Protein phosphatase 5 regulates titin phosphorylation and function at a sarcomere-associated mechanosensor complex in cardiomyocytes

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Serine/threonine protein phosphatase 5 (PP5) is ubiquitously expressed in eukaryotic cells; however, its function in cardiomyocytes is unknown. Under basal conditions, PP5 is auto-inhibited, but enzymatic activity rises upon binding of specific factors, such as the chaperone Hsp90. Here we show that PP5 binds and dephosphorylates the elastic N2B-unique sequence (N2Bus) of titin in cardiomyocytes. Using various binding and phosphorylation tests, cell-culture manipulation, and transgenic mouse hearts, we demonstrate that PP5 associates with N2Bus in vitro and in sarcomeres and is antagonistic to several protein kinases, which phosphorylate N2Bus and lower titin-based passive tension. PP5 is pathologically elevated and likely contributes to hypo-phosphorylation of N2Bus in failing human hearts. Furthermore, Hsp90-activated PP5 interacts with components of a sarcomeric, N2Bus-associated, mechanosensor complex, and blocks mitogen-activated protein-kinase signaling in this complex. Our work establishes PP5 as a compartmentalized, well-controlled phosphatase in cardiomyocytes, which regulates titin properties and kinase signaling at the myofilaments.

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During the lifetime of a beating heart, the cardiomyocytes must respond dynamically to a multitude of internal and external stresses. Such functional flexibility is supported at the level of the contractile units, the sarcomeres, by the expression of cardiac-specific isoforms of structural, contractile, and regulatory proteins. Some of them, such as cardiac troponin-I, myosin-binding protein-C, or titin, contain unique sequence motifs that can be phosphorylated and dephosphorylated by protein kinases and phosphatases, respectively. These selective biochemical events then help to quickly adjust the mechanical function of the cardiac sarcomere to altered physiological requirements, e.g., during exercise. In the diseased heart this fine-tuned mechanism can be disrupted. Whereas multiplex kinase signaling has been recognized as an important modifier of cardiac function at the level of sarcomeric proteins, much less is known about how this function is modulated by protein phosphatases.

Titin is a multifunctional protein giant, which determines the ‘passive’ elasticity of the sarcomere, and also modulates active contractile properties. Human titin encompasses up to ~36,000 amino acids encoded by the 346 exons of the TTN gene and probably is the protein with the most potential phosphorylation sites, but very few have been explored functionally. Only one region in titin, termed N2B (encoded by exon 49 in mouse and human), is unique to the cardiac isoforms. This region is located in the elastic (I-band) segment of the molecule and contains a 572-residue N2B-unique sequence (N2Bus), which is an important spring element. Moreover, N2Bus is a hub for protein-protein interactions and a major site for oxidation and phosphorylation. Several protein kinases (PKs) phosphorylate N2Bus, including PKA, PKG, the mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinase 2 (ERK2, encoded by MAPK1) and Ca/calmodulin-dependent protein kinase IIα (CaMKIIα). Functionally, enhanced phosphorylation increases the distensibility of N2Bus and lessens the force needed to stretch this spring element, which lowers cardiomyocyte passive tension. Whether this phosphorylation affects the force needed to stretch this spring element, which lowers cardiovascular passive stiffness, notably in HF with preserved ejection fraction (HFrEF).

In the present study, we identified serine/threonine protein phosphatase 5 (PP5, encoded by PPS5C) as a novel interaction partner of N2Bus and a physiological antagonist to the PKs that phosphorylate N2Bus. PP5 is established as a ubiquitous enzyme in eukaryotic cells involved in multiple signaling pathways important for cell cycle progression, glucocorticoid receptor activation, DNA damage repair, transcriptional activation, or apoptosis. PP5 is unique among serine/threonine phosphatases as it contains a tetracontapeptide repeat (TPR) domain at the N-terminus, which binds to the C-terminal catalytic domain and blocks substrate access to the catalytic site, such that ‘free’ PP5 has low activity. However, PP5 becomes activated when the kinase JNK/p38 pathway by binding and dephosphorylating apoptosis signal-regulating kinase-1 (ASK1, encoded by MAP3K5) in response to oxidative stress. Moreover, PP5 dephosphorylates Raf-1 and suppresses downstream signaling to MAPK/ERK kinase (MEK, encoded by MAP2K) and ERK1 (MAPK3)/ERK2 (MAPK1) when PP5 also interacts with ERK1/2, it does not dephosphorylate it. The relationship between PP5 and the MAPK/ERK branch is particularly interesting in connection with titin, since PP5 binds MAPK2, and ERK1 (MAPK3)/ERK2 (MAPK1) are recruited to N2Bus via a linker, four-and-a-half-LIM-domains protein-1 (FHL-1). The N2Bus-FHL-1-MAPK complex forms a putative sarcomeric mechanism involved in pro-hypertrophic cardiac signaling. Whereas in vitro evidence suggested that FHL-1 blocks phosphorylation of N2Bus by ERK2, phosphorylation of cardiac fibers by ERK2 readily lowered their titin-based passive tension.

Here we show that PP5 dephosphorylates titin specifically at N2Bus and reverses the effect of phosphorylation on titin-based passive tension. Through association with Hsp90 and components of the N2Bus-mechanosensor complex, PP5 operates in a compartmentalized manner at the sarcomere I-bands. Our work establishes PP5 as a well-controlled enzyme in cardiomyocytes, which regulates titin properties and MAPK signaling to support dynamic heart function.

Results
PP5 binds the titin N2Bus domain. In a yeast-2-hybrid screen (Y2H), we used full-length human titin N2Bus as bait and a human adult heart cDNA library as prey. Approximately 150 clones grew on selective media plates and 50 of them were tested as a binding partner of cardiac titin at the N2Bus.

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myofibril and binding detected by indirect immunofluorescence. Consistent with the results of the co-immunoprecipitation assay (Fig. 1c), exogenous PP5c bound only weakly to the myofibrils (Fig. 1d, lower left images). However, the binding was much intensified and clearly localized to the sarcomeric I-bands when the myofibril was phosphorylated (by catalytic subunit of PKA) prior to incubation with PP5c (Fig. 1d, lower right images). In control experiments, neither Cy3-conjugated secondary antibody alone (Fig. 1d, top) nor recombinant green fluorescence protein (GFP) interacting with the myofibrils, suggesting that PP5 binding was not due to a generally increased stickiness of stretched sarcomeres. Considering the effect of prior phosphorylation of N2Bus on PP5 binding, we performed GST-pulldown assays with recombinant human PP5 and N2Bus, in which the latter was phosphorylated by PKA catalytic subunit or cGMP-activated PKG, or was left unphosphorylated (Fig. 1e). PP5 bound 2–3 times stronger to N2Bus if the titin region was phosphorylated (Fig. 1e, bar graph). Furthermore, in a 162-residue C-terminal fragment of N2Bus we mutated a known PKG-dependent phospho-}

PP5 translocates to sarcomeres if induced in cardiomyocytes. In testing for PP5 expression in vivo, we found a relatively high level in embryonic (E18) and newborn (P1) rat hearts, which decreased to half in adult rat hearts (Fig. 2a). We thus prepared primary cultures of neonatal rat ventricular myocytes (NRVM) and studied the intracellular localization of PP5 before and after pharmacological manipulation using PP5-activator arachidonic acid (aa; 200 μM) or potent inhibitor okadaic acid (oa; 10 nM)33. Interestingly, PP5 activation by aa also increased the expression level of PP5, compared to untreated NRVM, whereas PP5 inhibition by oa decreased it (Fig. 2b). Confocal microscopy showed PP5 to be distributed in a diffuse pattern all over the cytoplasm in
control NRVM, with only an occasional hint at a more regular striation pattern (Fig. 2c). However, stimulation by aa typically caused some PP5 to translocate to the sarcomeric Z/disk/I-band region, as suggested by co-localization with α-actinin (Fig. 2c; note that due to the short sarcomere length (SL) of NRVM cultures, the I-band and Z-disk regions are hardly distinguishable by confocal microscopy). Treatment with aa always resulted in PP5 staining patterns with no signs of regular striation. Because PP5 negatively regulates MAPK signaling by dephosphorylating Raf127, we also measured the expression and activity (phosphorylation) of the MAPK effector kinases ERK1/2 in NRVM. Whereas ERK1/2 expression was unaltered by aa and oa treatment, phospho-ERK1/2 was reduced by 65% in aa-treated cells but little affected by oa, as compared to controls (Supplementary Fig. 1a). Inhibitor of Raf1 (Raf1-I; 20 µM) lowered phospho-ERK1/2 by ~50%. Even larger alterations in ERK1/2 activity were seen in NRVM when the MAPK pathway was first stimulated by angiotensin-2 (AngII) or endothelin-1 (ET-1), prior to treatment with aa, oa, or Raf1-I (Supplementary Fig. 1b). Following ERK1/2 activation by AngII, treatment with oa now had an additional stimulatory effect on phospho-ERK1/2. Taken together, these findings suggest a link between PP5 and MAPK signaling, for the first time also in cardiomyocytes.

We also studied PP5 localization in primary cultures of adult rat cardiomyocytes (ARC) (Fig. 2d). Before PP5 induction, the phosphatase was diffusely cytosolic in some cells, but appeared in a regular striated (sarcomeric) pattern in others. Quantitation of this distribution showed that the proportion of cells with regular PP5 striations increased substantially in aa-treated (PP5-stimulated) compared with control ARC (Fig. 2d, bar graph). In most aa-stimulated cells, PP5 co-localized with the Z/I-region of the sarcomere marked by α-actinin. Treatment with aa did not alter the proportion of cells with regular PP5 striations, in comparison to controls, and the diffuse PP5 distribution was seen more frequently than in aa-treated cells (Fig. 2d). We conclude that, particularly after induction in cardiomyocytes, PP5 is preferentially translocated to the sarcomeres.

PP5 dephosphorylates titin at the N2Bus element. In order to determine whether PP5 dephosphorylates titin, we first quantified the total titin phosphorylation level in ARC cultures. Induction of
PP5 by aa reduced titin phosphorylation by a small but significant amount, whereas oa treatment did not alter it, in comparison to non-treated control cells (Fig. 2e). Next we performed indirect immunofluorescence on ARC using a phospho-specific antibody against rat/mouse phosphoserine P-S3991 located in titin’s N2Bus element (equivalent to P-S4010 in human titin13), which is known to be phosphorylated by ERK214 and PKA14. In most control cells, the N2BusP-S3991 epitope appeared in a regular striation pattern, co-localizing with the Z1-region of the sarcomere marked by α-actinin (Fig. 2f). In PP5-stimulated (aa-treated) ARC, the proportion of cells with regular N2BusP-S3991 striations was reduced compared to controls, whereas oa treatment did not alter this proportion (Fig. 2f, bar graph). These results suggest that induction and translocation of PP5 to the cardiac sarcomeres may result in dephosphorylation of titin at N2Bus.

To substantiate this finding, we generated recombinant human N2Bus and tested whether recombinant PP5 dephosphorylates the construct after phosphorylation by a kinase. ERK2-phosphorylated N2Bus was dephosphorylated by full-length PP5 or (most effectively) by PP5c, which induced >90% reduction of phosphorylation (Fig. 3a). In contrast, enzymatic dead catalytic subunit of PP5 (PP5cH304A-ED) was unable to dephosphorylate the ERK2-phosphorylated N2Bus. Similar experiments were conducted using PKA catalytic subunit or cGMP-activated PKG as the phosphorylating kinases. Under these conditions, PP5c dephosphorylated N2Bus by 45–50%, whereas PP5c ED again showed no dephosphorylation effect (Fig. 3b). These results, obtained using ProQ Diamond/Sypro Ruby staining, were further validated in 32P-autoradiography experiments, which showed that full-length PP5 and PP5c dephosphorylated the PKG-phosphorylated N2Bus by ~55%
and ~65%, respectively (Fig. 3c). The relatively high phosphatase activity of full-length PP5 found in these experiments was unexpected but consistently observed; it might be due to partial cleavage (and thus, activation) of the phosphatase in vitro or incomplete autoinhibition.

Recombinant full-length PP5 or PP5c also dephosphorylated endogenous N2Bus in skinned (demembranated) human heart tissue (Fig. 3d). Here, titin phosphorylation was detected by western blot using phospho-specific antibodies to P-S4185, a PKG/PKA-phosphorylated serine in human N2Bus13. Alkaline phosphatase (AP) was used to initially dephosphorylate the heart samples, before exogenous cGMP-activated PKG was given to maximize N2Bus phosphorylation (~100% phosphorylation). Subsequently, full-length PP5 or PP5c were able to dephosphorylate N2Bus13, P-S4185 by 70% to 80% (Fig. 3d). These results demonstrate that PP5 dephosphorylates N2Bus in vitro and in heart tissue.

PP5 is increased in human and experimental heart failure. In keeping with our earlier findings34,35, western blotting using phospho-specific antibodies to human titin (for epitope positions, see Fig. 1a) at residues P-S4010, P-S4099 (a PKG-dependent phosphosite)34, and P-S4185 detected a phosphorylation deficit at all three of these N2Bus sites in human end-stage failing hearts vs. non-failing donor hearts, which amounted to ~25–50% (Fig. 3e). In contrast, phosphorylation at P-11878 and P-12022 within the titin PEVK element was unaltered in failing vs. donor hearts. Interestingly, PP5 expression was ~45% higher in the failing hearts (Fig. 3f). Furthermore, in hearts from elderly hypertensive dogs with diastolic dysfunction, we observed ~30% less phosphorylation at N2Bus site P-S4010, compared to healthy canine hearts (Supplementary Fig. 2a), whereas PP5 expression was ~60% higher in the diseased hearts (Supplementary Fig. 2b). We conclude that hypo-phosphorylation of N2Bus in failing myocardium from humans and animal models, which is known to elevate titin-based passive stiffness3, may be attributable, at least in part, to increased PP5-mediated dephosphorylation.

PP5 transgenic cardiomyocytes have increased passive tension. Passive force (Fpassive) measurements were performed on skinned single cardiomyocytes of WT and PP5 TG mouse hearts in relaxing buffer. Cell stretch over the range 1.8–2.4 µm SL resulted in the typical, quasi-exponential increase in Fpassive (Fig. 5a). Importantly, the Fpassive–SL curves were much steeper in PP5 TG than in WT cardiomyocytes, and mean Fpassive was significantly increased at SL ≥ 2.0 µm (Fig. 5b, c). When PP5 TG and WT cardiomyocytes were incubated with recombinant PP5c, WT cells showed an additional increase in Fpassive, whereas WT cells were not altered in their stiffness (Fig. 5b). Moreover, Fpassive could be reduced significantly in both WT and PP5 TG cardiomyocytes upon treatment with ERK2 (Fig. 5c, d), PKA catalytic subunit (Supplementary Fig. 3a), or cGMP-activated PKG (Supplementary Fig. 3b, c). In the presence of exogenous kinase, Fpassive was always lower in WT than in TG cells. Finally, incubation of WT myocytes with recombinant PP5c following treatment with ERK2 or PKG (and washout of the kinase) significantly increased Fpassive to a level well above that measured in untreated WT cells (Fig. 5d and Supplementary Fig. 3c). We conclude that PP5 is an antagonist to the various PKs that phosphorylate N2Bus, in terms of its effect on cardiomyocyte Fpassive.

PP5 transgenic mouse hearts are hypophosphorylated at N2Bus. The increase in PP5 expression observed in failing human hearts prompted us to study titin phosphorylation in a transgenic (TG) mouse model with cardiac-specific overexpression of PP5. The cardiac phenotype of the TG model is mild, but some impairment of cardiac contractility has been noted25. The PP5 cardiac phenotype of the TG model is mild, but some impairment of cardiac contractility has been noted25. The PP5 overexpression of PP5.

PP5 and N2Bus bind to components of an I-band mechanosensor. N2Bus interacts directly with small heat-shock proteins32 and FHL proteins, which on their part recruit metabolic enzymes (FHL-231) and the MAPKs Raf1, MEK1/2 and ERK2 (FHL-129) to N2Bus, thus forming a putative mechanosensor complex. We studied by GST-pulldown assay whether PP5 also interacts with FHL-1 and found binding with both full-length PP5 and PP5c (Fig. 6a, top). In cardiomyocytes, FHL-1 mainly localized to the sarcomeric I-bands (marked by PEVK antibody) and thus, at the expected N2Bus binding site (Supplementary Fig. 4a). There was no difference in FHL-1 localization between PP5 TG and WT hearts. Additional in vitro binding assays revealed that PP5 or PP5c also interacted with ERK2 and Hsp90 (Fig. 6a), both of which have previously been described as binding partners of PP528,36. Since PP1 and PP2a are important phosphatases in cardiomyocytes, we determined their expression levels, as well as that of ERK2, in PP5 TG vs. WT mouse hearts, but observed no differences (Supplementary Fig. 4b).
Fig. 4 PP5 and phospho-titin in PP5-overexpressing TG mouse hearts. a Expression level of PP5 (left) and phospho-Raf1\(\text{S338}\) (right) in PP5 TG vs. WT mouse hearts by western blot. Data are mean ± s.e.m., \(n = 4\) hearts/group (age 5–6 months); duplicate analysis/group. b Sarcomeric localization of PP5 in PP5 WT and TG hearts by immunogold electron microscopy. Bars, 500 nm. Bar graph shows average number of gold particles counted in 50-µm\(^2\)-sized regions-of-interest (ROI), either on the sarcomeric I-band or elsewhere in the cardiomyocyte (‘Not on I-band’). Data are mean ± s.e.m., \(n = 5\) ROIs from 2 hearts/group. c PP5 localization in cardiomyocytes from PP5 TG and WT mouse hearts by indirect immunofluorescence. PP5 antibody (secondary antibody: Cy3-conjugated IgG), counterstained with anti-PEVK (titin) antibody (secondary antibody: FITC-conjugated IgG). Bars, 2 µm (main) and 1 µm (insets). d PP5 overexpression specifically decreases titin phosphorylation at N2Bus in PP5 TG vs. WT hearts. Total titin phosphorylation measured by ProQ Diamond/ Sypro Ruby staining (upper left), site-specific titin phosphorylation detected by western blot using antibodies to P-S3991, P-S4043, P-S4080 (all N2Bus; right panels), P-S2080 (titin Z/I junction), and P-S12742 (PEVK region). Phospho-titin signals were normalized to total titin signals detected by WB using a panel of sequence-specific antibodies (Pan). Means were indexed to those of control (WT) groups. Data are mean ± s.e.m., \(n = 4\) hearts/group, samples analyzed in triplicate. e Localization of phospho-N2Bus\(\text{S3991}\) in cardiomyocytes from PP5 TG and WT hearts by indirect immunofluorescence. Anti-N2Bus\(\text{S3991}\) antibody (secondary antibody: Cy3-conjugated IgG), counterstained with anti-PEVK antibody (secondary antibody: FITC-conjugated IgG). Bars, 2 µm (main) and 1 µm (insets). f Sarcomeric localization of phospho-N2Bus\(\text{S3991}\) in PP5 WT and TG hearts by immunogold electron microscopy. Bars, 500 nm (main) and 100 nm (insets). Bar graph shows average number of gold particles counted in 50-µm\(^2\)-sized regions-of-interest (ROI), either on the sarcomeric I-band or elsewhere in the cardiomyocyte (‘Not on I-band’). Data are mean ± s.e.m., \(n = 5\) ROIs from 2 hearts/group. In a, b, d, and f, *\(p < 0.05\) and **\(p < 0.001\), by two-tailed Student’s t-test.

Focusing on interactors of N2Bus, we confirmed binding of this titin region to FHL-1\(^{29}\) and FHL-2\(^{31}\) by GST-pulldown assay (Fig. 6b). FHL-2 also binds ERK2\(^{35}\) and could thus play a role in the N2Bus-associated mechanosensor. Interestingly, N2Bus interacted in vitro with the \(\alpha\) and \(\beta\) isoforms of Hsp90 (Fig. 6b). Since Hsp90 activates PP5 through binding to the TPR region of the phosphatase, we performed in vitro ‘competition’ assays to test the impact of Hsp90 on the N2Bus-PP5 association. In these assays, pre-incubation of N2Bus-bound sepharose beads with PP5, followed by incubation with Hsp90, resulted in relatively weak binding of PP5 to N2Bus, whereas pre-incubation of N2Bus-bound sepharose beads with Hsp90, followed by incubation with PP5, resulted in significantly stronger binding of PP5 to N2Bus (Fig. 6c). Thus, binding of Hsp90 to N2Bus could promote subsequent PP5 binding to N2Bus. In this context, we immunostained against Hsp90 on myocardial tissue sections from PP5 TG and WT mice and found a diffuse cytosolic distribution of the chaperone in WT, with only an occasional hint at a more regular (sarcomeric) striation pattern (Fig. 6d). However, in PP5 TG hearts, Hsp90 consistently showed a regular striation pattern, in addition to the cytosolic localization, and co-localization with PEVK suggested binding to I-band titin, presumably N2Bus (Fig. 6d). Thus, Hsp90 and PP5 (Fig. 4c) may translocate to N2Bus in a coordinated manner. In conclusion, we confirmed known binary interactions within the N2Bus-associated mechanosensor and provided evidence for additional interactions involving PP5 and some of its binding partners.
FHL-1-deficient hearts show reduced N2Bus phosphorylation. In vitro data suggested that FHL-1 blocks ERK2-mediated phosphorylation of specific N2Bus residues, including S3991. However, FHL-1 may also promote N2Bus phosphorylation, because FHL-1 is needed to anchor MAPKs at N2Bus. To address this conundrum, we quantified cardiac titin phosphorylation in a mouse model deficient in FHL-1. Total phosphotitin was significantly lowered by ~30%, whereas N2Bus phosphorylation at S3991 was reduced by ~50% in FHL-1 knockout (KO) vs. WT hearts (Fig. 7a). As a control, we measured phosphorylation of S2080 at the Z/I-band junction of titin and found no difference between WT and FHL-1 KO hearts (Fig. 7a). On immunofluorescently stained tissue sections of both WT and FHL-1 KO hearts, P2c appeared mainly in the cytosolic space and sometimes at the sarcomeres; a difference in PP5-staining intensity and pattern was not consistently observed (Fig. 7b). However, FHL-1 supports phosphorylation specifically at N2Bus sites (but not I-band titin in general), presumably by targeting PKs to this spring element.

Discussion
Unlike the phosphatases PP1, PP2a, and PP2b (calcineurin), PP5 has gained little attention in the cardiac field, although it is readily expressed in cardiomyocytes. A reason may be the low basal activity of PP5 brought about by autoinhibition. However, PP5 is activated when Hsp90, Caβ/α1S00 proteins, arachidonic acid or LCACe bind to the enzyme (Fig. 8), to release the autoinhibitory mechanism. Our work now demonstrates an important role for this distinctly regulated phosphatase in cardiomyocytes, in that it binds and specifically dephosphorylates the N2Bus spring element in cardiac titin, thus acting antagonistic to the PKs that phosphorylate N2Bus and lower cardiomyocyte passive tension. PP5 performs this function as a novel constituent of the sarcomeric mechanosensor formed by N2Bus, FHL-1, and MAPK family members Raf1, MEK1/2, and ERK2. Unlike proposed earlier, FHL-1 does not block ERK2-mediated N2Bus phosphorylation in this complex structure, as demonstrated by us using WT and FHL-1 KO mouse hearts. Instead, FHL-1 supports efficient phosphorylation of specific serines in N2Bus, probably because it is important for the recruitment of MAPKs (and perhaps other kinases) to N2Bus.

One effect of PP5 on the N2Bus-associated mechanosensor complex is brought about by dephosphorylation of Raf1, which blocks downstream MAPK signaling. ERK2 cannot shuttle to

Fig. 5 Passive tension of cardiomyocytes of WT and PPS TG mouse hearts. a Representative image of permeabilized cardiomyocyte glued at the ends to a force transducer and micrometer, respectively, and experimental protocol. Bar, 20 μm. b Passive tension (FPassive) vs. SL curves of permeabilized single PPS TG and WT cardiomyocytes in relaxing solution, before and after treatment with recombinant PP5c. c FPassive-5L curves of permeabilized single PPS TG and WT cardiomyocytes before and after treatment with ERK2. d FPassive-5L curves of permeabilized single WT cardiomyocytes before and after treatment with ERK2 and additional exposure to PP5c. Data are mean ± s.e.m., n = 5 cells/condition (2 different hearts/condition). Curves are second-order polynomial fits to the means. *p < 0.05, TG vs. WT; †p < 0.05, WT + PP5c vs. WT in b and WT + ERK2 vs. WT in c and d. /p < 0.05, TG + ERK2 vs. TG in c and WT + ERK2 + PP5c vs. WT + ERK2 in d; all by two-tailed Student's t-test.

Fig. 6 Binary interactions of PP5 or N2Bus and role of Hsp90. a Interactions of PP5 or PP5c by GST-pulldown assay. The PP5-GST assay is a negative control. b Interactions of N2Bus by GST-pulldown assay. c Impact of Hsp90 on PP5-N2Bus binding in GST-pulldown ‘competition’ assays. ‘PP5→Hsp90’; N2Bus immobilized on GSH-beads, incubated with PP5, then washed, and Hsp90 added thereafter. ‘Hsp90→PP5’; N2Bus immobilized on GSH-beads, incubated with Hsp90, then washed, and PP5 added thereafter. Summary data in bar graph are relative values indexed to the ‘PP5→Hsp90’ control; mean ± s.e.m., n = 3 experiments/condition; *p < 0.05, by two-tailed Student’s t-test. d Localization of Hsp90 in cardiomyocytes from PPS TG and WT hearts by indirect immunofluorescence. Anti-Hsp90 antibody (secondary antibody; Cy3-conjugated IgG), counterstained with anti-PEVK (titin) antibody (secondary antibody; FITC-conjugated IgG). Bars, 5 μm (main) and 1 μm (inset).
the nucleus of the cardiomyocyte where it normally acts as a transcriptional co-factor\(^3\), and mechanosensor function will be compromised (Fig. 8). In support of this scenario, Raf1 activity was reduced in the cardiomyocytes of PP5-overexpressing TG mice (Fig. 4a). Another effect of PP5 on the mechanosensor is triggered by the dephosphorylation of N2Bus. In PP5 TG hearts, we found lowered site-specific N2Bus phosphorylation and increased cardiomyocyte passive tension, compared to WT. Since the rise in passive tension follows from reduced distensibility\(^3\), and considering that mechanosensor function will depend on N2Bus distensibility\(^3\), PP5 may inactivate the mechanosensor by stiffening N2Bus (Fig. 8). Taken together, PP5-mediated regulation of mechanosensor activity is possible, in principle, by dephosphorylation of Raf1 or N2Bus, or both.

MAPK/ERK signaling via G\(\alpha\)q is promoted by G-protein coupled receptor (GPCR) agonists, such as ET-1 or AngII, in cardiomyocyte cultures and intact hearts (Fig. 8)\(^3\)–\(^\text{3}\). Our results suggest that PP5 interferes with this pathway under physiological conditions in cardiomyocytes and regulates it in a compartmentalized manner. In cultured NRVM, we observed ERK1/2 activation by AngII or ET-1, hyper-activation of ERK1/2 in the presence of PP5-inhibitor okadaic acid, and very effective suppression of ERK activity by PP5-activator arachidonic acid.
Elevated PP5 expression was observed in end-stage failing human hearts and hypertensive dog hearts with diastolic dysfunction (Fig. 3f and Supplementary Fig. 2b). Pathological triggers could thus activate and recruit PP5 to the sarcomeric N2Bus-FHL-MAPK complex in heart disease. In the failing human and dog hearts, we confirmed the reduced site-specific titin phosphorylation at N2Bus, but not PEVK (Fig. 3e and Supplementary Fig. 2a), reported earlier. Such phosphorylation deficit at N2Bus is discussed as a main reason for the pathological increase in titin-based myocardial passive stiffness in HF, especially in HFpEF. Whereas hypo-phosphorylation of N2Bus in HFP EF has so far been explained mainly in terms of pathologically downregulated cGMP-PKG signaling, our findings suggest another mechanism, which involves PP5. The phosphatase most efficiently dephosphorylated ERK2-phosphorylated human N2Bus in vitro, but also dephosphorylated PKA- or PKG-phosphorylated N2Bus (Fig. 3). Moreover, recombinant PP5c reversed the softening effect of ERK2 or cGMP-activated PKG on isolated skinned cardiomyocytes (Fig. 5d and Supplementary Fig. 3c). In PP5 TG mouse cardiomyocytes, ERK2/PKA-dependent N2Bus phosphosite P-S3991 was much less phosphorylated than in WT, as was CaMKIIδ-dependent P-S4043 and PKG-dependent P-S4080, but not PKCo-dependent P-S2080 at the Z-line junction of titin (Fig. 4). Thus, the induction of PP5 in failing hearts and the activity of PP5 towards N2Bus reported in Supplementary Fig. 2a) can explain why there is a phosphorylation deficit specifically at N2Bus but not at the PEVK domain.

These findings implicate several potential treatment strategies to reduce the high myocardial passive stiffness of HFP EF patients, which are worthwhile to be tested. First, the inhibition of PP5 could increase N2Bus phosphorylation and lower titin-based myocardial passive stiffness. Second, PP5 can associate with the titin-based mechanosensor at N2Bus (right side). Thus, N2Bus (previously phosphorylated by ERK2, PKA, PKG, or CaMKII) is dephosphorylated, which reduces its distensibility and increases titin-based passive tension; the mechanosensor is now less sensitive. Raf-1 is also dephosphorylated and signaling to ERK2 is disabled, such that the mechanosensor function is additionally compromised. The process is embedded in signaling pathways activated via G-protein coupled receptor (GPCR) and Ras, and it can be reversed when PP5 is deactivated. (Molecules that have a color code were studied here, those with no color/white background were inferred from the literature)
PP2a, either directly or indirectly through a regulatory subunit that binds both phosphatases. PP2a is known to dephosphorylate multiple cardiomyocyte proteins and its altered activity is associated with heart failure. Site-directed mutagenesis was used to generate PP5 monoclonal antibody (60 µg/ml) for secondary tests. Uncropped images of gels and western blots are shown in Supplementary Fig. 2 and Supplementary Fig. 3.

In summary, we demonstrated that increased heart failure would promote titin phosphorylation and help reduce pathologically increased diastolic stiffness.

Methods

Signaling assay. Prior to transfection, 1 × 10^6 HEK cells were seeded in 24-well plates and incubated at 37°C and 3% CO2 for 24 h. Cells were transfected with chemical transfection method using BD-Tagging PP5 (5 ng DNA/well) and BD-Tagging N2Bus (2 ng DNA/well). After 4 h, the cells were washed twice with cold PBS (3x) and cultured for 1 day. For protein expression, the cells were washed twice with cold PBS and lysed by addition of 100 µl of lysis buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM DTT, 10% glycerol, 0.5% NP-40), and protein expression was measured using SDS-PAGE and western blotting.

Co-immunoprecipitation assay. Our results indicate that PP5 functions in cardiomyocytes, as the phosphatase binds and dephosphorylates titin properties in normal and failing hearts. Moreover, PP5 could play a role in regulating the activity of PP5 in heart failure would promote titin phosphorylation and help reduce pathologically increased diastolic stiffness.

Co-immunoprecipitation assay. To search for interactors of N2Bus we used human cDNA libraries (Clontech) serving as prey and a pACT2 vector, which was transformed into yeast strain Y187 (Clontech) using electroporation. Mating and screening procedures were carried out as described by the manufacturer (Clontech); 3-amino-1.2.4-triazol (70 mM) was used as a selective agent (3-AT). 200 ng of prey was incubated with 20 µg of N2Bus (in pGBKT7 and AH109) for 6 h at 4°C. After 6 h of incubation, the mixture was added to 95°C for 5 min. The eluate was additionally loaded at 7.5-fold excess, to reproducibly detect interaction. For western blotting, polyvinylidene difluoride (PVDF) membrane was blocked using 5% bovine serum albumin (BSA) at room temperature for 1 h. Primary antibodies were incubated at 4°C overnight, secondary antibodies were added for 1 h at room temperature for 1 h. Signal detection of HRP conjugates was visualized by ECL Western blotting detection kit (GE Healthcare). The following antibodies were used: anti-N2Bus (target, rat PP5 catalytic subunit), 1:2000 (PVDF) membrane was blocked using 5% bovine serum albumin (BSA) at room temperature for 1 h. Primary antibodies were incubated at 4°C overnight, secondary antibodies were added for 1 h at room temperature for 1 h. Secondary antibody was added for 1 h at room temperature for 1 h. Signal detection of HRP conjugates was visualized by ECL Western blotting detection kit (GE Healthcare). Immunochemistry labeling of streptavidin conjugated biotin (Vector Laboratories). The following antibodies were used: anti-PGK (target, peptide surrounding Asn300 of human PGK; 1:400) or anti-PP5c (catalytic subunit) antibody (target, rat PP5 amino acids 36–238; 3/PP5; BD Biosciences, 611021; monoclonal, rabbit; 1:2000) was used for detection, sometimes also anti-N2Bus (titin) antibody (custom-made by Eurogentec; affinity-purified polyclonal, rabbit; 2000 ng/µl). Anti-PGK (target, peptide surrounding Asn300 of human PGK; C9S5; Cell Signaling, #4877; monoclonal, rabbit; 1:1000). A given interaction test was performed at least three times. Uncropped images of gels and western blots are shown in Supplementary Fig. 5 and Supplementary Fig. 6.

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The heavy-chain promoter were generated as described previously. The transgene consisted of the α-heavy-chain heavy-chain promoter, the entire protein coding region for rat α-actin (aa 2-281) flanked with 3' untranslated sequence, and the SV-40 polyadenylation signal sequence. The transgene, isolated from the parent plasmid, was microinjected in fertilized mouse eggs. Mice positive for the transgene were identified via Southern blot and PCR of tail genomic DNA. In total, we studied the hearts of four TG and four litter-matched WT male mice aged 6–6 months, using a TG line with a 7.8-fold overexpression of the transgenic protein. These experiments showed a slightly compromised contractility in a previous study that provided phenotypic characterization of the model. Hearts from TG and WT animals were collected at the University of Münster in full accordance with the institutional guidelines. Approval for the study was granted by the State Office for Nature, Environment and Conservation North Rhine-Westphalia (LANUV NRW; reference number 8.87-50.36.09.006). Samples were prepared for immediate mechanical measurements of isolated cardiomyocytes, immunofluorescence, and immunoelectron microscopy, or deep-frozen for later biochemical analysis.

**FHL-1 knockout mouse hearts.** A mouse model deficient in FHL-1 (kind gift from Dr. Ju Chen, University of California-San Diego, La Jolla, CA, USA) was generated previously. From this model we obtained adult heart tissue (6–8 month-old mice) and analyzed three KO and three litter-matched WT hearts. All animal procedures were in full compliance with the guidelines approved by the UCSD Animal Care and Use Committee. The committee approved the procedure of heart extraction from the deceased animals for later biochemical and histochemical analysis.

**Neonatal rat cardiomyocyte cultures.** NRVM were isolated and prepared from 1-day-old to 2-day-old Wistar-Kyoto rat hearts, following the guidelines and under the approval of the Animal Care and Use Committee at Ruhr University Bochum. Cells were dispersed by incubation in pancratin solution containing 8 g NaCl, 2 g urea, 200 mg KCl, 57.5 mg NaH2PO4·H2O, 20 mg phenol red, 1 g Na-bicarbonate, supplemented with 20 mg pancratin (Sigma-Aldrich) and 150 μL collagenase (Roche) per 10 cm culture dish. After several digestion steps, the pellet was suspended in Dulbecco’s modified Eagles medium with 10% fetal calf serum (FCS) and antibiotics (10 mg/ml penicillin/streptomycin; Gibco). Pre-plating for 1 h removed unattached cells and fibroblasts and increased the content of cardiomyocytes. The cell suspension was then plated at a density of 1 × 10^6 cells/mm^2 and cultured at 37 °C and 5% CO₂. After 24 h, medium was replaced by fresh culture medium. Following another 24 h, some cells were treated with arachidonic acid (aa; Sigma-Aldrich; 200 μM, 2 h) or okadacic acid (oa; Sigma-Aldrich; 10 μM, 1 h), while others were left untreated (control). Additional cells were treated with inhibitor of Raf1 (cRaf1-I GW5074; Enzo Lifesciences; 20 μM, 1 h), or to stimulate the MAPK pathway, with angiotensin-II (AngII; 1 nM, 24 h), endothelin-1 (ET-1; Sigma-Aldrich; 1 nM, 24 h), or a combination of those. Cells were rinsed with warm PBS and subsequently harvested in 1.5 mL fresh PBS. After centrifugation, pellets were homogenized in modified Laemmli buffer (8 M urea; 2 M thiourea; 3 % SDS; 50 M Tris-HCl (pH 6.8); 0.5 % glutaraldehyde; 75 mM DTT; Serva Blue) and analyzed by SDS-PAGE and western blot for expression of PP5, ERK1/2, Phospho-ERK1/2, and GAPDH (loading control). Antibodies to PP5 (target, N-terminal of human PP5; Cell Signaling, #2289; polyclonal, rabbit; 1:50), anti-PP5 (target, N-terminal of human PP5; Cell Signaling, #2289; polyclonal, rabbit; 1:100), anti-VEPV-PP5 (custom-made by Eurogentec; affinity-purified polyclonal, rabbit; 1:400)30,46, anti-phospho-N2Bus of titin (P-S2080)31,42, and α-actinin, and indirect immunofluorescence was again performed using confocal laser scanning microscopy. On those samples, we counted the proportion of cells showing a clear striation pattern for either PP5 or titin P-S2080, relative to the total number of cells. Counting was done by a person blinded to the identity of the cells.

**Immunofluorescence microscopy.** Cardiomyocyte heart tissue sections were fixed in 4% paraformaldehyde (PFA) for 20 min, rinsed with PBS, and permeabilized with 0.5% triton X-100 for 5 min. After repeated washes with PBS, samples were blocked with 2% BSA for 20 min and subsequently stained with buffer (ph 7.4) overnight at 4 °C at a concentration of antibodies against titin isoform (target, peptide corresponding to amino acids at the COOH terminal sequence of human FHL-1; Abcam, #58067; monoclonal, mouse; 1:250), anti-Hsp90 (target, peptide surrounding Asn300 of human Hsp90; C45G5; Cell Signaling, #4877S; monoclonal, rabbit; 1:120) and anti-PP5 (target, N2BusS3991 of human Hsp90; C45G5; Cell Signaling, #4877S; monoclonal, rabbit; 1:100). Secondary antibodies for immunofluorescence were incubated for 1 h at room temperature at the following concentrations: Cy3-conjugated IgG (target, gamma immunoglobulins heavy and light chains; Invitrogen, #A10520; polyclonal, goat), 1:300, and FITC-conjugated IgG (target, mouse IgG, whole molecule; Rockland, #21-120-1204; polyclonal, goat), 1:300. Control experiments, secondary antibody alone or control antibody incubation, were performed but did not reveal any immunofluorescence signals above background. Immunostained samples were embedded in Mowiol (Sigma-Aldrich) and analyzed by confocal laser scanning microscopy (Nikon Eclipse Ti microscope). Immunofluorescence imaging was processed similarly in the experimental and control groups.

**Immunoprecipitation microscopy.** Adult rat cardiomyocyte cultures were homogenized in modified Laemmli buffer, stored on ice for 30 min and subsequently boiled for 3 min at 97 °C. Protein concentration was determined spectrophotometrically using the Bio-Rad Protein Assay. 10% whole cell protein extract was used for IPs. Briefly, 40 μg of total protein were immunoprecipitated with 2 μg of the respective antibody. Competing antibodies for titin analysis were used for titin immunoprecipitation (target, titin α-actinin; EA-53; Sigma-Aldrich, #A7811; monoclonal, mouse; 1:300) or phospho-N2Bus (custom-made by Eurogentec; affinity-purified polyclonal, rabbit; 1:400)30,46, anti-phospho-N2Bus of titin (P-S2080)31,42, and α-actinin, and indirect immunofluorescence was again performed using confocal laser scanning microscopy. On those samples, we counted the proportion of cells showing a clear striation pattern for either PP5 or titin P-S2080, relative to the total number of cells. Counting was done by a person blinded to the identity of the cells.

**Mature mouse hearts.** Ventricular cardiomyocytes were used for experiments from day 2 onwards. Cells were treated with arachidonic acid (aa; 200 μM, 2 h), or okadacic acid (oa; 10 μM, 1 h), while other cells were left untreated (control). Cells were then harvested as described above for NRVM. Total titin phosphorylation was detected on titin gels stained with the ProQ Diamond (phosphoprotein) vs. Sypro Ruby (total protein) system. Other cells were immunostained using antibodies against P55 (target, N-terminal of human PP5; Cell Signaling, #2289; polyclonal, rabbit; 1:50) and α-actinin (target, rabbit skeletal α-actinin; EA-53; Sigma-Aldrich, #A7811; monoclonal, mouse; 1:300) or phospho-N2Bus (custom-made by Eurogentec; affinity-purified polyclonal, rabbit; 1:400)30,46, and α-actinin, and indirect immunofluorescence was again performed using confocal laser scanning microscopy. On those samples, we counted the proportion of cells showing a clear striation pattern for either PP5 or titin P-S2080, relative to the total number of cells. Counting was done by a person blinded to the identity of the cells.

**SDS-PAGE and immunoblotting.** Deep-frozen cardiac tissues (or cardiomyocyte cultures) were homogenized in modified Laemmli buffer, stored on ice for 30 min and subsequently boiled for 3 min at 97 °C. Protein concentration was determined spectrophotometrically using the Bio-Rad Protein Assay. Whole cell protein extract was used for SDS-PAGE for titin analysis. Western blotting was performed as described. Antibodies to the following proteins were used: P5 (target, N-terminal of human PP5; Cell Signaling, #2289; polyclonal, rabbit; 1:100), PPs (target, rat PP5 amino acids residues 36–238; 1PPS; BD Biosciences, #1201; monoclonal, mouse; 1:2000), GAPDH (target, full-length protein corresponding to human GAPDH; Abcam, #a9484; monoclonal, mouse; 1:2000), PP1x (target, peptide corresponding to the N-terminal sequence of human PP1x; Cell Signaling, #2582; polyclonal, rabbit; 1:1000), αPP2a (α + β isoform; target, peptide corresponding to amino acids at the C-terminus of human PP2a; Cell Signaling, #2238; polyclonal, rabbit; 1:1000), phospho-Raf1 (Ser338; target, phosphopeptide corresponding to residues 333–345 of human Raf-1; Merck, 05–538; monoclonal, mouse; 1:1000), and monoclonal, mouse; 1:2000).
Hsp90 (target, peptide surrounding Aaa300 of human Hsp90; C45GS; Cell Signaling, #4877s; monoclonal, rabbit; 1:1000), ERK1/2 (target, peptide corresponding to a sequence in the C-terminus of rat MAP kinase; Cell Signaling, P19112; polyclonal, rabbit; 1:1000) and phospho-ERK1/2 (target, phosphopeptide corresponding to residues surrounding Thr202/Tyr204 of ERK/MAPK; Biaf, #AB-PERK-100; polyclonal, rabbit; 1:1000).

**Quantification of titin phosphorylation.** By 2D-p-autoradiography we measured phosphorylation of recombinant human N2B25. N2B2 fragment was incubated with PKG (4.7 U/µl + 10 mM cGMP, kindly provided by Dr. Elke Butt, Würzburg, Germany) or Protein kinase A (PKA) catalytic subunit (1 µU/µl; Biofilin) in presence of [γ-32P]ATP (250 µCi/µl; Hartmann Analytic, Braunschweig, Germany) for 30 min at 35 °C. Some samples were dephosphorylated overnight at 35 °C with recombinant full-length PPP or catalytic subunit of PPP (PPS) (12 µg/µl). Samples were loaded on 7.5% SDS-PAGE gels, which were dried and exposed to autoradiographic film (FujiFilm BAS-1800 II) overnight at room temperature. Signal were analyzed using Multi Gauge V3.2 software.

The ProQ Diamond/SypRo dual staining system (Molecular Probes) was used to measure titin phosphorylation in recombinant N2B2 rat cardiomyocytes, or adult mouse heart tissue. Recombinant human N2B2 was phosphorylated with PKG (4.7 U/µl, plus 10 mM cGMP), PKA catalytic subunit (1 µU/µl), or ERK2 (20.5 U/µl; ThermoFischer Scientific) in presence of ATP for 30 min at 35 °C and was dephosphorylated overnight at 35 °C using recombinant full-length PPP or catalytic subunit of PPP (PPS) (12 µg/µl). A separate batch of recombinant N2B2 was also treated (following phosphorylation) with enzymatic dead PPPc (ED H304A mutant) overnight at 35 °C. Proteins were separated on SDS-PAGE gels stained with ProQ Diamond and subsequently with Sypro Ruby overnight. Protein bands were analyzed densitometrically and phospho-signals on ProQ Diamond-stained gels were normalized to corresponding total protein signals on SypRo-stained gels.

By western blotting, we visualized site-specific titin phosphorylation in heart tissue, as described14,15. Following separation of titin on SDS-PAGE gels, immunoblotting was performed using custom-made, affinity-purified, phospho-specific antibodies against phosphoserines in titin (for details, see ref. 15). The following anti-mouse titin antibodies (affinity-purified, polyclonal, rabbit), generated by Eurogentec (Belgium), were used (amino acid positions according to UniProtKB identifier, A2AS6): anti-P-S2080 (Z/I-junctional region; 1:400); anti-P-S3055 (1:400); -P-S4010 (1:400), -P-S4043 (1:400); -P-S4080 (1:400) (all N2Bus); and anti-P12742 (corresponding pan-titin antibody recognizing both the phosphorylated and the non-phosphorylated site was used separately on western blots. For quantification of phosphorylation, phospho-titin signals were normalized to signals obtained with the corresponding pan-titin antibodies. Moreover, phospho-specific anti-human titin (UniProtKB identifier, Q8W242) antibodies (affinity-purified, polyclonal, rabbit) were used that had been generated by Eurogentec against P-S4010 (1:400), P-S4080 (1:400), both N2B2), P-S1878 (1:400), and P-12022 (1:400) (both PEVK)14,15. Another antibody (affinity-purified, polyclonal, rabbit) was custom-made by Immunoglobl (Hemmerdt, Germany) against P-S4185 in human N2B2 (1:5000)14, this site is present in human titin, but is not cross-species conserved16. Signals obtained with the anti-human antibodies were indexed to signals on (coomassie-stained) PVDF blotting membrane, to adjust for differences in protein load. In some experiments, endogenous titin in human heart samples was first dephosphorylated using alkaline phosphatase (AP, 10 U/µl; New England Biolabs) before back-phosphorylation with PKG, followed by dephosphorylation with PPPc or PPSc. Uncropped images of gels, autoradiograms, and western blots are shown in Supplementary Fig. 5 and Supplementary Fig. 6.

**Force measurements on isolated skinned cardiomyocytes.** Force measurements were performed on single, isolated cardiomyocytes, as described17. Cardiomyocytes were isolated from the hearts of PP5 TG (n = 3) and litter-matched WT (n = 3) mice. Cells were skinned in relaxing buffer by using 0.5% Triton X-100 and placed under a Solaro, R. J. Multiplex kinase signaling modulates cardiac function at the level of sarcomeric proteins. J. Biol. Chem. 283, 26829–26833 (2008).

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Author contributions

J.K. carried out most of the experiments and wrote a manuscript draft. A.U. performed immunofluorescence and immunoelectron microscopy. L.B. performed binding assays in cell culture. J.N. carried out mechanical measurements on cardiomyocytes. M.V.F.-S. performed protein gel electrophoresis and western blotting. C.G.D.R. and M.M.R. provided tissue from failing hearts. F.S., U.G. and P.B. generated and provided genetic mouse models and revised the manuscript. W.A.L. designed and supervised the study, analyzed data, wrote the manuscript, and provided funding.

Additional information

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