The cardiac syndecan-4 interactome reveals a role for syndecan-4 in nuclear translocation of muscle LIM protein (MLP)

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Running title: The cardiac syndecan-4 interactome reveals MLP connection

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ABSTRACT

Costameres are signaling hubs at the sarcolemma and important contact points between the extracellular matrix and cell interior, sensing and transducing biomechanical signals into a cellular response. The transmembrane proteoglycan syndecan-4 localizes to these attachment points and has been shown to be important in initial stages of cardiac remodeling, but its mechanistic function in the heart remains insufficiently understood. Here, we sought to map the cardiac interactome of syndecan-4 to better understand its function and downstream signaling mechanisms. By combining two different affinity purification methods with MS analysis, we found that the cardiac syndecan-4 interactome consists of 21 novel and 29 previously described interaction partners. Nine of the novel partners were further validated to bind syndecan-4 in HEK293 cells (i.e. CAVIN1/PTRF, CCT5, CDK9, EIF2S1, EIF4B, MPP7, PARVB, PFKM, and RASIP). We also found that 19 of the 50 interactome partners bind differently to syndecan-4 in left ventricle lysate from aortic banded heart failure (ABHF) rats compared with SHAM-operated animals. One of these partners was the well-known mechanotransducer muscle LIM protein (MLP), which showed a direct and increased binding to syndecan-4 in ABHF. Nuclear translocation is important in MLP-mediated signaling, and we found less MLP in nuclear enriched fractions from syndecan-4−/− mouse left ventricles, but increased nuclear MLP when syndecan-4 was overexpressed in a cardiomyocyte cell line. In the presence of a cell-permeable syndecan-4–MLP disruptor peptide, the nuclear MLP level was reduced. These findings suggest that syndecan-4 mediates nuclear translocation of MLP in the heart.
In response to biomechanical stress and extracellular matrix cues, the heart is able to remodel its form and function to cope with increased demands. Major sites of biomechanical stress-sensing and transduction are the costamere and z-discs, which are the merging points between sarcomere and sarcolemma in the cardiomyocytes, and focal adhesions, which are the linkage sites between the extracellular matrix (ECM) and cytoskeleton in fibroblasts (1-3). Molecules able to merge and coordinate signaling inwards at these sites are likely key players in the biomechanical stress-sensing apparatus in the heart.

 Syndecan-4 is a ubiquitously expressed transmembrane proteoglycan with a large ectodomain decorated with ECM binding glycosaminoglycan chains, a transmembrane domain and a short cytoplasmic tail which connects to intracellular binding partners and the actin cytoskeleton (4). Since syndecan-4 holds no intrinsic enzymatic activity, direct signal transduction must occur through interactions with its cytoplasmic tail or indirectly through coreceptors like the integrins (4). Due to its membrane-spanning quality and ideal position at z-discs (5) and focal adhesions (6), syndecan-4 has attracted much attention as a potential mechanosensing and transducing molecule in the heart. When syndecan-4−/− mice are subjected to mechanical stress by inducing pressure overload of the left ventricle (LV), a reduction of the expected hypertrophy and ECM stiffness is observed, placing syndecan-4 as a player in the biomechanical stress-sensing apparatus (7,8).

 Several direct or indirect syndecan-4 partners are implicated in cardiac remodeling and pathology, including PKCα (9), calcineurin (7) and the calcineurin-dependent transcription factor nuclear factor of activated T-cells (NFAT) (1,2).

 To better understand how syndecan-4 exerts its cellular function in the heart, we set out to map the cardiac syndecan-4 interactome. By combining two different large scale affinity purification (AP-MS) methods with mass spectrometry (MS), we identified 21 novel syndecan-4 interaction partners together with 29 partners previously described in the literature, which together make up the cardiac interactome. 38% of these 50 interactions were altered during LV pressure overload, including MLP, a known mechanotransducer directly involved in human cardiomyopathy (10,11). We found a direct interaction between MLP and syndecan-4. Nuclear translocation of MLP, which is important for its ability to initiate hypertrophic gene expression (12), was reduced in syndecan-4−/− mice and in H9c2 rat cardiomyoblast cells treated with a cell-permeable syndecan-4-MLP disruptor peptide. In contrast the nuclear MLP level increased when syndecan-4 was overexpressed in H9c2. Altogether, our data suggests syndecan-4 mediates nuclear translocation of MLP.

**RESULTS**

**Use of two different AP-MS approaches to capture syndecan-4 interaction partners**

To increase confidence in our results, two different affinity purification (AP) approaches were used to identify syndecan-4 protein partners. In the first approach, we used biotinylated peptides covering the cytoplasmic part of syndecan-4 as bait, with or without phosphorylation at serine 179 (SDC4cyt and pS179-SDC4cyt, respectively) (Fig. 1A i), left panel). A biotinylated scrambled peptide was used as a negative control (peptide sequences are given in Fig. 1B). The integrity of the peptides was first confirmed by immunoblotting. Both biotin-SDC4cyt and biotin-pS179-SDC4cyt were recognized by anti-syndecan-4cyt (custom made, with an internal epitope, Genscript) (Supporting Fig. S1A, upper panel), biotin-pS179-SDC4 was only recognized by anti-pS179-syndecan-4 (custom made, Genscript) (Supporting Fig. S1A, middle panel), whereas all three peptides were recognized by anti-biotin-HRP (Supporting Fig. S1A, lower panel). To confirm binding capacity of the bait, biotin-SDC4cyt was shown to bind to the syndecan-4 C1-domain partner ezrin (13) in an ELISA-based interaction assay, whereas the scrambled control peptide did not (Fig. 1C). For the mass spectrometry (MS) analyzes, equivalent amounts of the three peptides were separately incubated in rat LV lysate, and putative syndecan-4 interacting proteins were pulled out with anti-biotin-agarose beads. Nonspecific binders (light blue proteins in Fig. 1A) were removed by several wash steps and remaining bound proteins (light brown proteins) were eluted with 25mM biotin before precipitation with ice-cold acetone, trypsin digestion and identification by MS.
In the second approach, we used anti-syndecan-4 (KY/8.2) to precipitate endogenous syndecan-4 (Fig. 1A ii), middle panel. The specificity of the syndecan-4 (KY/8.2) antibody was first validated by epitope mapping. Anti-syndecan-4 (KY/8.2) was overlaid onto 20-mer peptides covering the full length syndecan-4 rat protein, and ALPDEDAGGL was recognized as the core epitope (Fig. 1D). Importantly, the ALPDEDAGGL sequence was unique to syndecan-4 and not present in the three other syndecans (Fig. 1E). Consistently, anti-syndecan-4 (KY/8.2) did not recognize syndecan-1, -2 or -3 (Supporting Fig. S1B). Immunoprecipitation experiments confirmed that anti-syndecan-4 (KY/8.2) specifically precipitated syndecan-4 from both isolated rat neonatal cardiomyocytes (CM) and cardiac fibroblasts (CFB) (Fig. 1F).

Identification of syndecan-4 interacting proteins from AP-MS

Both AP approaches were performed in large scale and in three biological parallels and each parallel were run in triplicates by MS. To eliminate potential false positives, we excluded common contaminants such as ribosomal proteins, uncharacterized proteins and pure nuclear and mitochondrial proteins. In the first approach using biotin-SDC4cyt or biotin-pS179-SDC4cyt versus biotin-scram (negative control) as bait, we identified 40 proteins which were significant in at least two experiments (n=2, p<0.05). No significant changes were observed when comparing the results of pull down experiments with biotin-SDC4cyt and biotin-pS179-SDC4cyt. In the second approach using anti-syndecan-4 against endogenous syndecan-4, we identified 368 putative syndecan-4 interacting proteins (n=3, p<0.01). 21 novel partners were identified in LV lysate by both AP-MS approaches (Fig. 2, Venn diagram, pink circle). These were AP-3 complex subunit delta-1 (AP3D1), caveola-associated protein 1 (CAVIN1/PTRF), t-complex protein 1 subunit epsilon (CCT5), cyclin-dependent kinase 9 (CDK9), cysteine and glycine-rich protein 3 (MLP/CSRP3), eukaryotic translation initiation factor 2 subunit 1 (EIF2S1), eukaryotic translation initiation factor 4B (EIF4B), 4.1 (EPB41), BRISC complex subunit Abraxas 2 (FAM175B/ABRAXAS2), glutathione peroxidase 1 (GPX1), growth hormone-regulated TBC protein 1 (GRTP1), MAGUK p55 subfamily member 7 (MPP7), protein LYRIC (MDTH), beta-parvin (PARVB), ATP-dependent 6-phosphofructokinase, muscle type (PFKM), ras-interacting protein 1 (RASIP1), ras-related GTP-interacting protein C (RRAGC), Na(+)/H(+) exchange regulatory cofactor NHE-RF2 (SLC9A3R2), tight junction protein ZO-2 (TJP2), tripartite motif-containing protein 72 (TRIM21), uveal autoantigen with coiled-coil domains and ankyrin repeats (UACA). RRAGC, TRIM72 and UACA interacted with syndecan-4 only in LV from rats with aortic banded heart failure (ABHF) (Fig. 2, denoted with **) and is discussed further below. The 21 novel partners are summarized in Table 1 and in more detail in Supporting Table S1. A complete list of all proteins identified in either AP-MS approaches is given in Supporting Table S2.

We also performed an extensive literature search and a summary of the 71 direct or indirect literature syndecan-4 partners reported in different species, tissues and cells is given in Supporting Table S3. Interestingly, 29 of the literature partners were also significant in our IP-syndecan-4 approach (Fig. 2, Venn diagram, green circle, and Supporting Table S3, p<0.05). The remaining literature partners were either not significant or showed no interaction with syndecan-4 in LV lysates.

Confirmation of syndecan-4 interaction partners in HEK293

Novel syndecan-4 protein partners were validated for syndecan-4 binding in HEK293 cells. MPP7, PARVB, CDK9, EIF2S1, PFKM, CCT5, RASIP1, EIF4B and CAVIN1 were cloned with either a 3xFLAG tag at N-terminus or a single FLAG tag at C-terminus and co-expressed with HA-syndecan-4 in HEK293 cells. The HEK293 lysates were further subjected to immunoprecipitation using anti-FLAG, thereby using the putative partner as bait and reversing the set-up used for the MS analysis. Immunoblotting with anti-HA or anti-syndecan-4 revealed that syndecan-4 co-precipitated strongly with MPP7 (Fig. 3A), PARVB (Fig. 3B), CDK9 (Fig. 3C), EIF2S1 (Fig. 3D), PFKM (Fig. 3E), CCT5 (Fig. 3F), RASIP1 (Fig. 3G), EIF4B (Fig. 3H) and CAVIN1 (Fig. 3I) (n=3). We detected binding between CAVIN1 and the 25kDa syndecan-4 when probing with anti–syndecan-4 and not anti-
HA, indicating that this band could represent the endogenous syndecan-4 core protein. Only non-specific binding was observed in the negative controls 3xFLAG-ADD3 (gamma-adducin) and 3xFLAG-STEK39 (STE20/SPS1-related proline-alanine-rich protein kinase) (Fig. 3J and K, respectively) (n=3). Thus, we conclude that the syndecan-4 binding in Fig. 3A-I was not due to simple overexpression. Previous studies have shown unsuccessful translocation of MLP-FLAG, indicating that the FLAG tag disturbs MLP function (14). The syndecan-4-MLP interaction was therefore confirmed directly in rat LV lysate (Fig. 6A).

The cardiac syndecan-4 interactome is enriched in cell communication/signal transduction, cardiovascular disease, stress response proteins, and PDZ domains

To get an overview of the cardiac syndecan-4 interactome, the 21 novel partners together with the 29 literature partners (Venn diagram in Fig. 2) were clustered according to biological process using the GO annotation data from The Human Protein Reference Database (HPRD) (Fig. 4). Most of the syndecan-4 partners were involved in cell communication/signal transduction, protein metabolism and cell growth/maintenance. Within “cell communication/signal transduction” (right in Fig. 4), “enzymatic activity”, which includes serine/threonine/tyrosine kinases and phosphatases, formed the largest cluster, whereas “receptor activity” and “GTPase activity” were the next biggest clusters. An in silico interaction analysis using STRING 10.5 (http://string-db.org, (15)) showed that the cardiac syndecan-4 interactome contained more interactions than expected from a random set of proteins, indicating that these proteins indeed form complexes (nodes: 50, edges: 166 (expected: 69), average node degree: 6.64, average local clustering coefficient: 0.621, PPI enrichment p-value: <1.0e-16) (Supporting Fig. S2). According to STRING, 42% of the proteins in the cardiac syndecan-4 interactome were coupled to stress response (Supporting Fig. S2, labelled in yellow).

The cardiac syndecan-4 interactome was also analyzed by the DAVID functional annotation tool (15). “Metabolic” (count 25, 50%, 5.5x10^2) and “cardiovascular” (count 22, 44%, 4.3x10^2) were amongst the most enriched disease classes (Table 2). The cardiovascular disease class included the novel partners AP3D1, EPB41, GPX1, MLP, MPP7, PARVB and RASIP1. Moreover, to get an insight into which signaling pathways the syndecan-4 interactome is involved in, we used the Kyoto Encyclopedia of Genes and Genome (KEGG) pathways. The most enriched pathways were “proteoglycans in cancer” (30%, p-value 9.7x10^-13) and “focal adhesion” (26%, p-value 4.9x10^-10) (Table 3). The syndecan-4 interactome was also enriched in pathways involved in different cardiomyopathies like arrhythmogenic right ventricular cardiomyopathy (10%), hypertrophic cardiomyopathy (6%) and dilated cardiomyopathy (6%). To detect enriched protein domains in the syndecan-4 interactome we used PFAM (16). PDZ was the most abundant PFAM domain and found in the novel partners MPP7, SLC9A3R2, TJIP2 and the literature partner SDCBP (Table 4).

Changes in the syndecan-4 interactome during development of heart failure

To identify changes within the syndecan-4 interactome during pressure overload, large scale IP-syndecan-4 experiments were performed in LV lysate isolated from ABHF and SHAM rats (Fig. 1A iii), right panel). In total, 19 syndecan-4 interactions, involving novel and literature partners, were found to be either up- or downregulated in ABHF compared to SHAM (Supporting Fig. S4 and Supporting Table S1 and S3, right columns, n=3, p<0.05). These 19 proteins clustered into 11 different syndecan-4 networks (Fig.5, novel partners in pink) according to STRING 10.5 and literature. The largest cluster contained the novel partners MLP and UACA and the literature partners PRKCD, PRKCA, ARF6, RHOA, RHOG, and ACTN2. The second largest cluster contained collagen alpha-1 (COL1A1) and other extracellular literature partners and is consistent with a role for syndecan-4 in matrix remodeling and fibrosis in heart (recently reviewed in Christensen et al., 2018).

Syndecan-4 mediates nuclear translocation of MLP

As MLP is a well-known mechanotransducer, we wanted to analyze its interaction with syndecan-4 in more detail. Consistent with the MS
dataset (Supporting Fig. S3K and S4E), we showed that MLP co-precipitated stronger with anti-syndecan-4 (KY/8.2) than non-relevant rabbit antibodies (rIgG) (Fig. 6A) and that the interaction was increased in ABHF compared to SHAM (Fig. 6B). Consistently, the total MLP protein level was increased in ABHF compared to SHAM (Fig. 6C), whereas the syndecan-4 expression was unchanged (Fig. 6D). An ELISA-based assay using biotin-SDC4cyt against recombinant MLP identified the syndecan-4-MLP interaction to be direct (Fig. 6E). To assess if syndecan-4 influenced nuclear translocation of MLP, MLP was analyzed in nuclear enriched fractions isolated from wild type (WT) and syndecan-4 knock-out (SDC4-/-) mouse hearts. The nuclear MLP level was reduced when syndecan-4 was absent (Fig. 6F, right panel), even though the total amount of MLP was equal in RIPA extracts of WT and SDC4-/- mouse hearts (Fig. 6G). On the other side, adenovirus-mediated overexpression of full length syndecan-4 in a H9c2 rat cardiomyoblast cell line resulted in an increased nuclear MLP level compared to null virus (control) (Fig. 6H). The amount of syndecan-4 overexpressed and the distribution in cytoplasmic, nuclear and membrane fractions is given in Supporting Fig. S5B. Since we found the syndecan-4-MLP interaction to be direct, breaking this bond should disrupt the biological function of this interaction. We generated a cell-permeable syndecan-4-MLP disruptor peptide by adding a TAT-tag to the N-terminus of the cytoplasmic tail of syndecan-4 (TAT-SDC4cyt) (upper panel in Fig. 6I) and tested its effect on isoprenaline (ISO) stimulated H9c2 cells. H9c2 cells show a similar hypertrophic response to ISO as primary neonatal rat cardiomyocytes in vitro (17). The time point of increased MLP nuclear entry in response to ISO was determined to be 6-12 h post-stimulation (Supporting Fig. S5C). In the presence of disruptor peptide (TAT-SDC4cyt), the nuclear fraction of MLP was reduced compared to the TAT-tagged scrambled control peptide (Fig. 6I, lower panel). As a physiological read-out we also measured the protein content. Consistent with a role of MLP in gene transcription and hypertrophic growth (12), we observed less protein in the cytoplasmic fractions of cells treated with TAT-SDC4cyt compared to cells treated with TAT-scram (Fig. 6J). The principle of this disruptor peptide experiment is illustrated in Fig. 6K. Altogether, our data suggest syndecan-4 to be a mediator for nuclear translocation of MLP.

**DISCUSSION**

**The cardiac syndecan-4 interactome**

An important step in understanding how syndecan-4 mediates its cardiac function is to determine its molecular interaction partners. In this study we have identified the cardiac syndecan-4 protein interaction network of 21 novel partners and 29 previously described in the literature. This is the first experimental syndecan-4 interactome and it is enriched with proteins involved in cell communication/signal transduction, stress response, metabolic and cardiovascular disease.

In our analysis, proteins had to be detected by two different AP-MS approaches to be regarded as a novel syndecan-4 partner. This strategy might have favored more stable syndecan-4 interactions and transient and weak interactions might have been lost. As input material we used LVs lysed in a buffer containing 1% Triton and thus included all cellular compartments, except the nucleus, from all cardiac cell types. The complexity of the input material might have introduced false protein-protein interactions not relevant in vivo. However, false positives seemed not to be a problem since all protein partners we tested also revealed syndecan-4 binding in HEK293, whereas non-relevant proteins did not. It has also been noted by others that the cytoplasmic domain of syndecan-4 has a high number of interaction partners compared to size (18). The large number of partners can be explained by different types of interactions (direct/indirect) in different cardiac cell types and subcellular locations. Syndecan-4 locates to both costamere and Z-disc (5), but is also reported to localize to Golgi structures, intracellular vesicle membranes and perinuclear during muscle differentiation (19).

**Changes in the syndecan-4 interactome during development of heart failure**

Interestingly, 19 proteins in the interactome (38%) showed an altered interaction with syndecan-4 in ABHF versus SHAM and clustered into 11 smaller syndecan-4 networks (Fig. 5). Altered interactions could be due to several factors e.g. changes in availability, post-translational
modifications, syndecan-4 oligomerization level and/or competition between the protein partners for syndecan-4 binding.

One network contained SDCBP, which binds directly through its PDZ2 domain to the C-terminal FYA-motif in syndecan-4 (20). SDCBP is involved in recycling of the syndecan to the plasma membrane (21) and exosome formation through an SDCBP-dependent recruitment of PDCD6IP (programmed cell death 6-interacting protein), another partner in the syndecan-4 interactome (Fig. 4), and the ESCRT-III complex (endosomal-sorting complex required for transport III) (22). Our findings indicate that these syndecan-4-dependent trafficking processes might change during pressure overload. SDCBP also negatively regulates syndecan-4 function in focal adhesion formation by reducing the syndecan-4-PKCα interaction and thus PKCα activation (20).

A second syndecan-4 network consisted of the two novel partners CAVIN1 and TRIM72, where the latter interaction was only identified in ABHF (Fig. 5). CAVIN1 plays an important role in caveolae formation and organization (23) and recruits TRIM72 in an emergency membrane repair response to protect against stress-induced loss of cardiomyocytes (24,25). Our findings of a novel syndecan-4-CAVIN1-TRIM72 axis might suggest a compensatory role for syndecan-4 in cell membrane repair during pressure overload. Mutations in CAVIN1 are reported to cause skeletal muscle hypertrophy, lipodystrophy and cardiac dysfunctions including arrhythmias (25,26).

A third syndecan-4 network contained the novel partner AP3D1 and the literature partner CLTC (27), a major protein of the polyhedral coat of coated pits. AP3D1, which showed an increased interaction with syndecan-4 (Fig. 5), is a component in the ubiquitous heterotrameric AP-3 (adaptor protein complex 3) involved in intracellular trafficking and generation of vesicles with different cargo or protein compositions. The literature partner CLTC is suggested to play a cargo-selective role in this process (reviewed in (28)). We speculate whether syndecan-4 is subjected to increased trafficking through an AP-3-dependent pathway during pressure overload and perhaps also plays a cargo-selective role. AP3D1 is reported to associate with myocardial infarction and atherosclerosis in a Japanese population (29).

A fourth syndecan-4 network contained the novel partner RRAGC and the literature partner serine/threonine-protein kinase mTOR (mammalian target of rapamycin) (Fig. 5). mTOR activity is regulated by assembly into two functionally distinct complexes mTORC1 and mTORC2. mTORC1 is a major driver of cell growth and protein synthesis, triggered by growth factors, stress signals, energy and nutrients (reviewed in (30)). In response to amino acids, RRAGC<sub>GDP</sub> targets mTORC1 to lysosomes where it is activated by Rheb (31,32). Our findings of a novel syndecan-4-RRAGC interaction only in ABHF hearts (Fig. 5), could suggest that syndecan-4 is more involved in mTORC1 signaling in heart failure development. On the other hand, activity of the mTORC2-Akt-eNOS axis depends on a syndecan-4-PKCα-dependent recruitment of mTORC2 to rafts in endothelial cells (33).

A fifth syndecan-4 network contained the two novel partners TJP2 and EPB41 (Fig. 5), where the latter partner links cell surface membrane proteins to the actin cytoskeleton. EPB41 and TJP2 are reported to form a complex at cell-cell junctions, which are structures known to be important in maintaining mechanical integrity of cardiomyocytes and resulting in arrhythmias when absent (34,35). Our findings of a decreased EPB41-syndecan-4 interaction (Fig. 5) indicate a reduction in the linkage between syndecan-4 and the actin cytoskeleton during pressure overload. Whether this interaction is lost at cell-cell junctions will have to be investigated in future studies. However, syndecan-4 showed an increased binding to the novel partner tight junction adaptor protein MMP7 (36), supporting a role for syndecan-4 at cell-cell junctions during pressure overload.

A sixth syndecan-4 network contained the two novel partners EIF4B and EIF2S1 (Fig. 5). EIF4B is involved in the initiation of translation and recruitment of the 40S ribosomal subunit to the mRNA, a process that is regulated by phosphorylation of EIF4B according to the cell’s need, through e.g. the mTOR pathway (37). EIF2S1 is also involved in translation initiation and can be regulated through phosphorylation. In response to cell stress, phosphorylation of EIF2S1
has been shown to inhibit protein synthesis except selected stress response mRNAs (38). More EIF2S1 and less EIF4B precipitated with syndecan-4 in ABHF, which could indicate competition between the two proteins and perhaps a role for syndecan-4 in directing translation in heart failure development.

The largest syndecan-4 network contained partners involved in actin remodeling, signal- and mechanotransduction (Fig. 5). PKCδ (PRKCD), PKCa (PRKCA), and UACA interacted only with syndecan-4 during pressure overload. PKCa is a well-known syndecan-4 partner and involved in adhesion formation and contractility (9,39). Upon dephosphorylation of pSer179, syndecan-4 oligomerizes and activates PKCa and can thereby regulate the time and place for PKCa activity (40). PKC activation is recently found to be upregulated at the costameres in heart disease (41), and we speculate whether this is due to an increased interaction with syndecan-4. In line with this, we have previously found reduced pSer179-syndecan-4 levels in aortic stenosis patients and in mice after aortic banding (7). Contrarily, the literature partner PKCδ phosphorylates syndecan-4 at serine 179, thus reducing PKCa activity and impairing endothelial cell function (42). The literature partners RHOA, RHOG and ARF6 are involved in actin cytoskeletal remodeling (4). Syndecan-4 controls both suppression and activation of these GTPases, for example it activates RHOG by first activating PKCa thereby triggering RHOG/caveolin-dependent endocytosis of integrins (4,18). ARF6, which is also implicated in syndecan and integrin recycling (18,21) interacts with the novel syndecan-4 partner UACA, another cytoskeletal remodeler (43). The network also revealed a novel syndecan-4-MLP-ACTN2 axis. The novel partner MLP is a well-known stress- and mechanotransducer in muscle cells and associates directly with human myopathies (11). The literature partner ACTN2 is a F-actin cross-linking scaffolding protein at Z-disc and cell-cell junctions, where it couples receptors, like syndecan-4, to the actin cytoskeleton (35). A human hypertrophic cardiomyopathy mutation in MLP has been shown to destabilize the interaction between MLP and ACTN2 and a third component N-rap leading to myocyte disarray (44). The increased interaction between ACTN2 and MLP with syndecan-4 during pressure overload supports a role for syndecan-4 in mechano signaling complexes.

Altogether, our data indicate that syndecan-4 associates with important cellular structures and functions, including focal adhesions, tight junctions, endocytosis, trafficking, mechanotransduction and cytoskeleton remodeling during development of heart failure induced by pressure overload. These wide-ranging functions are probably explained by its many intracellular binding partners.

**Syndecan-4 mediates nuclear translocation of MLP**

The large multi-protein structures of z-discs have been suggested as major sites for sensing and transducing mechanical stimuli (1), and of particular interest are LIM domain proteins, which are able to shuttle to the nucleus, thus connecting mechanical stimuli with gene expression (45,46). MLP, which we identified as a novel and direct syndecan-4 partner, contains two independent LIM domains that facilitate formation of multiprotein complexes (47). MLP interacts with structural and signaling proteins at the z-discs and translocates to the nucleus to initiate hypertrophic gene expression in response to biomechanical stress (11,12,14). Nuclear translocation of MLP is crucial for its mechano-transducing probabilities, and its nuclear accumulation associates with pathology (14). Interestingly, we found the syndecan-4-MLP interaction to be increased during pressure overload, suggesting a function for this complex in cardiac pathology. Our findings of a direct syndecan-4-MLP binding led us to develop a specific cell-permeable disruptor peptide to assess the biological function of this interaction. The syndecan-4-MLP disruptor peptide inhibited nuclear translocation of MLP and attenuated the increase in protein content following ISO stimulation of H9c2 cells, suggesting the nuclear translocation of MLP and the hypertrophic gene expression to be syndecan-4-dependent. The syndecan-4-MLP interaction also appears to be relevant in vivo, since less nuclear MLP was found in the syndecan-4−/− mouse LVs, an observation consistent with loss of concentric myocardial hypertrophy and rather LV dilatation and dysfunction during pressure overload (7). (7,12,48)
The increased syndecan-4-MLP interaction can be due to changes in availability, structures or post-translational modifications during pressure overload. We have previously shown reduced pSer179-syndecan-4 levels in human aortic stenosis patients and in mice after pressure overload, enabling calcineurin binding and activation of the hypertrophic NFAT pathway (7). Perhaps the pSer179 also works as a molecular switch for the MLP interaction and its nuclear translocation. Syndecan-4 has previously been found in the nuclear membrane and perinuclear compartment (19), and syndecan-1 has been suggested to translocate into the nucleus (49). Future studies are needed to investigate how syndecan-4 directs the translocation of MLP and whether syndecan-4 also goes into the nucleus. It will also be interesting to investigate whether the syndecan-4 binding overlaps with any of the MLP mutations associated with human hypertrophic cardiomyopathy (10,50), or dilated cardiomyopathy (11). Collectively, these data indicate that syndecan-4 act upstream of MLP, and mediates its nuclear translocation upon stress.

**EXPERIMENTAL PROCEDURES**

**Animal handling**

All animal handling was preapproved by the Norwegian National Animal Research Committee (FOTS ID 3820 for rats, FOTS ID: 6989 for SDC4−/− mouse) and conformed to the Guide for the Care and Use of Laboratory Animals (NIH publication 85-23, revised 2011, US). Animals were stored with 12:12 h light and dark cycles and were given access to food and water *ad libitum*. For neonatal cell isolation: Permit of approval number IV1-17U.

**Aorta banding in rats**

~170 gram male Wistar-Hannover rats (Taconic, Denmark) were anesthetized and ventilated with a mixture of 68% N₂O, 29% O₂ and 2-3% isoflurane through endotracheal intubation. The chest was opened and aorta banding was induced by 3.0 silk suture tightened around the ascending aorta proximal to the brachiocephalic trunk. SHAM animals were used as controls, and went through the same surgical procedure except tightening of the suture around the ascending aorta. Buprenorphine was used as analgesic. Echocardiography was performed with a Vevo 2100 (VisualSonics, Canada) six weeks after surgery, before myocardial tissue samples were harvested in deep surgical anesthesia. The left and right ventricle were rapidly dissected and subsequently put into liquid nitrogen and stored at -70°C for later molecular analysis. The criteria for congestive heart failure (ABHF) were increased lung weight (>2.0 g), increased posterior wall diameter (PWd, >1.9 mm) and left atrial diameter (>5.0 mm). Characteristics of the animals used in the large scale AP-MS experiments are given in Supporting Table S4. For the qPCR and western blot analysis 23 additional animals were included using the same criteria as in Supporting Table S4.

**Left ventricular lysates**

Frozen rat or mouse LVs were pulverized in liquid nitrogen before homogenization with a Polytron 1200 homogenizer in three series of 1 min in lysis buffer (150 mM NaCl, 20 mM -Hepes, pH 7.5, 1 mM EDTA, 0.5% Triton X-100 (Sigma-Aldrich, USA)) supplemented with complete protease inhibitor cocktail (05050489001, Roche, Switzerland) or in RIPA buffer (#R0278, Sigma-Aldrich) on ice. Following centrifugation at 100,000 x g for 60 min at 4°C, supernatants were stored at -80°C until analysis.

**Antibodies**

Anti-syndecan-4cyt (epitope: C-DLGKKPIYKKAPT) and anti-pS179-syndecan-4 (epitope: EGPYSYDLGKKPIYKCC) were custom made by Genscript Corp. (USA). Anti-syndecan-4 (KY/8.2, #550350) was obtained from BD Pharmingen™ (USA). Anti-biotin-HRP (A-0185) and anti-FLAG (F1804) were obtained from Sigma-Aldrich. Anti-MLP was obtained from Santa Cruz Biotechnology (USA) (sc-166930) or Thermo Scientific (USA) (PA5-19062). Anti-HA (#3724) and anti-histone H3 (#4499) were from Cell Signaling technology, Inc. (The Netherlands). Non-relevant rat IgG (#sc-2026) and anti-GADPH (#sc-20357) were from Santa Cruz Biotecology. Anti-FLAG (sc-20357) were from Santa Cruz Biotecology. Anti-mouse IgG HRP (NA931V) and anti-rabbit IgG HRP (NA934V) affinity-purified polyclonal antibody (both from GE Healthcare, USA) were used as secondary antibody.

**Peptides and recombinant proteins**
Peptides were synthesized to > 80% purity by Genscript.
Biotin-syndecan-4<sub>cyt</sub>: RMKKKDEGSYDLGKKPIYKKAPTNEFYA,
Biotin-pS179-syndecan-4<sub>cyt</sub>: RMKKKDEGSYDLGKKPIYKKAPTNEFYA,
Biotin-scram: GTKYPKMDRGKLFKYKAKPENASYIK,
TAT-syndecan-4<sub>cyt</sub>: RKKRRQRRRMMKKKDESYDLGKKPIYKKAPTNEFYA,
TAT-scram: RKKRRQRRRTGKYPKMDRGKLFKYKAKPENASYIK,
Recombinant 12xHis -Ezrin (1-333) (rat, Uniprot P31977) was made by Genscript. Recombinant MLP was obtained from Abova (Taiwan) (H0008048-P01).

**ELISA**
The ELISA plate was first coated with 0.46 µg/well of recombinant MLP or 10 µg/mL of recombinant ezrin in 1xPBS and rotated overnight (ON) at 4°C. The next day the wells were washed once in 1xPBS-T (0.1%) before blocking in PBS with 1% gelatin (G-1890, Sigma-Aldrich) for 1 h while rotating at room temperature (RT). After blocking, the coated wells were incubated with either biotin-syndecan-4<sub>cyt</sub> or biotin-scram (control peptide) in 1xPBS-T (0.1%) for 2 h at 37°C while rotating. Thereafter, the wells were washed five times in 1xPBS-T (0.1%) before incubating with anti-biotin-HRP (1:5000) for 30 min while rotating at RT. Then wells were washed five times in 1xPBS-T (0.1%) before adding Ultra TMB-ELISA solution (34028, Thermo Scientific) and incubated for 15-30 min while rotating at RT. Reaction was stopped by adding 2 N HCL and the signal measured by a plate reader at 450 nm (Hidex Sense multimodal microplate reader, Finland).

**Epitope mapping**
Rat syndecan-1-4 were synthesized as 20-mers with 3 amino acid offsets onto cellulose membranes by a Multipep automated peptide synthesizer (INTAVIS Bioanalytical instruments AG, Germany) as described in (51). After blocking, the membranes were incubated with anti-syndecan-4 ON at 4°C. After washing three times in TBS-T, membranes were incubated with secondary antibodies for 1 h at RT, before another three times wash in TBS-T and developed as an immunoblot.

**Primary rat neonatal cardiac myocytes and fibroblasts**
Primary cardiomyocytes and fibroblasts were isolated from 1-3 days old Wistar rats (Møllergaard Breeding and Research Center, Denmark). Hearts were excised, trimmed for atrial tissue, minced and digested with a collagenase solution (Worthington biochemical, USA). The cell suspension was transferred to uncoated culture flasks with serum containing medium in two rounds for 20 min at 37°C for fibroblasts to attach. After attachment, fibroblasts were kept in fibroblast medium (Dulbecco's modified Eagle's medium (DMEM) (41965-039, Gibco, Life Technologies, Inc., USA)) supplemented with penicillin/streptomycin (P0781, Sigma-Aldrich) and fetal bovine serum (FBS) (FB-1001/500, Biosera, France). Myocytes were isolated as non-adherent cells and seeded onto 6-well culture plates precoated with gelatin/fibronectin (Sigma-Aldrich) and kept in plating medium consisting of DMEM (D1152, Sigma-Aldrich), M-199 (M2520, Sigma-Aldrich), penicillin/streptomycin, horse serum (14-403E, BioWhittaker, USA) and fetal bovine serum (14-701F, BioWhittaker). The cells were cultured for up to one week in a 37°C, 5% CO₂ humidified incubator before lysis in RH buffer (1xPBS, 1% Triton (Sigma-Aldrich), 0.1% Tween (161-0781, Bio-Rad, USA) supplemented with cOmplete protease (Roche) and phosSTOP phosphatase inhibitor cocktail (04906837001, Roche)).

**Cell lines, constructs and transfection**
HEK293 (ATCC® CRL-1573™) and H9c2 (ATCC® CRL-1446™) cells were cultured in DMEM with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (PS) (P0781, Sigma-Aldrich) at 37°C in 5% CO₂ in a humidified incubator. After 24 h in PS free medium, HEK293 cells were transfected via the CaCl₂ method. For each transfection, 8 µg DNA in a CaCl₂ solution (248 mM) was mixed with 500 µl 2xHEPES (50 mM HEPES, 280 mM NaCl, 1.5 mM Na₂HPO₄, pH 7.0) in a drop-wise manner and incubated at RT for 20-30 min before being transferred onto the cells. 24 h after transfection the cells were harvested in IP buffer (150 mM NaCl, 20 mM...
HEPES pH 7.5, 1 mM EDTA, 1% Triton X-100 supplemented with complete protease inhibitor cocktail (Roche). Cloning was done by Genscript and all genes were tagged with either HA or FLAG tags in the pCPE4 or pcDNA3.1 vectors (Genscript). Constructs were HA-SDC4 mouse (O35988), 3xFLAG-MPP7 rat (NM_001100575), 3xFLAG-PARVB rat (NM_001134780), 3xFLAG-CDK9 rat (NM_001007743), 3xFLAG-EIF2S1 rat (NM_019356), PFKM-FLAG human (NM_000289.5), CCT5-FLAG human (NM_012073.4), RASIP1-FLAG human (NM_017805.2), EIF4B-FLAG human (NM_001417.6), PTFR-FLAG human (NM_012232.5), 3xFLAG-ADD3 rat (NM_006231599.3) (negative control), and 3xFLAG-STK39 rat (NM_019362) (negative control).

Small scale immunoprecipitation and immunoblotting
For all IPs, lysates were incubated with 2 μg antibody and protein A/G-agarose beads (sc-2003, Santa Cruz Biotechnology) ON at 4°C and unless otherwise stated washed three times in IP buffer (150 mM NaCl, 20 mM HEPES, pH 7.5, 1 mM EDTA, 1% Triton X-100 with complete protease inhibitor cocktail (Roche)) before elution by boiling in 2xSDS loading buffer. In Fig. 6A, 200 uL (6.1 ug/uL) of rat LV lysate was used per IP and 5 uL lysate was used as input (2.5% of IP lysate). After precipitation, this IP was washed three times in PBS buffer. In Fig. 6B, 200 uL (2.0 ug/uL) of rat LV lysate was used per IP.

Lysates, immunoprecipitations and peptides were analyzed on a 4-15% or 15% Criterion Tris-HCl precast gel (3450028 and 3450021, Bio-Rad) and blotted onto a PVDF membrane (#1704157, Bio-Rad). The membranes were blocked in 5% nonfat dried milk or 1% casein in TBS-T (Tris-buffered saline with 1% Tween-20 (161-0781, Bio-Rad)) for 1 h at RT. Membranes were thereafter incubated with primary antibodies for 1 h at RT or ON at 4°C, followed by three times 5 min wash in TBS-T before incubation with horseradish peroxidase-conjugated secondary antibody for 1 h at RT. Blots were developed using ECL Prime (RPN 2232, GE Healthcare). When the signal was too high, blots incubated with anti-MLP were washed in TBS-T before repeating development. For reprobing, the membranes were stripped in a Restore™ Western Blot Stripping Buffer (21059, Thermo Scientific). Densitometric analysis was achieved with ImageJ (NIH). Total protein concentration of the various samples was measured using the Micro BCA Protein Assay Kit (23235, Thermo scientific). Equal protein amounts were loaded per lane: 20 μg of nuclear fraction isolated from mice hearts, 35.5 μg of RIPA extracts isolated from mice hearts, 25 μg of nuclear fractions isolated from virus transduced cells and 11 μg of nuclear fractions isolated from ISO stimulated cells.

Large scale pull down experiments and immunoprecipitations
For the large scale pull down experiments, LV lysates were pooled and mixed with 0.01 mM biotinylated peptides before rotation at 4°C ON. Streptavidin coated dynabeads (Dynabeads™ M-270 Streptavidin, #65305, Life Technologies) were washed three times in PBS before adding the peptide containing LV lysate and rotated for 40 min at RT. The beads were washed five times in 1 mL PBS and protein complexes were eluted in 250 μL 25 mM biotin for 3 h at 60°C. Proteins were precipitated in 1 mL 4xice-cold acetone added glycobue ON at -20°C. After centrifugation, the pellets were air-dried before analysis by MS.

For the large scale immunoprecipitation experiments, 10 μg/mg beads of anti-syndecan-4 or anti-rat IgG were coupled to magnetic dynabeads (Dynabeads™ Antibody Coupling Kit, #14311D, Thermo Fisher technologies, USA) according to manufacturer’s protocol. The antibody coupled beads were incubated with LV lysates from WT, aortic banded (ABHF) or SHAM rat hearts and rotated ON at 4°C. After three times wash in ice-cold PBS and two times wash in ice-cold MQ water to remove salts (52), interacting proteins were eluted in 0.1% TFA in 50% acetonitrile for 30 min while rotating at RT. The elution step was repeated once with fresh TFA, before precipitation and centrifugation, and finally the pellets were air-dried.

Wild type and syndecan-4−/− mice
Hearts were harvested from 9 weeks old female WT littermates or syndecan-4−/− mice (53), snap-frozen in liquid nitrogen and stored at -70°C until fractionation with Compartment Protein Extraction by guest on April 27, 2019
Kit (2145, Merck, USA) according to manufacturer’s protocol.

**Viral transduction**

H9c2 cells were cultured to a confluency of about 80% before being transduced with adenovirus serotype 5 encoding mouse syndecan-4 (#ADV-271493, Vector Biolabs, USA) or empty vector (null virus) (#1300, Vector Biolabs). Virus titer used was 2x10^7 PFU/mL. After 24 h incubation the cells were washed in PBS and added fresh media. 48 h after induction the cells were fractionated as described above.

**Isoprenaline stimulation and treatment with cell permeable peptides**

H9c2 cells were serum starved for 24 h before stimulation with 10 µM isoprenaline sulfate (ISO) (#24 37 82, NAF, Norway) with or without cell permeable peptides for 12 h in Fig. 6I, 6-12 h in Fig. 6J and 1-24 h in Supporting fig. S5C and subsequently fractionated as described above.

**RNA isolation and quantitative real-time PCR (qRT-PCR)**

RNA was isolated from LV tissue using RNeasy mini (#74104, Qiagen Norge, Oslo, Norway). RNA concentration was determined using the Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). For cDNA synthesis iScript cDNA Synthesis Kit (#170-8891, Bio-Rad Laboratories, Inc., Hercules, CA) were used according to manufacturer’s protocol. Gene expression levels were determined using pre-designed TaqMan assays detected on a QuantStudio3 (Applied Biosystems by Thermo Fischer Scientific, Waltham, MA) and analyzed via the QuantStudio Design and Analysis Software. TaqMan assays used were: (Rn00561900_m1; Sdc4) and (Rn00820748_g1; Rpl32) (LifeTech by Thermo Fischer Scientific, Waltham, MA). SDC4 expression was normalized to RPL32.

**Mass spectrometry**

The proteins were precipitated using 2-D Clean Up-Kit (80-6484-51, GE healthcare) according to manufacturer’s instructions. The precipitated proteins were dissolved in 40 µL 0.2% ProteaseMAX™ Surfactant, Trypsin Enhancer (Promega) in 50 mM NH₄HCO₃ followed by protein reduction, alkylation and in-solution digestion with trypsin (Promega) ON in 37°C. After digestion, the samples were centrifuged at 14000 x g for 10 min, trypsin was inactivated by adding 100 µl 1% TFA, and the samples were again centrifuged at 14000 x g for 10 min. Peptides were desalted and concentrated before MS by the STAGE-TIP method using a C18 resin disk (3M Empore). The peptides were eluted with 80 µl 80% ACN/0.1% FA, dried, and solubilized in 7 µL 0.1% FA for MS analysis.

Each peptide mixture was analyzed on an Easy nLC1000 nano-LC system connected to a quadrupole – Orbitrap (QXactive) mass spectrometer (ThermoElectron, Bremen, Germany) equipped with a nanoelectrospray ion source (EasySpray/Thermo). For liquid chromatography separation we used an EasySpray column (C18, 2 µm beads, 100 Å, 75 µm inner diameter) (Thermo) capillary of 25 cm bed length. The flow rate used was 0.3 µL/min, and the solvent gradient was 5% B to 30% B in 120 min, then 90% B wash in 20 min. Solvent A was aqueous 0.1% formic acid, whereas solvent B was 100% acetonitrile in 0.1% formic acid. Column temperature was kept at 60°C.

The mass spectrometer was operated in the data-dependent mode to automatically switch between MS and MS/MS acquisition. Survey full scan MS spectra (from m/z 400 to 1,200) were acquired in the Orbitrap with resolution R = 70,000 at m/z 200 (after accumulation to a target of 3,000,000 ions in the quadruple). The method used allowed sequential isolation of the most intense multiply-charged ions, up to ten, depending on signal intensity, for fragmentation on the HCD cell using high-energy collision dissociation at a target value of 100,000 charges or maximum acquisition time of 100 ms. MS/MS scans were collected at 17,500 resolution at the Orbitrap cell. Target ions already selected for MS/MS were dynamically excluded for 30 seconds. General MS conditions were: electrospray voltage, 2.1 kV; no sheath and auxiliary gas flow, heated capillary temperature of 250°C, normalized HCD collision energy 25%. Ion selection threshold was set to 5e4 counts. Isolation width of 3.0 Da was used.

The resulting MS raw files were submitted to the MaxQuant software (version 1.5.2.8) for protein identification using the Andromeda search engine. Carbamidomethyl (C) was set as a fixed modification and protein N-acetylation and
methionine oxidation were set as variable modifications. First search peptide tolerance of 20 ppm and main search error 6 ppm were used. Trypsin without proline restriction enzyme option was selected, with two allowed miscleavages. The minimal unique+razor peptides number was set to 1, and the allowed FDR was 0.01 (1%) for peptide and protein identification. Label-free quantitation (LFQ) was employed with default settings. The Uniprot database with ‘rattus’ entries was used for the database searches. Perseus software was used for the statistical analysis of the label-free quantitation results. Known contaminants as provided by MaxQuant and identified in the samples were excluded from further analysis. Proteins had to be identified with at least two peptides to be considered for further analysis.

**Biostatistics**
For enrichment analysis, the Database for Annotation, Visualization and Integrated Discovery (DAVID), version 6.8, was used. Official gene names were imported into the search tool and *Homo sapiens* was chosen for both species and background with the following setting: count (2) and EASE score (0.1) (Modified Fisher Exact $p$-value) (54). For categorizing the interactome into Biological Process, the Human Protein Reference Database (HPRD) was used (55).

**Statistics**
For quantitative comparisons and determination of significance between the MS samples, LFQ intensities were loaded in Perseus (version 1.4.0.20). Those values were log2 transformed and a t-test was done. For the three large scale biotin-pS179/syndecan-4 pull down analyses (each run in triplicates), $p$ values of 0.05 or below were accepted as statistically significant. For the three large scale IP-SDCD4 analyses (each run in triplicates), $p$ values of 0.01 or below were accepted as statistically significant. To get more inclusive data for the six AP-MS analyses, statistics without any correction were performed (Supporting Table S1). However, to be regarded as a true syndecan-4 partner, the protein had to be identified in at least five of these AP-MS analyses. The probability of the same random false positives to occur in all five AP-MS analyses was regarded low. For the large scale IP-SDCD4 analysis in ABHF vs. SHAM (run in triplicates), $p$ values of 0.05 or below were accepted as statistically significant. ELISA-based assay and cell experiments data are presented with group means ± SD. Statistical analysis was done in GraphPad Prism 7.01 using unpaired t-test and $p<0.05$ was accepted as statistically significant.
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CONFLICT OF INTEREST
The authors declare that they have no conflict of interest with the contents of this article.
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The abbreviations used are: ABHF: aortic banded heart failure; AP: affinity purification; AP-MS: affinity purification coupled to mass spectrometry; CF: cardiac fibroblast; C: cardiomyocyte; ECM: extracellular matrix; HPRD: human protein reference database; ISO: isoprenaline; KY/8.2: commercially available anti-syndecan-4; LV: left ventricle; ON: overnight; rIgG: non-relevant rabbit antibodies; SHAM: control operated; SDC4_cyt: cytoplasmic domain of syndecan-4; pS179-SDC4_cyt: cytoplasmic domain of syndecan-4 with phosphorylation at serine 179; TAT-SDC4_cyt: cell permeable disruptor peptide.

AP3D1: UniProt # O14617
CAVIN1/PTRF: UniProt # Q6NZI2
CCT5: UniProt # P48643
CDK9: UniProt # P50750
EIF2S1: UniProt # P05198
EIF4B: UniProt # P23588
EPB41: UniProt # P11171
FAM175B/ABRAXAS2: UniProt # Q15018
GPX1: UniProt # P07203
GRTP1: UniProt # Q5TC63
MLP/CSRP3: UniProt # P50461
MTDH: UniProt # Q86UE4
PARVB: UniProt #Q9HB11
PFKM: UniProt # P08237
RASIP1: UniProt # Q5U651
RRAGC: UniProt # Q9HB90
SLC9A3R2: UniProt # Q15599
TJP2: UniProt #Q9UDY2
TRIM72: UniProt # Q6ZMU5
UACA: UniProt # Q9BZF9
Table 1. 21 novel syndecan-4 interacting proteins identified by two AP-MS approaches.

| Gene            | Protein (Uniprot)                                                                 | Evidence for interaction | Molecular function (HPRD)                      |
|-----------------|----------------------------------------------------------------------------------|--------------------------|------------------------------------------------|
| AP3D1*          | AP-3 complex subunit delta-1                                                    | MS                       | Transporter activity                            |
| CAVIN1/PTRF     | Caveolae-associated protein 1                                                    | MS, Co-IP               | Transcription regulator activity                |
| CCT5            | T-complex protein 1 subunit epsilon                                              | MS, Co-IP               | Chaperone activity                              |
| CDK9            | Cyclin-dependent kinase 9                                                       | MS, Co-IP               | Protein serine/threonine kinase activity        |
| EIF2S1*         | Eukaryotic translation initiation factor 2 subunit 1                             | MS, Co-IP               | Translation regulator activity                  |
| EIF4B*          | Eukaryotic translation initiation factor 4B                                      | MS, Co-IP               | Translation regulator activity                  |
| EPB41*          | Protein 4.1                                                                      | MS                       | Structural molecule activity                    |
| FAM175B/        | BRISC complex subunit Abraxas 2                                                 | MS                       | Unknown                                         |
| ABRAXAS2        |                                                                                  |                          |                                                 |
| GPX1            | Glutathione peroxidase 1                                                         | MS                       | Peroxidase activity                             |
| GRTP1*          | Growth hormone-regulated TBC protein 1                                           | MS                       | GTPase activator activity                       |
| MLP/CSRP3*      | Muscle LIM protein/cysteine and glycine-rich protein 3                           | MS                       | Cytoskeletal protein binding                    |
| MPP7*           | MAGUK p55 subfamily member 7                                                     | MS, Co-IP               | Receptor signaling complex scaffold activity    |
| MTDH            | Protein LYRIC                                                                    | MS                       | Unknown                                         |
| PARVB           | Beta-parvin                                                                      | MS, Co-IP               | Cytoskeletal protein binding                    |
| PFKM            | ATP-dependent 6-phosphofructokinase, muscle type                                | MS, Co-IP               | Catalytic activity                              |
| RASIP1          | Ras-interacting protein 1                                                        | MS, Co-IP               | Unknown                                         |
| RRAGC**         | Ras-related GTP binding protein C                                                | MS                       | GTPase activity                                 |
| SLC9A3R2        | Na(+)/H(+) exchange regulatory cofactor NHE-RF2                                  | MS                       | Receptor signaling complex scaffold activity    |
| TJP2            | Tight junction protein ZO-2                                                       | MS                       | Cell adhesion molecule activity                 |
| TRIM72**        | Tripartite motif-containing protein 72                                            | MS                       | Unknown                                         |
| UACA**          | Uveal autoantigen with coiled-coil domains and ankyrin repeats                   | MS                       | Unknown                                         |

Co-IP: co-immunoprecipitation in HEK
* Interaction with syndecan-4 significantly changed in ABHF (p<0.05, n=3).
** Interaction with syndecan-4 only detected in ABHF (p<0.05, n=3).
**Table 2**: Disease class enrichment for the syndecan-4 protein partners (DAVID).

| Disease class        | Gene name                                                                 | Count | p-value   |
|----------------------|---------------------------------------------------------------------------|-------|-----------|
| Metabolic            | AP3D1, APP, CALM1, CAMK2A, COL1A1, MLP/CSRPR3, CTNNB1, DNM2, FYN, GPX1, ITGA2, ITGB1, MMP2, MMP9, MPP7, MTOR, PDCD6IP, PFKM, PLG, PPP3CA, PRKCA, PRKCD, RHOA, SRC, TP2 | 25    | 5.5x10^-2 |
| Cardiovascular       | ACTN2, AP3D1, APP, CALM1, COL1A1, MLP/CSRPR3, DNM2, EPB41, FYN, GPX1, ITGA2, ITGB1, MMP2, MMP9, MPP7, NF1, PARVB, PLG, PDCD6IP, RASIP1, RHOA, RAC1 | 22    | 4.3x10^-2 |

**Table 3**: Enriched KEGG pathways in the syndecan-4 cardiac interactome.

| Term                                              | Gene name                                                                 | Count | p-value   |
|---------------------------------------------------|---------------------------------------------------------------------------|-------|-----------|
| Proteoglycans in cancer                           | CAMK2A, CTNNB1, CTN, EIF4B, ITGA2, ITGA5, ITGB1, MMP2, MMP9, MTOR, PRKCA, PXN, RAC1, RHOA, SRC | 15    | 9.7 x10^-13 |
| Focal adhesion                                    | ACTN2, COL1A1, CTNNB1, FYN, ITGA2, ITGA5, ITGB1, PARVB, PRKCA, PXN, RAC1, RHOA, SRC | 13    | 4.9 x10^-10 |
| Arrhythmogenic right ventricular cardiomyopathy   | ACTN2, CTNNB1, ITGA2, ITGA5, ITGB1 | 5     | 5.8 x10^-4  |
| Hypertrophic cardiomyopathy                       | ITGA2, ITGA5, ITGB1 | 3     | 6.8 x10^-2  |
| Dilated cardiomyopathy                            | ITGA2, ITGA5, ITGB1 | 3     | 7.7x10^-2   |

**Table 4**: Enriched PFAM domains in the syndecan-4 cardiac interactome.

| Term                                              | Gene name                                                                 | Count | p-value   |
|---------------------------------------------------|---------------------------------------------------------------------------|-------|-----------|
| PDZ domain (Also known as DHR or GLGF)            | SLC9A3R2, MPP7, SDCBP, TJP2 | 4     | 5.4 x10^-3 |
| SH3 domain                                        | CTNN, FYN, SRC | 3     | 3.2 x10^-2 |
| Fibronectin type II domain                        | MMP2, MMP9 | 2     | 3.7 x10^-2 |
| Integrin alpha                                    | ITGA2, ITGA5 | 2     | 4.7 x10^-2 |
Figure 1. Two different AP-MS strategies were used to identify novel syndecan-4 protein partners. (A) Schematic illustration of the experimental design. Precipitation of syndecan-4 interacting proteins was performed by i) fishing with a biotinylated peptide covering the syndecan-4 cytoplasmic part (biotin-SDC4cyt) or the syndecan-4 cytoplasmic part phosphorylated at serine 179 (biotin-pS179-SDC4cyt), or by precipitation of endogenous syndecan-4 using syndecan-4 specific antibodies in ii) WT LV lysate or iii) ABHF LV lysate. A scrambled syndecan-4 control peptide, non-relevant IgG, beads and lysate from SHAM LVs were used as respective negative controls. Illustrations of the full length transmembrane syndecan-4 and aortic banding are given to the left. (B) Sequences of the three biotinylated peptides used in the pull down approach in A. (C) Binding of biotin-SDC4cyt to ezrin was confirmed by ELISA (unpaired t-test, ***, p<0.0001) (D) Anti-SDC4 (KY/8.2) was overlayed arrays of immobilized overlapping 20-mer peptides covering the full length syndecan-4 rat protein sequence. The core epitope is underlined. Immunoblotting without any primary antibody was used as negative control (lower panel) (n=2, two independent peptide arrays syntheses). (E) The antibody core epitope is underlined in the alignment of rat syndecan 1-4 (DNA Star, Madison, Wisconsin). Black boxes indicate identical amino acids. (F) Anti-SDC4 (KY/8.2) (with an external epitope) precipitated endogenous syndecan-4 in lysates from both rat neonatal cardiomyocytes (CM) and cardiac fibroblasts (CFB). Non relevant rat IgG was used as negative control.
Figure 2. Venn diagram depicting the distribution of the putative syndecan-4 partners together with literature partners identified by the two different AP-MS strategies (p<0.01-0.05). *Interaction with syndecan-4 changed in ABHF (p<0.05, n=3). **Interaction with syndecan-4 only detected in ABHF (p<0.05, n=3). The syndecan-4 literature partners were detected at n=1-3 (p<0.01-0.05, see Supplementary Table 2). SDCBP, CALM1 and COL1A1 were also detected in the pull down approach, but only COL1A1 was significant.
Validation of syndecan-4 protein partners in HEK293

A

B

C

D

E

F

G

H

I

Negative controls

J

K

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Figure 3. Confirmation of syndecan-4 binding of selected putative partners in HEK293. HEK293 cells were co-transfected with HA-tagged syndecan-4 and (A) 3xFLAG-MPP7, (B) 3xFLAG-PARVB, (C) 3xFLAG-CDK9, (D) 3xFLAG-EIF2S1, (E) PFKM-FLAG, (F) CCT5-FLAG, (G) RASIP1-FLAG, (H) EIF4B-FLAG, (I) PTRF-FLAG, (J) 3xFLAG-ADD3 (negative control), or (K) 3xFLAG-STK39 (negative control). HEK293 lysates were subjected to immunoprecipititation using anti-FLAG. Immunoprecipitates (two upper most panels) and lysates (two lower most panels) were analyzed by immunoblotting with anti-HA or anti-FLAG. Expression of RASIP1 was too low to be detected without IP. The 25 kDa core syndecan-4 protein (arrow) interacted with PTRF-FLAG in I.
Figure 4. The cardiac syndecan-4 interactome. Proteins in the syndecan-4 interactome clustered according to biological processes using the Human Protein Reference Database (HPRD) (GO annotations). Proteins in “Cell communication/Signal transduction” are subdivided into molecular functions. The 21 novel syndecan-4 partners identified by the two AP-MS approaches are in pink. *Interaction changed in ABHF (p<0.05, n=3). **Interaction only detected in ABHF (p<0.05, n=3).
Figure 5. Changes in the cardiac syndecan-4 interactome during pressure overload. 11 syndecan-4 networks were changed during pressure overload. Arrows denotes whether the syndecan-4 interaction was increased (↑) or decreased (↓). Novel partners identified are in pink. *Interaction significantly changed in ABHF (p<0.05, n=3). **Interaction only detected in ABHF (p<0.05, n=3). The STRING database 10.5 (https://string-db.org/) was used to generate small string maps.
Figure 6. Syndecan-4 binds directly to MLP and mediates its nuclear translocation. LV lysates from (A) WT rat and (B) ABHF or SHAM rats (six individual rat hearts) were subjected to immunoprecipitation using anti-syndecan-4 (KY/8.2, which has an extracellular epitope). Levels of immunoprecipitated MLP and syndecan-4 were analyzed by immunoblotting with anti-MLP and anti-syndecan-4cyt (which has an intracellular epitope). Non relevant rat IgG was used as negative control. (C) Total MLP protein level in SHAM and ABHF lysates was analyzed through semiquantified densitometry analysis of immunoblots shown in Supporting Fig. S5A. (D) Syndecan-4 expression in SHAM and ABHF was analyzed by qPCR. (E) Recombinant MLP bound directly to syndecan-4 in an ELISA-based assay. A scrambled peptide was used as negative control. Immunoblot analysis of MLP levels in (F) nuclear enriched fractions and (G) RIPA extracts of LV from WT and syndecan-4/- mice. Immunoblot analysis of MLP levels in (H) H9c2 cells transduced with a syndecan-4 adenovirus or null virus, and (I) H9c2 cells treated with a disruptor peptide (TAT-SDC4cyt) or a scrambled control peptide (TAT-scram) in presence of isoprenaline (ISO). The ~10-15 kDa syndecan-4 band represent the intracellular fragment after shedding of the extracellular domain and is visible when overexpressing syndecan-4 (52). Histone H3 were used as a loading marker for the nuclear enriched fractions in F, H and I. (B, F-I) Scatter plots show MLP levels semiquantified by densitometry analysis. (J) Protein content in cytoplasmic fractions of H9c2 cells treated with TAT-SDC4cyt and TAT-scram control peptide (n=8). (K) Schematic illustration of the disruptor peptide experiment. Differences were tested using unpaired t-test (*, p < 0.05; **, p < 0.01; ***, p < 0.001, ns indicates not significant) (n = 3-8). Error bars, SD.
The cardiac syndecan-4 interactome reveals a role for syndecan-4 in nuclear translocation of muscle LIM protein (MLP)
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