron AJ, Arimura T, Bonne G, Worman HJ. Activation of MAPK pathways links LMNA mutations to cardiomyopathy in Emery-Dreifuss muscular dystrophy. *J Clin Invest.* 2007; 117:1282–1293. [PubMed: 17446932]
40. Emerson LJ, Holt MR, Wheeler MA, Wehnert M, Parsons M, Ellis JA. Defects in cell spreading and ERK1/2 activation in fibroblasts with lamin A/C mutations. *Biochim Biophys Acta.* 2009; 1792:810–821. [PubMed: 19524666]
41. Favreau C, Delbarre E, Courvalin JC, Buendia B. Differentiation of C2C12 myoblasts expressing lamin A mutated at a site responsible for Emery-Dreifuss muscular dystrophy is improved by inhibition of the MEK-ERK pathway and stimulation of the PI3-kinase pathway. *Exp Cell Res.* 2008; 314:1392–1405. [PubMed: 18294630]
42. Bollati M, Barbiroli A, Favalli V, Arbustini E, Charron P, Bolognesi M. Structures of the lamin A/C R335W and E347K mutants: implications for dilated cardiomyopathies. *Biochem Biophys Res Commun.* 2012; 418:217–221. [PubMed: 22266370]
43. Muchir A, Pavlidis P, Bonne G, Hayashi YK, Worman HJ. Activation of MAPK in hearts of EMD null mice: similarities between mouse models of X-linked and autosomal dominant Emery Dreifuss muscular dystrophy. *Hum Mol Genet.* 2007; 16:1884–1895. [PubMed: 17567779]
44. Muchir A, Wu W, Worman HJ. Reduced expression of A-type lamins and emerin activates extracellular signal-regulated kinase in cultured cells. *Biochim Biophys Acta.* 2009; 1792:75–81. [PubMed: 19022376]
45. Mohamed JS, Hajira A, Li Z, Paulin D, Boriek AM. Desmin regulates airway smooth muscle hypertrophy through early growth-responsive protein-1 and microRNA-26a. *J Biol Chem.* 2011; 286:43394–43404. [PubMed: 21903578]
46. Gard JJ, Yamada K, Green KG, Eloff BC, Rosenbaum DS, Wang X, Robbins J, Schuessler RB, Yamada KA, Saffitz JE. Remodeling of gap junctions and slow conduction in a mouse model of desmin-related cardiomyopathy. *Cardiovasc Res.* 2005; 67:539–547. [PubMed: 15913582]
47. Piercy RJ, Zhou H, Feng L, Pombo A, Muntoni F, Brown SC. Desmin immunolocalisation in autosomal dominant Emery-Dreifuss muscular dystrophy. *Neuromuscul Disord.* 2007; 17:297–305. [PubMed: 17329105]

Highlights

- Connexin43 phosphorylation plays a role in laminopathy-associated conduction defects.
- Loss of A-type lamin activity results in release of pERK1/2 from the nucleus.
- Increased cytoplasmic localization of pERK1/2 acts to phosphorylate S279/282 of Cx43.
- Phosphorylation of S279/282 on Cx43 decreases gap junction activity in cell culture.
- Mice lacking A-type lamins have increased phosphorylation on S279/282 of Cx43.

Figure 1. pS279/282 Cx43 increased in *Lmna*^{-/-} MEFs

(A) Representative Western blot analysis of *Lmna*^{+/+} (+/+) and *Lmna*^{-/-} (-/-) MEFs show increased levels and appearance of high-molecular weight isoform of pS279/282 Cx43 relative to total Cx43 (NT1) in cells deficient for lamin A/C. Levels of pERK1/2 normalized to total ERK1/2 are not significantly changed. α-tubulin is shown as a loading control. Boxes representing the areas used for quantification are shown in lane 1. (B) *Lmna*^{+/+} and *Lmna*^{-/-} lysates treated with alkaline phosphatase (+AP) collapse and reduce pS279/282 Cx43 bands. (C) Co-immunoprecipitation of *Lmna*^{+/+} and *Lmna*^{-/-} MEFs using the IF1 and CT1 Cx43 antibodies to precipitate total Cx43 and probed with pERK1/2 show an increased amount of pERK1/2 interacting with Cx43.

Figure 2. Localization of pERK1/2 shifts towards cytoplasm in *Lmna*^{-/-} cells and increases S279/282 phosphorylation of Cx43

(A) Immunofluorescence staining of *Lmna*^{+/+} and *Lmna*^{-/-} MEFs with pERK1/2 antibody show an increased presence of pERK1/2 in the cytoplasm of *Lmna*^{-/-} MEFs as indicated by white arrows. Note we are not implying this potential membrane staining would co-localize with Cx43. Scale bar denotes 10 μ m. (B) Scoring of cytoplasmic pERK1/2 staining in immunofluorescence images of *Lmna*^{+/+} (n = 398) and *Lmna*^{-/-} (n = 397) cells show a significant increase in frequency of cytoplasmic pERK1/2 in *Lmna*^{-/-} cells compared to *Lmna*^{+/+} (p < 0.001). (C) Immunofluorescence staining of *Lmna*^{+/+} and *Lmna*^{-/-} MEFs with IF1 Cx43 and pS279/282 Cx43 show no significant change in Cx43 staining by IF1, but increased levels of pS279/282 Cx43, with a cross-reactive signal in the nucleus. Areas of notable colocalization are indicated by white arrows. Scale bar denotes 10 μ m. (D) Quantitation of pS279/282 Cx43 by excluding non-IF1 Cx43 signal shows a significant 2-fold increase of pS279/282 Cx43 in *Lmna*^{-/-} cells (n = 209) vs *Lmna*^{+/+} (n = 118) (p < 0.001).

Figure 3. Cell communication is deficient in *Lmna*^{-/-} MEFs as measured by calcein/DiI transfer assay

(A) *Lmna*^{-/-} cells transfer significantly less dye than *Lmna*^{+/+} cells as illustrated by representative images of calcein/DiI transfer assay showing potential acceptor cells (DiI-positive) and donor cells (calcein-positive). Scale bar denotes 25 μ m. (B) Scoring of calcein/DiI images from *Lmna*^{+/+} or *Lmna*^{-/-} cells. *Lmna*^{-/-} cells (n = 1344) had significantly less cell communication compared to *Lmna*^{+/+} (n = 1388) in all cases (p < 0.001).

Figure 4. pS279/282 Cx43 is increased in *Lmna*^{-/-} mouse heart

(A) Representative Western blot analysis of heart lysates from either *Lmna*^{+/+} or *Lmna*^{-/-} mice demonstrating increased pERK1/2 relative to total ERK1/2 and increased pS279/282 Cx43 relative to total NT1 Cx43 signal. α-tubulin is shown as a loading control. Boxes representing the areas used for quantification are shown in lane 1. (B) Quantification of pS279/282 signal normalized to NT1 Cx43 reveals a significant 1.7-fold increase in *Lmna*^{-/-} mice (*Lmna*^{+/+} n = 5, *Lmna*^{-/-} n = 7, p < 0.01). (C) Immunofluorescence of ventricle sections from *Lmna*^{+/+} and *Lmna*^{-/-} mice stained with pS279/282 Cx43 and IF1 Cx43, which predominantly recognizes functional gap junctions at the intercalated disk, show localization and increased presence of pS279/282 at the intercalated disk in *Lmna*^{-/-} heart. Scale bar denotes 10 μm.