A Novel Mechanism Involving Four-and-a-half LIM Domain Protein-1 and Extracellular Signal-regulated Kinase-2 Regulates Titin Phosphorylation and Mechanics*§

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Background: Titin is critical for cardiac muscle function; however, limited knowledge exists of mechanisms important for its regulation.

Results: A four-and-a-half LIM domain protein-1/extracellular signal-regulated kinase-2-associated complex modulates titin-N2B levels, phosphorylation, and mechanics.

Conclusion: We reveal new mechanisms underlying titin mechano-signaling.

Significance: We advance our understanding of how titin-associated complexes/mutations can impact cardiac muscle function and disease.

Understanding mechanisms underlying titin regulation in cardiac muscle function is of critical importance given recent compelling evidence that highlight titin mutations as major determinants of human cardiomyopathy. We previously identified a cardiac biomechanical stress-regulated complex at the cardiac-specific N2B region of titin that includes four-and-a-half LIM domain protein-1 (Fhl1) and components of the mitogen-activated protein signaling cascade, which impacted muscle compliance in Fhl1 knock-out cardiac muscle. However, direct regulation of these molecular components in mediating titin N2B function remained unresolved. Here we identify Fhl1 as a novel negative regulator of titin N2B levels and phosphorylation-mediated mechanics. We specifically identify titin N2B as a novel substrate of extracellular signal-regulated kinase-2 (Erk2) and demonstrate that Fhl1 directly interferes with Erk2-mediated titin-N2B phosphorylation. We highlight the critical region in titin-N2B that interacts with Fhl1 and residues that are dependent on Erk2-mediated phosphorylation in situ. We also propose a potential mechanism for a known titin-N2B cardiomyopathy-causing mutation that involves this regulatory complex. These studies shed light on a novel mechanism regulating titin-N2B mechano-signaling as well as suggest that dysfunction of these pathways could be important in cardiac disease states affecting muscle compliance.

Titin is a large ~3.0-MDa myofilament protein that spans the sarcomere from the Z-line to the M-line (1). The importance of titin in human cardiac disease was recently highlighted when titin truncation mutations were identified as a major genetic cause of human dilated cardiomyopathy; however, the precise signaling and functional consequences of titin dysregulation and disease-causing mutations remain unresolved (2). Despite the complex nature of titin, it is the major determinant of passive tension and muscle compliance within cardiomyocytes over the physiological sarcomere length range (3). The I-band region of titin in particular is a complex molecular spring consisting of PEVK and tandem Ig segments as well as N2A and N2B elements, which extend sequentially and generate passive tension after stretch (4). Cardiac muscle expresses two major titin isoforms that include the larger and more compliant N2BA splice isoform, which consists of both N2A and N2B elements as well as the shorter, stiffer N2B splice isoform, which consists of N2B alone (5, 6). The phosphorylation status of titin plays a critical role in mediating muscle compliance in a titin isoform-dependent manner. For example, the cardiac-specific N2B element of titin is a substrate of protein kinase A (PKA) and PKG, and its phosphorylation can affect diastolic muscle mechanics and muscle compliance after stretch (7–10). Titin N2B phosphorylation has also been suggested to influence cardiomyocyte stiffness in disease states (11). Targeted deletion of titin N2B in mice highlights its importance in diastolic mechanics in vivo (12); however, mechanisms regulating its phosphorylation in muscle function as well as whether other kinases can target titin N2B remains unresolved.

We identified a novel complex within the cardiomyocyte sarcomere, which involves the association of titin (N2B) with four-and-a-half LIM domain protein-1 (Fhl1) and the mitogen-ac-
tivated protein kinase signaling cascade (Raf1/Mek1/2/Erk2) (13). Fhl1 is a member of the Fhl subfamily, which is structurally characterized by four complete LIM domains and an N-terminal half LIM domain (14). LIM domain proteins play key roles in mediating protein-protein interactions, and their functions are largely dependent upon their associated binding partners (15–17). Previous studies have suggested a role for LIM domain proteins in biomechanical stress responses and as structural adaptors as well as subcellular localizers (18–20). Using Fhl1 knock-out (FHL1KO) mice we showed that Fhl1 plays an important role in sensing biomechanical hypertrophic stress responses in the sarcomere via titin N2B, which in turn regulates MAPK/hypertrophic signaling and muscle compliance (13). The Raf1-Mek1/2-Erk1/2 signaling pathway and multiple downstream effectors of this pathway have been shown to play an important role in regulating cardiac hypertrophy/stress in vitro and in vivo (21–24); however, how the molecular interaction of these components modulate Fhl1 and its influence on titin N2B mechanics and cardiac stress remained unresolved.

In this study, we identify Fhl1 as a novel negative regulator of titin N2B levels, phosphorylation, and mechanics. To further establish a mechanistic link between titin and MAPK signaling components (Raf1/Mek1/2/Erk2), we identified titin N2B as a novel substrate of Erk2. We also demonstrate that a dose-dependent increase in Fhl1 can interfere with Erk2-mediated titin N2B phosphorylation in vitro, suggesting the possibility that Fhl1 binds and competes with Erk2 to mask phosphorylation sites on titin N2B. We further identify the critical Erk phosphorylation sites important for human titin N2B phosphorylation in situ and a potential mechanism for a known titin N2B cardiac disease-causing mutation that directly impacts Erk2-mediated titin N2B phosphorylation. Dysfunction of these pathways could be important in regulating muscle compliance in disease states, such as pathological hypertrophy as well as diseases affecting diastolic stiffness as well as understanding novel functions of the ERK pathway.

EXPERIMENTAL PROCEDURES

Ex Vivo Intact Adult Mouse Cardiac Muscle Mechanics Model—FHL1KO mice have been previously described and characterized (13). Right ventricular papillary muscles were isolated from adult FHL1KO and wild-type littermate or age-matched mice and mounted horizontally in a cardiac tissue culture chamber as previously described (13). Cardiac muscles from FHL1KO mice do not display any significant differences in cross-sectional area as well as slack cardiomyocyte sarcomere length when compared with wild-type mice, as previously shown (13). For titin N2B phosphorylation-mediated compliance experiments, isoproterenol (Sigma; 1 μM) or vehicle was added to media of muscle preparations. To skin the cardiac muscle preparations, muscles were perfused for 2 h in a relaxing solution (pH 7.0) containing 7.8 mM ATP, 20 mM creatine phosphate, 20 mM imidazole, 4 mM EGTA, 12 mM magnesium proprionate, 97.6 mM potassium proprionate, 40 μg/ml leupeptin, 30 mM 2,3-butanedione monoxime, with pCa 8.0 and 2% Triton X-100 at a temperature of −10 °C as previously described (8). Muscles were then perfused with relaxing solution without Triton X-100 to remove the detergent. Preparations were continuously stretched (three times) from slack muscle length up to a diastolic stress of 8 kPa, which is a level of stress previously shown to stretch the sarcomere within physiological limits (3, 13). Stress-strain data were obtained as previously described (13). Local Lagrangian uniaxial strains ($E = 1/(2λ^2 - 1)$, where $λ$ is the stretch ratio) were calculated from the muscle stretch ratios using slack length of each muscle as its reference. The stretch ratio was determined from the displacement of two of the applied surface markers located along the long-axis fiber direction of the tissue. Cauchy stresses were calculated by dividing force by the initial cross-sectional area of each muscle. All animal procedures were approved by the University of California-San Diego Animal Care and Use Committee.

Protein Gel and Blot Analysis—For titin isoform studies, protein was isolated from left ventricular tissues and separated by vertical agarose gel electrophoresis (1%) as previously described (25) and were visualized by Coomassie staining according to manufacturer’s instructions (Sigma). Gels were scanned, and the isoform ratios were quantified by densitometry using OneDScan software and Excel (Microsoft) for statistical analysis. For Western blot analyses of phospholamban expression, total protein was isolated from the hearts as previously described (13). Immunodetection using anti-phosphorylated phospholamban (Serine-16), Upstate Biotechnology, 1:10,000) and total phospholamban (Upstate Biotechnology, 1:1000) antibodies was performed on protein lysates as previously described (13). Sypro-Ruby and Pro-Q Diamond phosphoprotein stains were used for the detection and semiquantitative analysis of total and phosphorylated myofilament protein levels in myofibrils. Myofibril homogenates from hearts were prepared, stained, and analyzed as previously described (26). Briefly, homogenates were loaded at total protein amounts of 4, 8, and 12 μg onto 10% Bio-Rad Criterion polyacrylamide gels, and electrophoresis was performed at 175 V for 62 min. Gels were removed, washed twice with deionized water and fixed 3 times for 30 min each time in fixative solution (50% methanol, 10% glacial acetic acid) with shaking. For detection of phosphorylated proteins, gels were stained with 75 ml of Pro-Q Diamond (Molecular Probes: P-33300) for 90 min in an opaque container. Gels were then washed twice with deionized water, and de-stained 4 times for 25 min each time with Pro-Q Diamond De-staining solution (Molecular Probes: P-33311). Pro-Q diamond fluorescence images were captured using a Molecular Imager F/X (Bio-Rad) with laser excitation at 532 nm and long pass filter at 555 nm. Gels were then post-stained with 60 ml of Sypro-Ruby (Molecular Probes: S12000) overnight and de-stained 4 times for 30 min each time with Sypro-Ruby de-staining solution (10% methanol, 7% glacial acetic acid). Sypro-Ruby fluorescence images were captured using a UVP EC3 imaging system at the UV source with a 302-nm excitation filter and long pass filter at 560 nm emission filter. Bio-Rad Laser-Pix software was used to convert images of stained gels to quantitative values of total protein and phosphorylation levels.

Histological Analysis—Cardiac ventricular sections were post-fixed in 10% zinc-formalin and then stained with picrosirius red using standard procedures (Electron Microscopy Sciences).
Hydroxyproline Assay to Measure Collagen Content—Assays were performed essentially as previously described (27) with minor modifications. Heart tissue (300 mg wet weight) was dried at 50 °C for 6–8 h. Dry weight of heart tissue was measured and hydrolyzed for 36 h in 6 ml of 6 M HCl at 80 °C with occasional mixing. The hydrolyzing liquid was subsequently allowed to evaporate from samples overnight at 80 °C. Hydrolyzed tissue was resuspended in 2 ml of deionized water and stored at –20 °C. Hydroxyproline standards were prepared in assay buffer (172 mM citric acid, 139 mM glacial acetic acid, 975 mM sodium acetate, 570 mM sodium hydroxide, 0.1% toluene, and 20% isopropyl alcohol, pH 6.5). Subsequently, 10 μl of hydrolysate was combined with 190 μl of assay buffer, 100 μl of a hydroxyproline standard, and 150 μl of freshly prepared chloramine T reagent (0.141g of chloramine T hydrate in 10 ml of deionized water) followed by incubation at room temperature for 20–25 min. Ehrlich’s reagent (1M p-dimethylamino benzaldehyde, 60% isopropyl alcohol and 18.2% perchloric acid) was prepared within 5 min of use, 150 μl was added to each sample, and samples were incubated at 60 °C for 15 min. Absorbance of each sample was read at 550 nm, and concentration of hydroxyproline, representative of collagen content was determined based on the plotted standard curve and expressed as the number of arbitrary units per total dry weight of sample. Statistical analysis was done using Excel (Microsoft).

Generation of Constructs—Human titin N2B us3 region (amino acids 3750 – 4019; Ref. 27) was subcloned into a modified pGEX-2TK bacterial expression vector (Stratagene) where the multiple cloning site was replaced with the pEGFP-C1 (Clontech) multiple cloning site, resulting in an in-frame fusion of N-terminal GST tag with this region of titin. Construction of GFP-tagged titin N2B us3 (residues 3750 – 4019) and FLAG and GST-tagged FHL1 constructs have been described previously (28). Site-directed mutagenesis of wild-type human titin N2B us3 was done using a modified version of the QuikChange protocol (Stratagene). Briefly, oligonucleotides carrying the desired mutation (see supplemental Table 1) were used to generate single-stranded linear constructs incorporating the mutation using PCR. PCR products were incubated with T4 DNA ligase for 2 h at room temperature to circularize single-stranded PCR products, and T4 Ligase was inactivated at 75 °C for 20 min. After DpnI digestion of ligated PCR products for 4 h at 37 °C, the mixture containing circular single-stranded undigested mutated DNA constructs was transformed into XL1-blue cells. Sequence and correct integration of the mutation was verified by sequencing. All residue numbers correspond to NP_003310 (NCBI accession number, Homo sapiens titin (TTN) N2B transcript variant).

Protein Expression and in Vitro Kinase Assay—Bacterial expression and purification of GST fusion constructs was done as previously described (28). Briefly, GST fusion proteins were eluted off glutathione-Sepharose beads (Amersham Biosciences) using GST elution buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 150 mM reduced glutathione). Subsequently purified GST fusion proteins were dialyzed overnight at 4 °C against dialysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.4, 1 mM DTT), and protein concentration was determined using a Bradford protein assay (Bio-Rad). For in vitro kinase assay, 2 μg of either titin N2B us3, GST, or myelin basic protein in the absence or presence of Fhl1 or GST (2, 6, 10 μg) were incubated with 40 units of activated Erk2 enzyme (New England Biolabs) and radioactively labeled [γ-32P]ATP (Amersham Biosciences) for 30 min at 30 °C in kinase buffer (50 mM Tris-HCl, 10 mM MgCl2, 2 mM DTT, 0.1 mM EDTA, 0.01% Brij 35, pH 7.5). After SDS-page, gels were Coomassie-stained and dried using a BioRad gel dryer. Dried gels were scanned for total protein and exposed to x-ray films for autoradiography to detect the presence of radioactive labeled phosphorylated proteins. For densitometric quantification of phosphorylation levels, band intensity was measured using Imagej (NIH), and statistical analysis was done using Excel (Microsoft).

Cell Culture, Transfection, and Phos-tag SDS-PAGE Analysis—COS-1 cells were cultured and transfected as previously described (28). Transfected cells were either incubated with 50 μM Mek1 inhibitor PD98059 (Cell Signaling Technology) for 1 h or 0.1 μM phenylephrine (Sigma) for 30 min. Cells were washed with 1× PBS and directly lysed into SDS sample buffer. After SDS-PAGE of cell extracts and blotting onto nitrocellulose membranes using standard procedures, the presence of GFP-tagged fusion proteins, activated Erk1/2, and total Erk2 was analyzed using monoclonal mouse anti-GFP antibodies (Roche Applied Science, 1:1000), rabbit anti-phenol p44/42 antibodies (Thr202/204, 1:1000), and rabbit anti-Erk2 antibodies (Cell Signaling Technology, 1:500). For analysis of N2B us3 phosphorylation levels, SDS gels containing 10 μM Phos-tag-modified acrylamide (29; Wako) was used. After equilibration of gels in blotting buffer containing 10 mM EDTA for 10 min, proteins were transferred onto nitrocellulose and analyzed using monoclonal GFP antibodies as described earlier.

Yeast-two Hybrid Assay—A forced yeast two-hybrid assay was done as previously described (13, 28). pLexA-FHL1 was cotransformed with either empty Act2 plasmid or Act2-titin N2B us3 fragments encompassing amino acids 3750 – 4019, 3750 – 3858, or 3859 – 4019 into the L40 yeast strain and plated onto SD-LW minimal medium (lacking amino acids leucine and tryptophan), indicating the presence of both plasmids. For identification of minimal titin N2B binding site in FHL1, yeast were cotransformed with Act2 or Act2-titin N2B us3 (3750 – 4019) and either LexA-FHL1 full-length or LIM domains 0.5–1, 2–3, and 1–3. After selection and growth of positive yeast transformants, yeast were examined using HIS3 reporter gene activity on SD-HLW minimal medium (lacking amino acids leucine, tryptophan, and histidine).

Statistical Analysis—Data presented in the text and figures are expressed as mean values ± S.E. Significance was evaluated by the two-tailed Student’s t test or repeated measures of analysis of variance. Statistical analysis for muscle mechanics data were done as previously described (13). p < 0.05 was considered statistically significant.

RESULTS

FHL1KO Cardiac Muscles Display Reduced Diastolic Stiffness Compared with Wild-type Cardiac Muscles—Fhl1 can directly interact with the cardiac-specific element titin N2B (13), which is localized within the I band region of titin and is a major determinant of diastolic muscle compliance (7–10). As a
result, we assessed the diastolic mechanical properties in isolated FHL1KO cardiac muscles by subjecting muscles to a maximum extension of ~20% stretch. Passive Cauchy stress in isolated RV papillary muscles was plotted as a function of Lagrangian strain. FHL1KO cardiac muscles displayed a significant reduction in diastolic stress at a given strain, reflecting a decrease in diastolic stiffness when compared with wild-type cardiac muscles (Fig. 1A). These findings are consistent with previous studies demonstrating greater compliance and sarcomere extensibility of FHL1KO cardiac muscles when compared with wild-type cardiac muscles (13).

**Titin N2B Isoform Expression Is Altered in FHL1KO Hearts**—Two major variables influencing the degree of muscle compliance are titin content as well as the expression and ratio of specific titin isoforms (e.g. N2BA versus N2B ratio). Isoform expression differences are altered in failing hearts and thus have been used as an indicator of the extent of muscle stiffness in disease (5, 25, 30, 31). To examine whether cardiac titin content and/or the ratio of titin isoforms is changed in FHL1KO cardiac muscles, we assessed titin expression by vertical agarose gel electrophoresis and Coomassie staining (Fig. 1B). Total titin to myosin heavy chain (MHC) ratio was not significantly different between wild-type and FHL1KO hearts (Fig. 1B, C), suggesting that loss of Fhl1 does not affect titin content. However, a significant decrease in titin N2BA/N2B ratio, reflective of an increase in titin N2B levels, was detected in FHL1KO hearts (Fig. 1C), suggesting that loss of Fhl1 could directly regulate the expression of titin N2B isoforms.

**Isolated Adult FHL1KO Cardiac Muscles Are Unresponsive to Titin N2B Phosphorylation-mediated Reduction in Diastolic Tension**—To determine whether loss of Fhl1 affects titin N2B phosphorylation and function, we assessed diastolic mechanical properties within isolated FHL1KO cardiac muscles by subjecting them to continuous stretch of 15–20% in the absence and presence of isoproterenol (Fig. 1D). β-Adrenergic stimulation via isoproterenol is known to target and increase/saturate titin N2B phosphorylation-dependent muscle compliance in wild-type cardiac muscles (7). As expected, isoproterenol-treated wild-type controls displayed a significant reduction in diastolic stress at a given diastolic strain and thus increased compliance compared with untreated controls (Fig. 1D), which is consistent with previous observations in rat, human, and cow heart tissues (7–9). However, FHL1KO cardiac muscles were unresponsive to β-adrenergic-mediated changes in diastolic stress (Fig. 1D). The diastolic stress-strain curves for isoproterenol-treated wild-type cardiac muscles was not significantly different from those of untreated controls (Fig. 1D).
different from untreated FHL1KO cardiac muscles \((p = 0.99)\) (Fig. 1D), suggesting the possibility that titin N2B phosphorylation may be intrinsically increased/saturated in FHL1KO muscles and may be reflective of the increase in titin N2B levels observed in FHL1KO hearts.

To determine whether FHL1KO cardiac muscles were functionally insensitive to \(\beta\)-adrenergic stimuli, we assessed systolic mechanical function in isoproterenol-treated FHL1KO and wild-type cardiac muscles and showed that FHL1KO cardiac muscles were able to significantly increase developed systolic stress at a maximum extension of \(\sim\)20% stretch, similar to wild-type controls (Fig. 2A), suggesting that FHL1KO muscles do not have intrinsic defects in their ability to functionally respond to \(\beta\)-adrenergic agonists. To further determine if loss of Fh1 alters intracellular \(\beta\)-adrenergic signaling and myofilament targets, we also assessed baseline expression of phospholamban, a \(\beta\)-adrenergic signaling target that is a substrate of PKA (32), in wild-type and FHL1KO hearts. No significant differences in phosphorylated and total levels of phospholamban were observed in FHL1KO hearts when compared with wild-type hearts (Fig. 2B). The total and phosphorylated levels of myosin-binding protein C and cardiac troponins (I, T), which are myofilament targets of \(\beta\)-adrenergic signaling (26, 32), including cardiac troponymosin are also not significantly different between wild-type and FHL1KO hearts (Fig. 2, C–F), further suggesting that intrinsic modifications to cardiac myofilament proteins are likely not contributing factors to the effects of loss of Fh1 on diastolic muscle compliance.

**Collagen Content and Mechanics Are Not altered in FHL1KO Cardiac Muscles When Compared with Wild-type Cardiac Muscles—**Fibrillar collagen is thought to be a major contributor to diastolic stiffness at high sarcomere lengths and stress (33); however, no significant differences in overall collagen content was observed between wild-type and FHL1KO cardiac ventricles as demonstrated by qualitative analysis of picrosirius stain (Fig. 3A) and quantitative analysis of collagen content via hydroxyproline concentration analyses (wild-type heart: 1.2 \(\pm\) 0.12 \((n = 3)\) versus FHL1KO heart: 1.5 \(\pm\) 0.28 \((n = 3)\); \(p = 0.33\)). To determine if collagen mechanics were altered in FHL1KO cardiac muscles, we treated skinned muscles with high concentrations of potassium chloride (KCl) and potassium iodide (KI), which abolishes titin as a major source of diastolic tension from within the tissue (8) but does not alter collagen-related mechanisms of passive stiffness. Wild-type and FHL1KO cardiac muscles treated with KCl/KI both displayed a significant reduction in diastolic stress and thus increased compliance compared with untreated controls (Fig. 3B). The diastolic stress-strain curve of wild-type and FHL1KO cardiac muscles treated with KCl/KI was not statistically different, demonstrating that no intrinsic differences in collagen-based diastolic stress exist between wild-type and FHL1KO cardiac muscles.

**Titin N2B Is a Novel Phosphorylation Target of Erk2—**Our results altogether suggest the possibility that titin N2B phosphorylation may be saturated in FHL1KO cardiac muscles, as no functional changes in cardiac muscle diastolic stress/muscle compliance could be observed in the presence of isoproterenol (Fig. 1D), a \(\beta\)-adrenergic stimuli known to increase/saturate titin N2B phosphorylation-dependent muscle compliance in wild-type cardiac muscles (7). To further gain insights into how titin N2B phosphorylation is regulated in FHL1KO hearts (Fig. 4A), we sought to determine whether Erk2, a serine/threonine kinase and integral component of the Fhl-associated complex binding to titin N2B that is also dysregulated from signaling in FHL1KO hearts after hypertrophic stress (13), has the potential to directly phosphorylate human titin N2B. In vitro phosphorylation assays revealed that the minimal titin N2B element (amino acids 3750–4019) is directly phosphorylated by Erk2, similar to myelin basic protein, which is a known target of Erk2 kinase (Fig. 4B). No phosphorylation of this minimal titin N2B element could be detected in the absence of Erk2 kinase or with GST alone in the presence of Erk2 kinase, demonstrating the specificity of titin N2B as a target of Erk2 phosphorylation (Fig. 4B).

**Fhl1 Interferes with Erk2-mediated Titin N2B Phosphorylation in Vitro—**To determine the influence of Fhl1 on Erk2-mediated titin N2B phosphorylation, in vitro phosphorylation assays were performed on the minimal titin N2B element in the presence and absence of Erk2 kinase and increasing levels of recombinant Fh1 (Fig. 4C), the latter of which was used to reflect the up-regulated levels of Fh1 in mouse and human hearts after cardiac biomechanical stress (13, 34–36). We demonstrate that a dose-dependent increase in Fhl1 levels can significantly reduce Erk2-mediated titin N2B phosphorylation in vitro (Fig. 4, C and D). No significant changes in Erk2-mediated titin N2B phosphorylation could be detected in the presence of increasing concentrations of a GST control, demonstrating the specificity of this competition (Fig. 4, C and D). Interestingly, our studies also suggest that Fh1 specifically targets titin N2B and not Erk2 kinase itself, as a dose-dependent increase in Fhl1 does not have a major impact on Erk2-mediated myelin-basic protein phosphorylation (supplemental Fig. S1).

**Site-directed Mutagenesis of Titin N2B Residues Reveals Key Residues Important for Erk2-mediated Titin N2B Phosphorylation in Situ and a Mechanism for a Known Human Cardiomyopathy-causing Mutation—**To determine the critical residues important for titin N2B phosphorylation by Erk2 kinase, a bioinformatic analyses for putative Erk1/2 serine/threonine phosphorylation consensus sites was performed on the minimal human titin N2B-us3 domain. We identified and focused on five putative phosphorylation sites (Thr-3836, Ser-3854, Ser-3873, Ser-3915, Ser-3965) in titin N2B domain (Fig. 5A). To determine the functional importance of these residues in Erk2-mediated titin N2B phosphorylation, we generated single (T3836A, S3854A, S3873A, S3915A, S3965A), double (S3873A/S3965A, S3873A/S3915A), and triple (S3873A/S3915A/S3965A) serine/threonine-to-alanine titin N2B phosphorylation mutants. The impact of these mutants on loss of Erk2-mediated titin N2B phosphorylation; however, no single mutation alone was sufficient to completely abrogate endogenous titin N2B phosphorylation by Erk2 (Fig. 5B). Analyses of double and triple phosphorylation mutants revealed that mutations of the Ser-3873 residue in combination with Ser-3915 or Ser-3915/Ser-3965 residues is sufficient to completely abolish Erk2-mediated
titin N2B phosphorylation in vitro (Fig. 5C), thus, identifying Ser-3873, Ser-3915, and Ser-3965 as critical Erk1/2 phosphorylation residues in htitin N2B. Interestingly, we show that in vitro phosphorylation assays utilizing a known human hypertrophic cardiomyopathy causing titin N2B mutation (S3799Y, which is now S3754Y) that has been previously shown to affect Fhl binding (37, 38) can also directly and significantly impact Erk2-mediated titin N2B phosphorylation (Fig. 5, B and C). Although mutagenesis of Ser-3873, Ser-3915, and Ser-3965 impact titin phosphorylation negatively, the S3754Y mutation results in significantly increased phosphorylation.

To verify the functional importance of the three identified serine residues as critical for Erk-mediated signaling in a cellular context, we transfected mammalian cells with wild-type human titin N2B us3 (hN2B) and the triple serine-to-alanine titin N2B mutant (hN2B tSA; S3873A/S3915A/S3965A) and treated them with PD98059, which is a mitogen-activated protein kinase kinase-1 inhibitor used to block endogenous Erk1/2 phosphorylation or phenylephrine, which is known α1-adrenergic agonist that increases Erk1/2 phosphorylation. We show that PD98059 has the ability to block both endogenous and adrenergic-mediated Erk1/2 phosphorylation in cells (supplemental Fig. S2). Our results show that although phenylephrine treatment caused a dramatic increase in Erk1/2 phosphorylation when compared with PD98059 in both groups of transfected cells, only hN2B tSA mutant-transfected cells showed a clear reduction in both endogenous and adrenergic-stimulated titin N2B phosphorylation (note the absence of double and triple N2B us3 phosphorylation states in Phos-tag SDS-PAGE Western blots) when compared with wild-type titin N2B-transfected control cells (Fig. 6), further highlighting that these three sites are critical for Erk-mediated titin N2B phosphorylation in cells in situ. Intriguingly, the minimal region of N2B us3 (amino acids 3859–4019) that encompasses these sites is also responsible for binding FHL1 (supplemental Fig. S3A).

In summary, we identify titin N2B as a bona fide novel target of ERK2-mediated phosphorylation in vitro and in a cellular context, with residues Ser-3873, Ser-3915, and Ser-3965 as targets of posttranslational modification. Moreover, changed phosphorylation levels of a human mutation S3754Y known to cause hypertrophic cardiomyopathy highlights the importance of this regulatory complex and reveals a potential mechanism for this mutation in human cardiac disease.

**DISCUSSION**

Recent studies have demonstrated a critical role for titin in human cardiac health, as pathological titin truncation mutations were found to be a major genetic cause of human cardiomyopathy (2). However, limited knowledge exists of the precise mechanisms important for its regulation and underlying basis for disease. We previously identified a novel complex in the cardiomyocyte sarcomere involved in sensing cardiac bio-

**FIGURE 2.** Assessment of β-adrenergic receptor-mediated systolic mechanics as well as β-adrenergic signaling and myofilament targets within adult wild-type and FHL1KO cardiac muscles. A, developed systolic stress in wild-type and FHL1KO muscles after vehicle and isoproterenol (1 μM) treatment is shown. *, p < 0.05; **, p < 0.01. B, protein (20 μg) from wild-type (n = 3) and FHL1KO ventricles (n = 3) was subjected to SDS-PAGE to detect total and phosphorylated levels of cardiac myosin-binding protein C (cMyBP-C), cardiac troponin T (cTn-T), troponymosin (cTm), and troponin I (cTn-I) proteins. **MyHC** represents myosin heavy chain protein. C and D, representative blots for Sypro-Ruby Red (C and D) as well as Pro-Q diamond stain (E and F) was used to detect total and phosphorylated levels of cardiac myosin-binding protein C (cMyBP-C), cardiac troponin T (cTn-T), troponymosin (cTm), and troponin I (cTn-I) proteins. **MyHC** represents myosin heavy chain protein. C and D, representative blots for Sypro-Ruby Red and Pro-Q diamond stains of myofibrillar proteins are shown. D and F, semiquantitative analysis of total and phosphorylated levels of cardiac myosin-binding protein C, cardiac troponin T, cardiac tropomyosin, and cardiac troponin I from wild-type (n = 4) and FHL1KO (n = 4) ventricles was also performed. n.s., represents not significant.

**FIGURE 3.** Assessment of collagen expression- and collagen-based diastolic mechanics within adult wild-type and FHL1KO cardiac muscles. A, detection of collagen expression in wild-type and FHL1KO left ventricles after picrosirius stain is shown. Representative left ventricular endocardial sections from mice are shown. Scale bar, 20 μm. B, shown is a diastolic stress-strain relationship in wild-type (top) and FHL1KO (bottom) muscles after chemical skinning via high potassium chloride (KCl) and potassium iodide (KI) treatment and after stretch. ***, p < 0.01; ***, p < 0.0001.
Our studies demonstrate that saturation of titin N2B phosphorylation is likely the main contributor to the increased compliance observed in FHL1KO cardiac muscles after stretch. These effects were accompanied by a significant increase in titin N2B levels (decreased titin N2B/NA2B ratios) in FHL1KO versus wild-type hearts (Fig. 1, B and C), which might reflect the increased or hyperphosphorylated state of titin N2B. In addition, we provide evidence through functional studies that FHL1KO cardiac muscles were unresponsive to changes in muscle compliance after isoproterenol treatment and exhibited a diastolic stress-strain curve at base line, which was not significantly different from isoproterenol-treated wild-type controls (Fig. 1D), an agonist known to saturate titin N2B phosphorylation (7–9). The effect of Fh1 on muscle compliance was not influenced by other factors known to affect muscle stiffness, such as changes in titin content (Fig. 1C), intrinsic β-adrennergic responsiveness or signaling (Fig. 2, A and B), and collagen content and diastolic mechanics (Fig. 3, A and B) as well as intrinsic modifications to β-adrenergic myofilament targets (Fig. 2, C–F), further refining an influence of titin N2B phosphorylation on passive mechanics in FHL1KO cardiac muscles.

Phosphorylation of titin N2B has been shown to play a critical role in mediating muscle compliance in cardiomyocytes; however, previous studies have confined the mechanisms to a role for protein kinases A and G (7–10). Our studies shed new light on additional molecular complexes associated with titin regulation/phosphorylation and muscle compliance involving the association of titin N2B with Erk2 and Fh1. We now provide direct evidence that titin N2B is a novel substrate of Erk2 kinase (Fig. 4B) and identify three critical serine residues (Ser-3873, Ser-3915, Ser-3965) in titin N2B that are required for its phosphorylation by Erk2 (Fig. 5C). It is currently unknown how phosphorylation increases compliance of the N2B element. Previously proposed mechanisms (9) include changes in the bending rigidity of the N2B element due to the addition of negative charges and the existence of local structures that undergo a structural transition when phosphorylated (although the N2B element is thought to be a largely random coil, the presence of structured local regions can currently not be excluded). This important issue needs to be experimentally addressed in future work.

ERK-mediated titin N2B phosphorylation is also evolutionarily conserved, as the same region within mouse titin N2B is phosphorylated by ERK2, adding to the biological relevance of this modification. Moreover, we were able to demonstrate that ERK2-mediated titin phosphorylation occurs in a cellular context, as mammalian cells transfected with a specific triple serine-to-alanine human titin N2B mutant (hN2B tSA) displayed a loss of phosphorylation both at base line and in response to adrenergic-mediated ERK1 signaling/phosphorylation (Fig. 6). We additionally show that FH1 can interfere with ERK2-mediated titin N2B phosphorylation in a dose-dependent manner (Fig. 4, C and D), highlighting the potential dynamics of this regulatory complex in the event of cardiac biomechanical
stress, an event associated with up-regulated levels of Fhl1 (13, 34–36). Our studies also suggest that this reduction in Erk2-mediated phosphorylation of N2B likely pertains to the ability of Fhl1 to now interact with the minimal N2B fragment, which is also evidenced by our previous studies demonstrating this interaction (13), and our results demonstrating that the minimal domain of N2B (3859–4019) containing critical Erk phosphorylation sites is responsible for Fhl1 binding (supplemental Fig. S3A). MAPKs and specifically ERK phosphorylation have been implicated in mechanotransduction and in some cases mechanically induced hypertrophy of various cell types, including skeletal and cardiac myocytes (39–44), thus suggesting a potential direct role for the Erk pathway in muscle mechanics. Our studies show that FHL1KO cardiac muscles display...
increased titin N2B levels and suggest that these muscles display saturation in phosphorylation-mediated compliance. These results along with our previous findings suggest the possibility that in the event that Fhl1 is lost, titin N2B may be more "open" to phosphorylation by kinases, such as Erk2, which then render the muscle more compliant (Fig. 7). Although extensive knowledge exists about the ERK pathway, previously unknown substrates and functions continue to be discovered (45). Thus, loss of Fhl1 may uncover a novel non-transcriptional role for Erk2 activity on N2B phosphorylation levels. Note the persistence of an additional unidentified Erk2-independent phosphorylation site in this region of titin. Please note that the black dotted lines between lanes denotes that lanes were run on the same gel but were non-contiguous. Results were consistent among two independent experiments.

FIGURE 6. ERK2 phosphorylates human titin N2B us3 in a cellular context. Shown are SDS-page and Phos-tag SDS-page analyses of protein lysates from COS-1 cells transfected with either GFP-tagged wild-type human htitin N2B us3 (hN2B wt, residues 3750 – 4019) or serine-alanine triple mutant (hN2B tSA, Ser-3873/3915/3965A) in the presence of MAPKK inhibitor (50 µM PD98059; PD) or an α1-adrenergic agonist (phenylephrine hydrochloride 0.1 µM, PE). Western blot analysis to assess for expression of htitin N2B, phosphorylated ERK1/2, and total ERK2 was performed using specific GFP, phosphorylated ERK1/2 and ERK2 antibodies. Phos-tag SDS-PAGE analysis (Ref. 29; 10 µM, second panel from top) identified the presence of four distinct N2B us3 phosphorylation states (the arrowhead indicates unphosphorylated N2B us3, and the arrowhead with the asterisk indicates single, double, or triple phosphorylated N2B), not visible in standard Western blot analysis (top panel). Intensity of wild-type N2B phosphorylation states is dependent on activity of ERK2 (indicated by phospho-specific ERK1/2 antibody; third panel from top). No change in total ERK2 level was observed (bottom panel). Analysis of triple serine-alanine N2B us3 mutant demonstrates the absence of double and triple N2B us3 phosphorylation states in Phos-tag SDS-PAGE Western blots, with abrogated influence of ERK2 activity on N2B phosphorylation levels. Note the persistence of an additional unidentified Erk2-independent phosphorylation site in this region of titin. Please note that the black dotted lines between lanes denote that lanes were run on the same gel but were non-contiguous. Results were consistent among two independent experiments.

increased titin N2B levels and suggest that these muscles display saturation in phosphorylation-mediated compliance.

New Titin-N2B Mechano-signaling Mechanisms

A  
B

FIGURE 7. Schematic presentation of identified titin N2B phosphorylation sites, domain structure, and interaction network in a static and dynamic setting. A, minimal FHL binding sites in titin are indicated. Known (Refs. 13, 28) and novel titin phosphorylation sites and responsible protein kinases are displayed. B, shown is a working hypothesis for a signaling mechanism mediating titin-based cardiac muscle compliance and disease. Top, with stretch, FHL1 limits titin N2B phosphorylation by interfering with kinases, such as ERK2, which can directly bind to titin N2B and lock the range at which physiological sarcomere length can extend after stretch, producing basal stiffness and "physiological" compliance. Instead, FHL1 acts as a MAPK scaffold to set up signals important for hypertrophic signaling. Bottom, in the event of stretch, loss of FHL1 reveals phosphorylation sites on titin N2B that are now open to kinases, such as ERK2, which increase the range at which sarcomere length can extend after stretch and increase muscle compliance. In addition, the MAPK scaffold is "destabilized," resulting in the loss of hypertrophic signaling. PKA and PKG, protein kinase A and G. MMCK, muscle isoform of creatine kinase; AK, adenylate kinase-1; PFK, phosphofructokinase.

phosphorylation sites on titin N2B, rendering kinases such as Erk2 inaccessible to phosphorylate titin N2B, which would ultimately maintain basal stiffness of wild-type cardiac muscles (Fig. 7). Intriguingly, similar to our previous studies on understanding Fhl1 interactions (13), our current experiments also suggest that binding of Fhl1 to N2B is not dependent on a spe-
New Titin-N2B Mechano-signaling Mechanisms

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New Titin-N2B Mechano-signaling Mechanisms


A Novel Mechanism Involving Four-and-a-half LIM Domain Protein-1 and Extracellular Signal-regulated Kinase-2 Regulates Titin Phosphorylation and Mechanics


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