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Molecular mechanisms of heart failure; Nuclear Factor of Activated T-cell (NFAT) signaling in myocardial hypertrophy and dysfunction

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To my grandfather Knut Dagfinn Lunde,

85 year old heart failure patient.

Contents

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Ida Gjervold Lunde

Oslo, Norway, December 16th 2011

List of papers

This thesis is based on the following papers which are referred to by Roman numerals:

I. **Angiotensin II and norepinephrine activate specific calcineurin-dependent NFAT transcription factor isoforms in cardiomyocytes**
Ida G. Lunde, Heidi Kvaløy, Bjørg Austbø, Geir Christensen, Cathrine R. Carlson.
J Appl Physiol, 2011 Nov;111(5):1278-89. Epub 2011 Apr 7.

II. **Calcineurin-dependent NFAT isoforms are activated in the myocardium of aortic stenosis patients and their activation is reversed by relief of pressure overload in a murine model of reverse remodeling**
Ida G. Lunde, Biljana Skrbic, Theis Tønnessen, Heidi Kvaløy, Ulla H. Enger, Ivar Sjaastad, Geir Christensen, Cathrine R. Carlson. *In preparation, 2011.*

III. **Syndecan-4 is essential for development of concentric myocardial hypertrophy via stretch-induced activation of the calcineurin-NFAT pathway**
Alexandra V. Finsen[#], Ida G. Lunde[#], Ivar Sjaastad, Even K. Østli, Marianne Lyngra, Hilde O. Jarstadmarken, Almira Hasic, Ståle Nygård, Sarah A. Wilcox-Adelman, Paul F. Goetinck, Torstein Lyberg, Biljana Skrbic, Geir Florholmen, Theis Tønnessen, William E. Louch, Srdjan Djurovic, Cathrine R. Carlson, Geir Christensen.
PLoS One, 2011 Dec 02. [#]These authors contributed equally.

IV. **Cortisol response to stress is associated with myocardial remodeling in salmonid fishes**
Ida B. Johansen, Ida G. Lunde, Helge Røsjø, Geir Christensen, Göran E. Nilsson, Morten Bakken, Øyvind Øverli. *J Exp Biol, 2011; 214: 1313-1321.*

V. **Cardiac O-GlcNAc signaling is increased in hypertrophy and heart failure**
Ida G. Lunde, Jan Magnus Aronsen, Heidi Kvaløy, Eirik Qvigstad, Ivar Sjaastad, Theis Tønnessen, Geir Christensen, Line M. Grønning-Wang, Cathrine R. Carlson.
Physiol Genomics, 2011 Nov 29 [Epub ahead of print].

Summary

Heart failure is a leading cause of morbidity and mortality in the Western world, yet the underlying molecular processes in the heart are incompletely understood. Calcineurin-nuclear factor of activated T-cell (NFAT) signaling, a central intracellular pathway in development of pathological hypertrophy and cardiac dysfunction, has been investigated in several projects.

A hallmark of cardiac hypertrophy and subsequent heart failure is activation of the sympathetic adrenergic and the renin-angiotensin-aldosterone systems. We have identified that both activate specific calcineurin-dependent NFAT isoforms in neonatal murine cardiomyocytes (**Paper I**). Furthermore, we have found that all four calcineurin-dependent NFAT isoforms are substantially up-regulated in the myocardium of aortic stenosis patients, and that pressure overload-induced NFAT activation in the heart can be reversed after relief of the stimulus (**Paper II**). The role of syndecan-4, a transmembrane proteoglycan localized to the Z-disc of cardiomyocytes and regulated in cardiac disease, was investigated in the pressure-overloaded heart. Syndecan-4 was found to be essential for development of myocardial hypertrophy through mechanical stress-induced activation of the calcineurin-NFAT signaling pathway (**Paper III**). Cardiac hypertrophy is associated with the neuroendocrine stress response, and in a salmonid fish model we identified a correlation between heart size, post-stress plasma cortisol, fibrosis and NFAT activation, emphasizing an evolutionary conservation of NFAT activation in cardiac disease (**Paper IV**). Finally, we have investigated a novel signaling system in the heart which has been connected to NFAT signaling; the reversible post-translational protein modification called O-GlcNAc. We found that O-GlcNAc signaling was increased in various etiologies of cardiac hypertrophy and failure (**Paper V**).

Selected abbreviations

AB/DB	aortic banding/aortic debanding
AID	autoinhibitory domain
ANGII	angiotensin II
ANP	atrial natriuretic peptide
AS	aortic stenosis
BNP	brain natriuretic peptide
β-MHC	β-myosin heavy chain
CAD	coronary artery disease
CaM	calmodulin
CnA/CnB	calcineurin A/calcineurin B
CsA	cyclosporin A
ECM	extracellular matrix
EF	ejection fraction
ERK 1/2	extracellular signal-regulated kinase 1/2
GAG	glycosaminoglycan
GSK-3β	glycogen synthase kinase 3β
HR/LR	high-responsive/low-responsive
JNK 2/3	jun-amino terminal kinase 2/3
KO	knock-out
MAPK	mitogen-activated protein kinase
MEF2	myocyte enhancer factor 2
MI	myocardial infarction
NE	norepinephrine
NFAT	nuclear factor of activated T-cell
O-GlcNAc	O-linked β-N-acetylglucosamine
PTM	post-translational modification
RAAS	renin-angiotensin-aldosterone system
RCAN1	regulator of calcineurin 1
SHR	spontaneously hypertensive rats
TRPC	transient receptor potential canonical
WKY	Wistar Kyoto rats

Introduction

Heart failure

Heart failure is today the leading cause of morbidity and mortality in the Western world with a prevalence increasing every year[1-4]. Estimations predict that 2-3% of the population translating into 23 million people worldwide are living with heart failure[3, 5]. Economically, heart failure comprises the most costly illness with health care costs twice as high as that of cancer[6], and is now the single most common reason for adults to be admitted to the hospital in the United States, with a lifetime risk of one to five[3, 4, 7, 8]. Thus, the socioeconomic impact of heart failure is alarming and has epidemic proportions[3, 9, 10].

Heart failure can be defined as a syndrome in which the heart is unable to pump blood sufficiently to meet the requirements of the metabolizing tissues[11]. Thus, heart failure is not a single disease, but a gradually developing, final common stage of many diseases of the heart. Any form of heart disease can lead to heart failure which is typically caused by risk factors such as myocardial injury, hypertension, obesity, smoking, ischemia, diabetes, mutations, valvular disease, pregnancy, viral infection or toxic substances as during for instance chemotherapy. Age, sex and ethnicity further influence on the development of heart failure such that prevalence is higher in men, in elderly and in black individuals[3]. Due to the aging population and improved management of acute heart disease, the prevalence of heart failure is increasing although it seems that age-adjusted incidence is stabilizing and the length of survival after diagnosis is increasing[3, 7, 12-14]. Moreover, growth in both incidence and prevalence of heart failure is expected in the years to come as developing countries shift from acute to chronic cardiac disease and the pervasiveness of risk factors in these countries increases[15, 16].

Heart failure manifests itself as impaired cardiac function, structural alterations, neurohormonal activation and increased venous pressure. Patients are usually diagnosed by shortness of breath, edema and/or signs of inadequate tissue perfusion, e.g. fatigue, poor exercise tolerance and renal failure. Based on the severity of the symptoms, heart failure patients are grouped into four classes made by the New York Heart Association (NYHA I-IV)[17], ranging from mild to severe. Functionally, heart failure patients may be separated into two major groups; one with reduced ejection fraction (EF) and a large cardiac chamber (i.e. systolic heart failure/impaired contractility) and one with preserved EF and a small cardiac

chamber (i.e. diastolic heart failure/impaired relaxation). Heart failure with preserved EF is believed to represent more than half of heart failure cases[3].

Despite advances in management of patients and development of therapeutic strategies such as β-adrenergic blockers and angiotensin-converting enzyme inhibitors, heart failure remains a deadly syndrome with 5-year mortality of 40-60%[3, 7]. These statistics reflect that the underlying disease mechanisms are poorly understood and that the important pathological mechanisms are not modified by present treatments modalities[18, 19].

Also for the aquaculture industry, in which millions of tons of fish are processed every year, myocardial remodeling, abnormalities, dysfunction and cardiac-related mortality have become an increasing problem[20-25]. Farmed fish generally grow faster than wild fish, they are fatter and poorer swimmers. The underlying causes of the observed pathological remodeling in farmed fish, however, are poorly investigated and largely unknown.

Molecular mechanisms of heart failure

Myocardial remodeling, which underlies the progressive development of clinical heart failure, comprises changes in mass, shape, volume and composition of the left ventricle. Molecular and cellular mechanisms are the main players orchestrating these changes, which encompass myocyte growth, myocyte death, alterations in extracellular matrix (ECM) deposition and fibroblast function, inflammation, alterations in myocyte energetics and changes in excitation-contraction coupling[26, 27].

Most types of heart failure are preceded by global or regional myocardial hypertrophy[11]. Based on the Framingham Heart Study in which 5000 inhabitants of Framingham, Massachusetts, were followed for four decades, left ventricular hypertrophy was defined as an important and independent risk factor for development of heart failure and premature death[1, 8, 28]. For instance, the incidence of heart failure in men younger than 65 years of age was increased 15-fold by presence of left ventricular hypertrophy. This is in contrast to the conventional belief of myocardial hypertrophy constituting an adaptive and compensatory mechanism to sustain the performance of the diseased heart[29-33]. Today, most evidence favors a model of both maladaptive and adaptive components of the myocardial hypertrophic response during remodeling in response to pathological stimuli.

Left ventricular hypertrophy may have a concentric or eccentric morphology, causing either a thickening or an elongation of the cardiomyocytes and the ventricle as such, in

response to pressure-overload and volume-overload, respectively[26, 27, 34]. The defining features of hypertrophy in addition to increased cardiomyocyte size, are increased protein synthesis and increased organization of the sarcomeres, which during concentric hypertrophy are added in parallel, while during eccentric hypertrophy, are added in series. These changes in cellular phenotype are accompanied by re-introduction of the so-called fetal gene program, i.e. expression of genes that were active in the fetal heart. This includes a change to carbohydrate metabolism, induction of natriuretic peptides, isoform switching of contractile proteins such as myosin, actin and troponin and re-employment of developmental transcription factors[35].

At the molecular level, several intracellular signaling pathways have been identified as central in the ventricular remodeling process. Briefly, these include G-protein signaling, the main three branches of the mitogen-activated protein kinase (MAPK) cascades, protein kinase A (PKA) and C (PKC) signaling, glycogen synthase kinase 3β (GSK-3β) and Ca^{2+}-regulated pathways such as calcineurin and Ca^{2+}/calmodulin-dependent kinase II (CaMKII)[19, 26, 27, 31, 32, 34]. Z-disc proteins and membrane integrins have been proposed as essential for coupling mechanical signals to intracellular kinases and phosphatases in cardiomyocytes[36, 37].

Many of the aforementioned enzymes alter gene expression by communicating their signals to the nucleus. Five families of transcription factors have been found to co-regulate approximately 90% of the 10 000 expressed genes in the heart, and especially those involved in pathological cardiac remodeling, i.e. the GATA family, the myocyte enhancer factor 2 (MEF2) family, the homebox family NKX, the nuclear factor of activated T-cell (NFAT) family and the forkhead family (FOX)[38, 39]. Gene expression is also regulated at the epigenetic level, and histone acetyl transferases (HATs) and deacetylases (HDACs) have been found to be central in pathological remodeling by regulating cardiac chromatin acetylation[40]. Recently, microRNAs (miRNAs), short *non*-coding RNAs which promote degradation of complementary mRNAs, have also emerged as potent regulators in cardiac disease and may be part of the fetal gene program[41].

Calcineurin-NFAT signaling in myocardial hypertrophy and dysfunction

Dysregulation of cardiomyocyte Ca^{2+} is a hallmark of cardiac disease, and several Ca^{2+} -responsive signaling pathways have been causally linked to heart failure progression[42]. Calcineurin, the Ca^{2+}-sensitive phosphatase regulating the activity of the NFAT transcription factor family, was first shown to be sufficient for cardiac hypertrophy by Molkentin *et al.* in 1998[43]. Later, calcineurin-NFAT has been established as a critical intracellular signaling pathway in pathological cardiac hypertrophy and failure[43-48] (illustrated in Fig. 1).

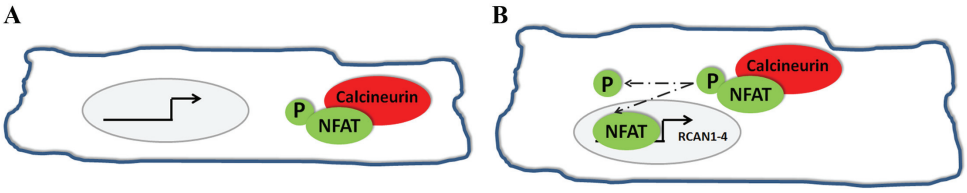

Fig. 1 Schematic of pro-hypertrophic calcineurin-NFAT signaling in cardiomyocytes.
In normal cardiomyocytes, nuclear factor of activated T-cell (NFAT) is phosphorylated (P) and localized to the cytoplasm, rendering calcineurin-NFAT signaling inactive (A). In hypertrophic cardiomyocytes, NFAT is dephosphorylated by calcineurin and subsequently translocates to the nucleus and activates pro-hypertrophic, NFAT-responsive genes such as the regulator of calcineurin 1-4 (RCAN1-4) (B).

Although initially discovered in T-cells, thereof the name, almost all mammalian tissues express one or more isoforms of the NFAT family. In cardiomyocytes, all four NFAT isoforms, termed NFATc1-c4, are expressed[45, 49, 50] (Fig. 2A). The characteristic feature of NFATc proteins that distinguishes them from other vertebrate transcription factors, is their ability to move rapidly into the nucleus in response to Ca^{2+} and calcineurin[51]. NFATc proteins contain three distinct domains: the NFAT homology region (NHR), the Rel-homology region (RHR) and the C-terminal domain[52, 53] (Fig. 2B). The NHR is conserved between the c1-c4 isoforms and contains a transactivating domain, a serine/threonine-rich regulatory domain to which calcineurin (PIxIxIT sequence) and NFAT-regulatory kinases dock and a nuclear localization signal. The RHR is also highly conserved between c1-c4 and is homologous with the Rel-domain of the nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) family. It mediates DNA-binding and protein-protein interactions such as dimerization. When

NFATc proteins are dephosphorylated by calcineurin, they translocate to the nucleus and activate NFATc responsive genes[53-56].

A

NFAT isoform:	Cardiomyocyte expression:	Other names:	Known sub-isoforms:
NFATc1	Yes	NFATc, NFAT2	5
NFATc2	Yes	NFATp, NFAT1	3
NFATc3	Yes	NFATx, NFAT4	4
NFATc4	Yes	NFAT3	5

B

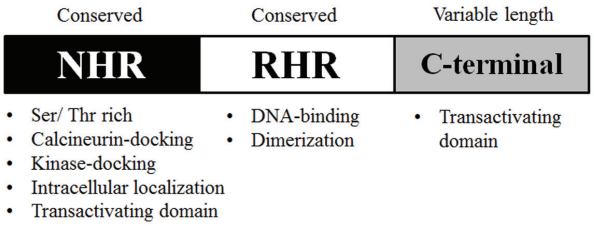

Fig. 2 The NFATc family and their protein structure. Table of NFATc isoform members expressed in cardiomyocytes, their alternative names and the number of known human sub-isoforms (A). Schematic presentation of the NFATc functional domains (B). NHR; NFAT homology region, RHR; Rel-homology region. The figure is modified from [52, 53].

NFATc knock-out (KO) mice have revealed differential remodeling in response to various pathological stimuli depending on the isoform being experimentally modified[45, 49, 57-59], indicating an importance of analyzing the four isoforms separately in the heart. Despite this, their differential regulation and effects seem somewhat a mystery, as the target gene consensus site is the same for all isoforms[53, 60]. Accordingly, most studies report NFATc activation in a one-isoform-represents-all manner. For instance, they all activate the exon 4 promoter of the regulator of calcineurin 1, RCAN1-4, in cardiomyocytes[45, 61] (Fig. 1).

In the human heart, 13% of the approximately 10 000 expressed genes have a consensus NFAT binding site in their promoter region[38]. In advanced stage heart failure, expression of 20-40% of these genes is altered, and the highest odds ratio for differential expression is found for genes having promoter regions with a combination of NFAT and MEF2 binding sites[38], emphasizing the importance of NFAT signaling in cardiac pathology.

Neurohormonal activation, in particular activation of the sympathetic nervous system and the renin-angiotensin-aldosterone system (RAAS), is a hallmark of cardiac hypertrophy and subsequent heart failure, in which norepinephrine (NE) and angiotensin II (ANGII) are the primary effectors mediating hypertrophic, apoptotic and fibrotic events in the heart[62].

Although the main therapies for heart failure today aim at antagonizing the RAAS and adrenergic systems, the intracellular responses to ANGII and NE in cardiomyocytes are not completely understood. Both NE and ANGII have been shown to affect intracellular Ca^{2+} in cardiomyocytes and to activate calcineurin, indicating that they may activate one or more of the NFATc isoforms. However, measuring isoform-specific activation in cardiomyocytes has been difficult[45, 49], and accordingly, little is known about endogenous cardiac NFAT isoform protein expression, differential regulation or their possible connection to ANGII or NE-mediated signaling. Thus, we validated antibodies for detection of endogenous NFATc and phospho-NFATc proteins and investigated which isoforms were activated by ANGII and NE in isolated neonatal mouse cardiomyocytes (Paper I).

Currently, although the importance of calcineurin activation has been established in human cardiac disease[63, 64], there is little knowledge regarding NFAT isoform activation in the human myocardium. Activation of the NFATc4 isoform has been reported in both hypertrophied and end-stage failing hearts[65, 66] and a c4 polymorphism correlates to left ventricular mass[67], however it is unclear whether the other NFATc isoforms, i.e. NFATc1, NFATc2 and NFATc3, are activated in human cardiac disease. We therefore investigated expression and activation of NFATc isoforms and components of the calcineurin signaling cascade, such as RCAN1-4, in the hypertrophic myocardium of patients with aortic stenosis (AS). As AS patients are treated with surgical aortic valve replacement (AVR), we investigated whether relief of pressure overload in a murine model could reverse NFAT activation in the heart (Paper II).

Syndecan-4 is a transmembrane proteoglycan localized to the Z-disc of cardiomyocytes[68] and has previously been shown to be up-regulated in cardiac disease[69, 70], and to be important for intracellular signaling in other cell types, e.g. fibroblasts and endothelial cells[71-73]. We have investigated potential syndecan-4-mediated signaling in cardiomyocytes, among them the calcineurin-NFAT pathway. In particular, the role of syndecan-4 and syndecan-4-mediated NFAT activation in development of pressure overload-induced cardiac hypertrophy and failure was investigated in a syndecan-4 KO mouse model (Paper III).

The Ca^{2+}/calcineurin-regulated NFATc family is thought to have arisen about 500 million years ago, producing a new group of transcription factors involved in signaling which is found only in the genomes of vertebrates. It has been proposed that this enabled Ca^{2+}

signals to be redirected to a new transcriptional program which provided part of the groundwork for vertebrate morphogenesis and organogenesis, including the cardiovascular system[51]. Thus, we investigated whether NFAT activation also occurred in a comparative model of cardiac hypertrophy, by measuring RCAN1-4 gene expression in hypertrophied fish hearts (Paper IV).

Finally, we investigated a novel signaling system in the heart which has been connected to NFAT signaling in immune cells[74] and hyperglycemic cardiomyocytes[75]; the reversible post-translational modification (PTM) called O-GlcNAc (O-linked β-N-acetylglucosamine), in cardiac hypertrophy and failure. Subsequent to the discovery by Torres and Hart[76], O-GlcNAc signaling has been implicated in a diverse array of physiological and pathological functions in the cell[77]. Yet, although there is a growing recognition that O-GlcNAc alterations are involved in cardiovascular pathophysiology in diabetes and acute ischemia[78-81], regulation in the heart is currently not well understood. We therefore investigated whether O-GlcNAcylation of the cardiac proteome was altered in AS patients and in animal models of three common etiologies of heart failure; i.e. hypertension, myocardial infarction (MI) and aortic constriction (Paper V).

Aims of the thesis

The overall aim of this thesis is to expand the knowledge of molecular signaling pathways underlying development of heart failure, particularly the role of calcineurin-NFAT signaling in hypertrophic and dysfunctional hearts. It is a hope that the basic knowledge that this thesis provides may be part of the extensive groundwork needed for understanding the underlying mechanisms of cardiac hypertrophy and dysfunction, a prerequisite for development of new therapeutic strategies for heart failure.

The specific aims of the separate studies were:

Paper I

Angiotensin II and norepinephrine activate specific calcineurin-dependent NFAT transcription factor isoforms in cardiomyocytes

- To validate methodology for measuring endogenous NFATc isoforms and their activity in cardiomyocytes.
- To investigate which NFATc isoforms were activated by ANGII and NE in cardiomyocytes.

Paper II

Calcineurin-dependent NFAT isoforms are activated in the myocardium of aortic stenosis patients and their activation is reversed by relief of pressure overload in a murine model of reverse remodeling

- To investigate activation of the four NFATc isoforms in the pressure-overloaded, hypertrophic myocardium of AS patients, and to assess activation of various components of the calcineurin-NFAT pathway in these hearts.
- To investigate the possible reversal of cardiac NFAT activation following relief of pressure-overload in mice.

Paper III

Syndecan-4 is essential for development of concentric myocardial hypertrophy via stretch-induced activation of the calcineurin-NFAT pathway

- To investigate the role of syndecan-4 in pressure overload-induced development of cardiac hypertrophy and failure using a syndecan-4 KO mouse model.
- To investigate the role of syndecan-4 in regulation of the calcineurin-NFAT signaling pathway during cardiac hypertrophy and failure.
- To investigate the role of syndecan-4 in mechanical stress-induced activation of NFAT in cardiomyocytes.

Paper IV

Cortisol response to stress is associated with myocardial remodeling in salmonid fishes

- To investigate the influence of stress-induced plasma cortisol on cardiac hypertrophy in salmonid fish.
- To investigate NFAT activation in neuroendocrine stress-induced cardiac hypertrophy in fish.

Paper V

Cardiac O-GlcNAc signaling is increased in hypertrophy and heart failure

- To investigate the regulation of O-GlcNAcylation of cardiac proteins in various etiologies of cardiac hypertrophy and failure; in AS patients and in models of hypertension, MI and aortic constriction in rat.
- To investigate the role of O-GlcNAcylation in the regulation of myocardial contractility.

Methodological considerations

The findings in this thesis result from molecular biology techniques employed on tissue and cells from human patients and animal models of cardiac disease such as mice, rats and fish. We have performed *in vivo* animal experiments, obtained myocardial biopsies per-operatively from patients, and employed cell lines and primary cell cultures for various *in vitro* experiments. In this section, methodological considerations of central experiments will be discussed.

Human myocardial biopsies

To study molecular signaling in human heart disease directly, we have utilized myocardial biopsy tissue taken during open heart surgery (Fig. 3). More specifically, our transmural biopsies were obtained from the left ventricle of patients with severe, symptomatic AS during elective AVR, and from a *non*-ischemic area of patients undergoing coronary artery bypass graft (CABG) operation due to coronary artery disease (CAD). Alternatively, we could have used tissue from explanted hearts yielding considerably more tissue, however, the explanted hearts represent severe, end-stage heart failure, and not hypertrophy in-transition-to failure, which is the case for AS hearts undergoing AVR. Thus, myocardial tissue from AS patients is more suitable for studying signaling pathways underlying the progressive development of failure in the human heart. In both cases, the main limitations concern ethical and experimental restrictions and a restricted number of samples, not to mention the control tissue, which can not be obtained from healthy controls.

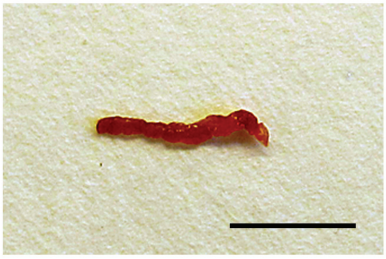

Fig. 3 Human myocardial biopsy.
Myocardial biopsy from a patient taken per-operatively using a biopsy needle. Scale bar: 1 cm. Photo: Ida G. Lunde.

Animal models of cardiac disease

To allow for *in vivo* gain and loss-of-function studies, genetically modified mouse models are extensively used in basic cardiac research. To study the role of a specific protein, the gene is overexpressed or knocked-out in the whole mouse or in the heart specifically, and it can even be controlled conditionally. In our studies, we have investigated the role of syndecan-4 *in vivo* in the pressure-overloaded hearts of mice lacking syndecan-4 in all cells[82]. We have also employed neonatal cardiomyocytes from syndecan-4 KO mice, NFAT-luciferase reporter mice[47] and syndecan-4 KO-NFAT-luciferase reporter mice (generated in our laboratory for Paper III). This approach is limited by the fact that the genetic modification is *non*-natural, it may influence on development and thus not be suited to study adult cardiac disease, and one might study phenotypes caused by off-target effects. However, these genetically modified mice are widely used by the cardiac research community and constitute a powerful tool to study the role of a specific protein in cardiac disease.

To mimic human cardiac diseases, *in vivo* animal models are well established and widely used in the field of basic cardiac research. In our project, we have employed spontaneously hypertensive rats (SHR), a model of MI made by ligation of a coronary artery, and a model of AS made by aortic banding (AB; Fig. 4), in mice and rats. These experiments are limited by the shortcomings of these models in resembling the human disease, the reproducibility of the models and also by species/line differences, sex and diet. Importantly, the relative age of the animals used is younger than that of the human patients, and as many disease-related processes are age-dependent, this constitutes a limitation to studies such as ours. Nevertheless, the coronary artery ligation and AB models have been shown to be valid models of myocardial remodeling following infarction and of concentric hypertrophy, respectively[83]. To study aspects of reverse left ventricular remodeling such as seen after AVR in AS patients[84-86], we have employed a mouse model where AB is followed by aortic debanding (DB)[87-89]. To control for effects of the operation itself, experiments were accompanied by sham-operated control animals. SHR develop increased blood pressure from the age of five to six weeks and progressive cardiac hypertrophy from the age of 20 weeks, and have been widely used as a model of cardiac remodeling following hypertension[90]. Normotensive Wistar-Kyoto (WKY) rats were included as controls for this study.

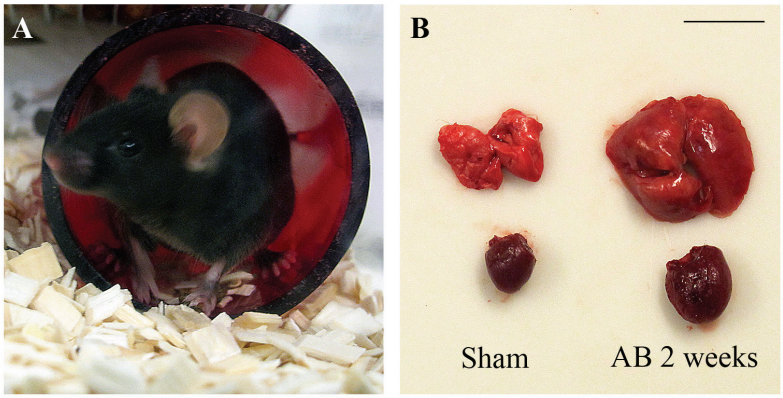

Fig. 4 Left ventricle and lungs of sham-operated and aortic-banded mice.
Wild type mouse (A), lungs and left ventricles following sham operation (left) and aortic banding (AB; right) (B). Scale bar: 1 cm. Photo: Ida G. Lunde.

The studies in salmonid fish were performed on hearts from a genetic model of cortisol stress-responsiveness, in two lines of rainbow trout (*Oncorhynchus mykiss*) selected for high and low post-stress plasma cortisol levels (HR and LR, respectively)[91]. In our study, these lines provided a tool to study cardiac effects of transiently elevated plasma cortisol levels. However, as the HR and LR fish have been bred for several generations during the selection regime, mutations and random genetic drift could have accounted for the observed effects. This is, however, unlikely as our findings were confirmed in a wild salmonid population (*Salmo trutta*; European brown trout) (Fig. 5). As stress and cardiac disease are both major problems in the fish farming industry[20-23], the HR and LR lines may actually be of direct commercial interest, making our results not only interesting in a comparative view.

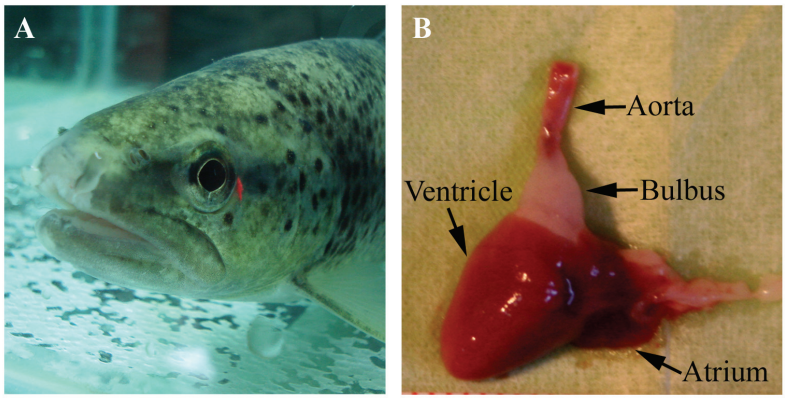

Fig. 5 European brown trout (*Salmo trutta*) and its heart.
European brown trout (A) and its heart, showing the single atrium, the single ventricle, the aorta and the bulbus (B). Photo: Lars-Petter Skillestad and Ida G. Lunde.

Cardiomyocyte cell cultures

As cardiomyocytes are terminally differentiated, primary cell cultures have been the most commonly used cell system in the field. Although techniques for cardiomyocyte isolation were developed 35 years ago[92], isolation of high quality/high yield cultures remains somewhat of an art and often unpredictable difficulties occur, limiting the experiments[93]. When studying signaling in cardiomyocytes, cells isolated from neonatal mice and rats have been preferred, as these survive in culture for a longer period than adult cells, and have a greater potential for morphological changes. Thus, we have employed primary cultures of neonatal cardiomyocytes to a large extent in our studies (Fig. 6). Although there are obvious shortcomings of using neonatal rodent cardiomyocytes to model what is happening in adult human cardiomyocytes in the intact heart during disease, many of the molecules found to be involved in hypertrophy and failure *in vitro* have been confirmed *in vivo*[94]. For instance, cyclic mechanical stretching of neonatal cardiomyocytes, such as performed in our studies (Papers II-III), have been shown to induce hypertrophy and expression of genes in a way similar to *in vivo* pressure overload[95]. As an alternative to transfection, cultured cardiomyocytes can be treated with arginine-tagged, cell permeable peptides such as the syndecan-4-derived peptides we used in Paper III[96]. Thus, neonatal cardiomyocytes represent a useful *in vitro* tool in basic cardiac research to study direct effects on cardiomyocytes.

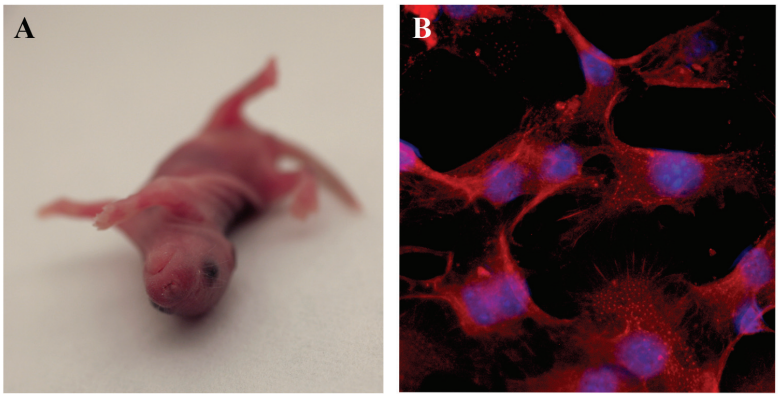

Fig. 6 Neonatal mouse and cultured primary neonatal cardiomyocytes.
Neonatal mouse (A) and cultured primary cardiomyocytes stained for nuclei in blue and actin filaments in red (B). Photo: Lars-Petter Skillestad and Geir Florholmen/Ida G. Lunde.

General techniques to study cardiac dimensions and function

To assess the state of cardiac hypertrophy and dysfunction, post-mortem analyses, echocardiography and left ventricular catheterization were performed to measure cardiac dimensions, hemodynamics and function in mice and rats. Standard patient management including echocardiography was performed in human patients. In fish, cardiac dimensions were assessed by post-mortem measurements. Expression of commonly used cardiac marker genes associated with hypertrophy and failure such as atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), β-myosin heavy chain (β-MHC) and α-skeletal actin (Acta1) were assessed by reverse transcription real-time polymerase chain reaction (RT-PCR) from myocardial tissue of humans, rodents and fish. These methods are detailed in the individual papers.

General molecular biology techniques

We have employed numerous molecular biology techniques and among them, the most central are summarized briefly in this section. For quantitative gene expression analyses, RT-PCR was performed. For protein and phospho-protein analyses, immunoblotting (i.e. Western blotting) and immunohistochemistry were performed. Luciferase activity was measured as a reporter of NFAT activation. General histochemistry and microscopy were performed to study tissue morphology. To study protein interactions, immunoprecipitations from tissue and *in*

vitro pull down and overlay peptide array techniques were employed. Overexpression of proteins was obtained by transfection using plasmid vectors, and primary cardiomyocytes were treated with cell-permeable peptides. To study protein synthesis in cells, incorporation of radioactive leucine was assessed. To validate antibody specificity, peptide arrays and blocking peptides were used. These methods are detailed in the individual papers.

Molecular techniques to study cardiac NFAT activity

NFAT mRNA, protein and phosphorylation status

Measuring endogenous cardiac NFATc isoform-specific activation has been difficult especially due to low antibody specificity[45, 49] and extensive alternative splicing resulting in numerous sub-isoforms[97]. Accordingly, little is known about their protein levels or their differential regulation in the heart. In Paper I we evaluated antibodies for doing so in isolated, neonatal murine cardiomyocytes. Thus, we evaluated antibody specificity and identified NFAT isoform protein bands by immunoblotting in combination with antibody blocking peptides in order to measure the level of phosphorylated, i.e. inactive NFATc, and the *non*-phosphorylated/total NFATc protein. In Paper II, we further validated their specificity by overexpressing NFATc1-c4 in human endothelial kidney 293 (HEK 293) cells, and employed the validated antibodies to detect NFATc1-c4 and phopsho-NFATc1-c4 in the human myocardium. In Paper III, we used these antibodies to detect activation of NFATc4 in murine myocardium and cells. Although there are limitations regarding *non*-specific protein bands, we believe we have identified antibodies that can be used to detect endogenous cardiac NFATc isoforms specifically in myocardial tissue from mice and humans (Papers I-III). We also believe that the phospho-NFAT antibodies can be used to detect isoform activation, at least during acute stimulation or short-term remodeling. As shown in Paper II, chronic remodeling such as in the myocardium of AS patients, phospho-levels become difficult to interpret as the proteins themselves are highly regulated, perhaps representing sub-isoform expression[97] and PTMs other than phosphorylation.

NFAT isoform mRNA measurements constitute another approach to assess isoform specificity and activation, however increased NFAT isoform protein levels do not correlate to increased NFAT mRNA (Paper II). As it has been proposed that NFAT expression in T-cells is regulated by proteolysis, and only partially at the transcriptional level[98], we do not believe

that measuring NFAT isoform mRNA should be used alone to describe NFAT isoform activity in the heart (Paper II).

NFAT target gene expression

Proteins within the RCAN family (also called modulatory calcineurin-interacting protein 1 (MCIP1), adapt 78, calcipressin 1 or down syndrome critical region 1 (DSCR1)) bind and inhibit calcineurin activity[99]. The human RCAN1 gene comprises seven exons, the first four of which are alternative first exons, resulting in different isoforms with specific expression patterns and regulation. Exons 1 + 5-7 give rise to RCAN isoform 1-1, while exons 4 + 5-7 give rise to RCAN1-4[100]. Expression of the exon 4 isoform, RCAN1-4, has been shown to be extremely sensitive to calcineurin activity *in vivo*[61, 101]. RCAN1-4 is a direct NFATc target in the heart due to a dense cluster of 15 consensus binding sites for NFAT in the exon 4 promoter[61, 102, 103]. Thus, RCAN1-4 transcript level, as well as protein level, have been used in numerous studies as a sensitive indicator of changes in calcineurin-NFAT activity. However, as there are no yet known differences in consensus target site for the NFATc isoforms[45, 53, 60, 61], this method of evaluating calcineurin-NFAT activity is not NFAT isoform-specific. RCAN1-4 can be used to measure both exogenous and endogenous NFAT activation in the heart, and have in our studies been measured by RT-PCR and immunoblotting in human and murine cardiac tissue, as well as in fish (Papers II-IV).

NFAT reporter systems

NFAT-luciferase reporter constructs and transgenic mice provide yet another sensitive method for measuring NFAT activity. NFAT-luciferase mice[47] have nine copies of an NFAT binding site from the interleukin (IL)-4 promoter (5`-TGGAAAATT-3`) inserted upstream of the luciferase gene. Thus, luciferase activity serves as a reporter of NFAT activity. This model has been widely employed for *in vivo* studies in combination with other genetically modified mouse models, and provides a relatively easy method for evaluating calcineurin-NFAT activity, although not providing information regarding NFATc isoform activation. In our studies, we have employed cardiomyocytes from neonatal NFAT-luciferase mice to investigate the regulation of calcineurin-NFAT signaling by ANGII, NE and several syndecan-4-derived cell-permeable peptides (Papers I and III). To measure syndecan-4-mediated NFAT activation *in vivo*, we generated syndecan-4 KO-NFAT-luciferase reporter

mice in our laboratory (Paper III). Furthermore, we employed the NFAT-luciferase mice to investigate reversal of NFAT activation following relief of pressure overload (Paper II).

Recently, a red fluorescent protein (RFP)-based reporter assay to measure NFAT activity was introduced by Rinne and Blatter[104]. This new tool allows for continuous monitoring by fluorescence microscopy of transcriptional NFAT activity in living cells, contrasting the more traditional luciferase assay which requires cell lysis and substrate addition. However, the RFP-assay requires transfection into cardiomyocytes as genetically modified mice have not yet been made, and for our studies, we have not used this method.

Intracellular NFAT translocation

The nuclear-cytoplasmic ratio of NFAT reflects the summation of nuclear import and export rates. Fusion constructs of NFATc proteins and green fluorescent protein (GFP) have been used in numerous studies of NFAT activation in living cells by measuring translocation between the cytoplasm and nucleus. Although isoform-specific, they do not provide a method for measuring endogenous NFATc activity as they require transfection into cardiomyocytes. In Paper II, we have fused NFATc1-c4 to enhanced GFP (EGFP) in expression vectors, and transfected these into HEK 293 cells, routinely done by lipofection, in order to validate antibody specificity. We have not, however, used these expression vectors in cardiomyocytes to quantitate NFATc translocation.

In order to measure endogenous NFATc isoform activation by translocation, subcellular protein fractionation can be performed. Thus, we measured the amount of NFATc found in the cytoplasmic and nuclear fractions in cardiomyocytes and HEK 293 cells following ANGII or NE stimulation and syndecan-4 transfection, respectively (Papers I and III), using the NFATc antibodies validated in Paper I.

NFAT DNA binding

Other molecular techniques to study NFAT activation in cardiomyocytes include NFAT-binding to the consensus target DNA sequence, which can be measured for instance by using the Transcription Factor Assay Kit available for NFATc1 from TransAm (Active Motif Europe, Belgium), which we used in Paper I, or electrophoretic mobility shift assays (EMSA)/gel shift assays. Moreover, chromatin immunoprecipitation (ChIP) could also be performed for this purpose. All these methods are antibody-dependent, and thus require

specific NFATc antibodies. As discussed in Paper I, we were successful in applying the NFATc1 Transcription Factor Assay Kit mentioned above to measure nuclear NFATc4 activation in murine hearts using the antibodies that we validated. However, the kit was not compatible, in our hands, with the small yield of nuclear protein from neonatal cardiomyocytes in combination with the c2, c3 and c4 antibodies.

Protein interactions

Calcineurin-NFAT signaling can be activated through protein-protein interactions. For instance, as we found that NFAT signaling was altered in syndecan-4 KO hearts, we investigated the potential regulation of cardiac calcineurin-NFAT signaling by direct interaction between syndecan-4 and calcineurin (Paper III). Similarly, it was recently shown that the Ca^{2+}- and integrin-binding protein 1 (CIB1) binds directly to the regulatory calcineurin B subunit (CnB) in the heart, anchoring calcineurin to the sarcolemma and regulating its catalytic activity towards NFAT[105]. Such an activation of calcineurin has previously also been shown for the muscle LIM protein (MLP) which anchors calcineurin to the Z-disc and regulates NFAT activation[106]. Thus, calcineurin-NFAT protein interactions can also reflect NFAT activation in the heart.

Calcineurin activity

In assessment of cardiac NFAT activation, studying the activity of phosphatases and kinases regulating level of NFAT phosphorylation are relevant approaches. Especially, calcineurin activation reflects the level of NFAT activation. The active calcineurin comprises the catalytic calcineurin A subunit (CnA) and the two Ca^{2+}-binding regulatory proteins, CnB and calmodulin (CaM). When activated by Ca^{2+}, CnB and CaM bind to CnA, displacing the autoinhibitory domain (AID) and rendering calcineurin in an active conformation allowing access of substrates, such as NFATc, to the catalytic domain[107]. Thus, immunoprecipitations of CnA revealing CnB and/or CaM binding, and the tissue levels of CnA, CnB and CaM, serve as indications of calcineurin activity. In Paper II, we measured the levels of these three proteins in the hearts of AS patients to assess NFAT activation level. Also commonly used to determine the relevance of calcineurin activity in a process are inhibitors of calcineurin activity, such as cyclosporine A (CsA), FK506[108, 109] and the NFAT-derived peptide VIVIT[110]. To this end, we used CsA to measure calcineurin-dependent NFAT activation in

neonatal NFAT-luciferase cardiomyocytes in Paper I. Finally, biochemical assays employing the phosphorylated regulatory subunit II (RII) of PKA as substrate, the most efficient and selective substrate known for calcineurin are used in numerous studies to assess calcineurin activity (e.g. Calcineurin Cellular Activity Assay Kit, Calbiochem).

GSK-3β activity

GSK-3β has been shown to promote nuclear export and thus inactivation of NFATc transcription factors by serine/threonine phosphorylation, antagonizing calcineurin action and development of cardiac hypertrophy[111, 112]. Serine 9 phosphorylation by Akt (also called protein kinase B (PKB)) in the phosphatidylinositol 3-kinase (PI3-K) pathway[113] has been found to inhibit GSK-3β activity[111]. Thus, increased levels of GSK-3β phosphorylation indirectly suggest increased NFATc activity. We have measured the level of GSK-3β and its phosphorylation level in myocardial tissue from AS patients and in syndecan-4 KO hearts as a means of approaching the mechanisms behind NFAT activation (Papers II-III). Furthermore, we have measured Akt phosphorylation (i.e. activation) as an indication of GSK-3β inactivation, and thus indirectly of NFAT activation.

MAPK activity

It has been shown that each of the three main branches of MAPK signaling cascades, i.e. the extracellular signal-regulated kinase 1/2 (ERK 1/2), p38-MAPK and jun-amino terminal kinase 2/3 (JNK 2/3), directly modulate calcineurin-NFAT signaling in the heart[114]. ERK 1/2 is believed to activate NFATc4 in cardiac myocytes and T-cells[114-116]. JNK and p38, on the other hand, directly phosphorylate NFAT, inhibit nuclear translocation, and thereby antagonize calcineurin-dependent NFAT activation and hypertrophy[114]. Thus, the protein and phosphorylation levels of these kinases (i.e. activation) could serve as indirect indications of NFAT activity in the heart. In Papers II-III we approached the level of NFAT activation indirectly by measuring protein levels and phosphorylation of ERK1/2, p38 and JNK 2/3.

NFAT dimerization partners

Investigating the level of, interaction with and activity of NFAT dimerization partners are useful approaches to further assess the degree of NFAT activation indirectly. NFATc proteins bind DNA weakly and it has thus been proposed that they need a partner protein to

transactivate genes[117]. For instance, NFATc proteins are known to transactivate target genes in heterodimers with MEF2, AP-1 and GATA4[26, 117], in addition to forming NFAT homodimers[118]. We investigated the levels of MEF2, AP-1 and GATA4 in Paper II to indirectly approach NFAT activation in the myocardium of AS patients.

Upstream activators of NFAT signaling; TRPC channels

Ca^{2+} function is likely compartmentalized into microdomains in cardiomyocytes since the substantial alterations in intracellular Ca^{2+} during contraction-relaxation do not trigger Ca^{2+}-dependent signaling such as NFAT. One important intracellular pool of Ca^{2+} is generated by Ca^{2+} influx into the cell through transient receptor potential canonical (TRPC) channels in the sarcolemma[26]. These are store-operated, i.e. their opening is coupled to a decline in SR Ca^{2+} stores, and they are believed to play an important role in development of hypertrophy[119, 120]. Importantly, of the seven family members, TRPC1, 3 and 6 are known to influence Ca^{2+}-dependent calcineurin-NFAT activity[121-124]. Thus, assessing the level of these three TRPC channels and their activity is yet another approach to indirectly describe NFAT activation level in the heart. We measured the level of TRPC 1, 3 and 6 in the biopsies from AS patients (Paper II).

Ethical considerations

The central ethical considerations of the projects constituting this thesis concern utilizing tissue from patients and experiments on animals. The human myocardial biopsy protocol was approved by the Norwegian regional ethics committee and conformed to the Declaration of Helsinki. Importantly, informed written consent was obtained from each patient included. Experiments on mice and rats were approved by the Norwegian National Animal Research Committee and conformed to the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1996, US). The transport and study procedures of fish were reviewed and approved by the Norwegian Food Safety Authority (www.mattilsynet.no) and the Norwegian National Animal Research Committee, respectively, while the fish studies performed in Bosnia and Herzegovina were approved by the State Veterinary Office of Bosnia and Herzegovina (http://www.vet.gov.ba). Certainly, care was taken to reduce, replace and refine (RRR) when employing animals in our studies.

Summary of results

The main results from this thesis are summarized briefly in this section.

Paper I

Angiotensin II and norepinephrine activate specific calcineurin-dependent NFAT transcription factor isoforms in cardiomyocytes

We identified antibodies for specific detection of endogenous NFATc isoforms and their phosphorylation level in cardiomyocytes. This was accomplished by validating antibody specificity towards murine NFATc isoforms by immunoblotting using isoform-specific peptide arrays and antibody peptide inhibitors. Both ANGII and NE increased calcineurin-dependent NFAT activation when measuring NFAT-luciferase activity, and they both stimulated hypertrophy of neonatal cardiomyocytes as assed by radioactive leucine incorporation. More specifically, we showed that both ANGII and NE activated NFATc1 and c4 in cardiomyocytes, while NFATc2 was activated by NE. Neither ANGII nor NE activated NFATc3 in cardiomyocytes. These results show that ANGII and NE, so central in the development of cardiac hypertrophy and failure, activate specific NFATc isoforms in cardiomyocytes, suggesting that mechanisms exist for NFATc isoform-specific activation in these cells.

Paper II

Calcineurin-dependent NFAT isoforms are activated in the myocardium of aortic stenosis patients and their activation is reversed by relief of pressure overload in a murine model of reverse remodeling

Using the antibodies for NFATc detection validated in Paper I on human myocardial biopsies, we showed substantially increased protein levels of all four NFATc isoforms in left ventricular tissue from AS patients. Despite increased protein levels, we found no difference in NFATc1-c4 mRNA between AS patients and controls, suggesting post-transcriptional regulation of NFATc isoforms in the diseased human heart. RCAN1-4 mRNA and protein were significantly increased, as expected, indicating increased NFATc activity in AS, although a substantial fraction of NFAT remained phosphorylated. We also showed regulated activity and protein level of GSK-3β, Akt and the MAPKs modulating calcineurin-NFAT activity, p38, ERK 1/2 and JNK 2/3, as well as increased levels of NFAT interaction partners

AP-1, MEF2 and GATA4, and of the NFAT-activating TRPC channels, in AS. Our results on the regulation of NFATc protein, mRNA and phosphorylation were confirmed in the murine AB model. Collectively, these results show multi-leveled activation of calcineurin-NFAT signaling in AS and substantially increased protein levels of the four calcineurin-dependent NFATc isoforms, suggesting that all four play a role in development of hypertrophy in the human heart. Moreover, a murine AB-DB model mimicking AVR in AS patients, relieving the pressure overload, showed that NFAT activation could be reversed, suggesting that the detrimental effects of this pathway in the diseased heart may be a target of pharmacological or surgical intervention.

Paper III

Syndecan-4 is essential for development of concentric myocardial hypertrophy via stretch-induced activation of the calcineurin-NFAT pathway

We showed that syndecan-4 KO mice do not develop concentric hypertrophy after AB, as do wild type mice, but rather show signs of cardiac dilation, dysfunction and failure. In agreement with lack of hypertrophy, syndecan-4 KO cardiomyocytes exhibited no increase in width following AB, as seen in wild type cells. Syndecan-4 KO mice showed reduced calcineurin-NFATc4 activation following AB, and cardiomyocytes lacking syndecan-4 showed minimal activation of NFAT following cyclic mechanical stretch. We identified that calcineurin bound directly to the intracellular part of syndecan-4 through its AID when syndecan-4 was dephosphorylated at serine 179. Experiments assessing NFAT activation suggested that the binding of calcineurin to the cytoplasmic part of syndecan-4 activated calcineurin. We found increased levels of syndecan-4 mRNA and protein in the hypertrophic myocardium of AS patients, and showed that overexpression of the membrane-localized syndecan-4 in cells activated NFATc4. Finally, we showed that syndecan-4 was dephosphorylated after AB in mice and in myocardial tissue from AS patients, favoring calcineurin-NFAT activation. Altogether, these results suggest that syndecan-4 is essential for development of concentric hypertrophy in the pressure-overloaded heart by activating the calcineurin-NFATc4 pathway.

Paper IV

Cortisol response to stress is associated with myocardial remodeling in salmonid fishes

We found that fish responding to stress with high plasma cortisol (HR line and wild salmonids) had bigger hearts than fish responding with low plasma cortisol (LR line and wild salmonids). HR hearts showed signs of hypertrophy of the outer, compact layer of the ventricle and increased focal fibrosis and collagen expression, indicating pathological cardiac remodeling. We showed increased RCAN expression in the HR hearts, suggesting increased NFAT activity in hearts of fish responding to stress with high levels of plasma cortisol. These results indicate that the cortisol response to stress is associated with pathological remodeling of the fish heart, and that NFAT activation in the diseased heart is an evolutionary conserved response.

Paper V

Cardiac O-GlcNAc signaling is increased in hypertrophy and heart failure

We observed increased levels of O-GlcNAcylation of numerous cardiac proteins in myocardial tissue from AS patients and rat models of chronic heart disease including hypertension, MI and aortic constriction. O-GlcNAc levels were increased in both hypertrophied and failing hearts. We showed increased levels of the enzymes regulating the O-GlcNAc PTM, O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA), in pressure-overloaded hearts, yet not in hearts post-MI. Pharmacological inhibition of OGA, increasing O-GlcNAc, reduced contractility in muscle strips from post-MI failing hearts, suggesting that O-GlcNAcylation is a possible mediator of cardiac dysfunction. Our results support the novel concept that O-GlcNAc signaling is involved in development of cardiac hypertrophy and failure of various etiologies, and to our knowledge, we were the first to show regulation of this PTM in the hypertrophic human heart.

Discussion

In the following, some specific and central topics from this thesis will be discussed.

The four NFATc isoforms in the heart – functional specification or redundancy?

The NFAT protein was discovered in 1988[125] as a nuclear protein regulating the IL-2 promoter in activated T-cells. The four genes encoding the four calcineurin-dependent NFATc isoforms are thought to have arisen 500 million years ago in the vertebrate genome[51, 54], and it is now evident that almost every cell type expresses at least one NFATc family member[53, 54, 56, 117]. Murine cardiomyocytes express all four[45, 49, 50], and in Paper II we show that all of them are expressed and up-regulated in the human hypertrophic myocardium. Extensive alternative splicing of the NFATc genes further complicates the picture, predicting for instance as much as 24 different NFATc4 sub-isoforms[97]. Thus, to understand the functional specification or redundancy of the NFATc isoforms is a particular challenge.

In *non*-cardiac tissues, murine KO models of single NFAT isoforms have revealed mild phenotypes suggesting relatively overlapping functions[56]. Given the central role of NFAT in gene regulation in the heart, it is interesting that the pathological significance of removing each individual member to the development of cardiac hypertrophy, is varied. This implies that although they are usually reported in a one-isoform-represents-all manner, we believe that NFATc isoforms should be analyzed separately in the heart as they probably have specific functions.

Lack of NFATc1 is embryonically lethal with defects in the cardiac septum and valves resembling common congenital heart defects seen in the human population[57, 58], and thus has not been investigated in the adult heart. Mice lacking NFATc2, on the other hand, are viable, and do not develop calcineurin-dependent hypertrophy and heart failure[49]. Likewise, NFATc3 null mice demonstrate a significantly reduced hypertrophic response following diverse stimuli[59]. Somewhat surprisingly, NFATc4 KO mice do not show a compromised ability to undergo hypertrophic growth[45, 59], although overexpression of NFATc4 induces extensive hypertrophy[43]. NFATc4 has also previously been shown to be activated in human heart failure[65, 66] and there is a c4 polymorphism which correlates to left ventricular mass of humans[67]. Collectively, these results point towards functional specification of the NFATc isoforms in cardiac remodeling.

Nevertheless, their differential regulation and effects are mysterious. The consensus target site[53, 60] and the DNA-binding domain (within the RHR) are highly conserved among all isoforms[51] such that they all activate RCAN1-4 and BNP in cardiomyocytes[45, 61]. Our finding that all four isoforms are up-regulated in the myocardium of AS patients could also imply co-regulation of the NFATc isoforms (Paper II).

In Paper I we show that ANGII and NE activate NFATc in an isoform-specific manner in murine cardiomyocytes, which is in line with the findings of Rinne *et al.*[126, 127]. These authors show isoform-specific activation in atrial and ventricular myocytes, and also indicate tissue-specific NFATc regulation within the heart[127]. Interestingly, it was recently shown that NFATc4, but not c2 and c3, is a direct target for the anti-hypertrophic miR-133 in the heart, suggesting activation of an NFATc isoform-specific mechanism in development of heart failure[128]. In skeletal muscle, a closely related tissue where all four isoforms are expressed, specific NFATc proteins show nuclear localization at specific developmental stages[129]. Furthermore, specific combinations of the isoforms determine transcription of fiber type-specific genes[130]. Interestingly, NFAT isoforms were found to be differentially sensitive to Ca^{2+} transients elicited by electrical activity. Differences in export/import mechanisms for the c1 and c3 isoforms have also been shown in skeletal muscle[131] as well as in cardiomyocytes[126, 127]. Collectively, these results suggest that cellular mechanisms exist for functional specification of NFAT isoforms in tissues where all four NFATcs are expressed, such as the myocardium. This strengthens our hypothesis that these four should be analyzed separately during cardiac remodeling.

NFATc-DNA binding is relatively weak such that they need a dimerization partner to transactivate genes. Some degree of isoform-specificity has been suggested to be product of their associated nuclear partners, despite the RHR being highly conserved among the c1-c4 isoforms[53, 117]. For instance in skeletal muscle it was shown that GATA bound NFATc1 but not c2[132]. Furthermore, NFAT isoforms may be localized to different intracellular pools and thus activated by local Ca^{2+} signals. It was noted by Rinne and co-workers that NFATc3 particularly accumulated around the nucleus in *non*-stimulated cardiomyocytes[127]. Moreover, the various isoforms may have a different sensitivity towards Ca^{2+} and calcineurin such as seen in skeletal muscle[130]. Yet another possibility is that calcineurin and other NFAT-modifying enzymes are localized to specific subcellular domains through association with binding proteins. For instance, calsarcins are localized to the sarcomere where they bind

calcineurin[133]. Interestingly, we (Paper III) and others[106] show that syndecan-4 and MLP, respectively, which are localized to the Z-disc, also bind calcineurin and affect NFATc4 activation.

Of notice, more pronounced effects are seen when several NFATc genes are lacking[53], however generation of mice lacking all isoforms have failed due to lethality[45]. For instance, mice lacking both c3 and c4 show embryonic lethality and a thin ventricle with reduced myocyte proliferation[134]. Interestingly, these embryos could be rescued by re-expression of constitutively active NFATc4, further complicating our present attempt to understand the functional relationship between NFATc members.

In conclusion, mechanisms for isoform-specific regulation of calcineurin-dependent NFAT isoforms exist in cells expressing more than one of the NFAT genes, such as cardiomyocytes expressing all four. Expression of these four genes over 500 million years of evolution point to functional specificity, along with differential expression in various cell types and at various developmental stages, and interestingly, the pathological significance in the heart of removing each member individually, varies. Nevertheless, there is evidence for overlapping functions such that they all regulate the same disease-related genes in the heart, and are co-regulated themselves in the hypertrophic human heart.

Is calcineurin-NFAT signaling a therapeutic target in heart failure?

Although it was not the purpose of the work constituting this thesis, the ultimate aim of our research is to improve basic knowledge such that therapy for heart failure may be improved. I will therefore discuss the current standing of calcineurin-NFAT modulation in heart disease. Heart failure therapy has over the last 40 years moved from bedrest to diuretics, inotropes, vasodilators, renin-angiotensin-aldosterone inhibitors and β-blockers which slow, but not reverse, the progression[18]. Today, several lines of evidence indicate that any degree of hypertrophy is detrimental for cardiac function and survival[30], suggesting benefits from inhibition of hypertrophy. Although several therapies that improve outcome are associated with a regression of hypertrophy[135-138], few clinical therapies target hypertrophic remodeling directly today.

The naturally occurring and widely used immunosuppressive drugs CsA and FK506 inhibit calcineurin[109]. Early animal studies showed that CsA/FK506 could inhibit and reverse hypertrophy following pressure overload, infarction, hypertension, adrenergic stimulation and

genetic modification[43, 139-144]. Thus, it is generally accepted that inhibition of calcineurin can reduce development of pathological hypertrophy, making calcineurin-NFAT intervention promising.

Despite these results, some studies have reported that lack of hypertrophy following pathological stimuli may result in failure and premature death. Accordingly, it was shown that although CsA attenuated pressure overload hypertrophy, at the same time it enhanced the susceptibility to failure[145]. This resembles our finding from mice lacking syndecan-4 in which pressure overload-induced concentric hypertrophy was inhibited, but at the same time, dysfunction and failure was induced (Paper III).

Supplying to the extensive evidence showing that calcineurin-NFAT is activated in the hypertrophic heart, we show that all NFATc isoforms are substantially up-regulated in the hearts of AS patients (Paper II). Since calcineurin-NFAT studies have relied heavily on murine models in which genetic manipulations have been initiated before birth, little was until recently known about the reversibility of this system, which is relevant for instance for our AS patients undergoing AVR. Berry *et al.* engineered mice with an inducible, constitutively active calcineurin gene in cardiomyocytes[146]. Activation of calcineurin in adulthood triggered pathological hypertrophy preceding systolic dysfunction, fetal gene activation, fibrosis and clinical heart failure. Interestingly, cardiac hypertrophy and fetal gene expression reversed when calcineurin activity was turned off, although fibrosis only partially reversed. Furthermore, these authors showed that systolic dysfunction is second to hypertrophy and not vice versa, supporting the view that hypertrophy itself could be a target for therapy. Along these lines, we show that NFAT activation induced by AB in mice is reversed when the pressure overload is relieved by DB (Paper II), suggesting that it is possible to pharmacologically or surgically reverse the pathological calcineurin-NFAT signals in the heart. Furthermore, extrapolating from these results, it is likely that some of the deleterious effects of calcineurin-NFAT signaling in the AS hearts are reversed when treated with AVR.

To inhibit the calcineurin-NFAT system in the heart, various approaches other than employing FK506/CsA are possible. For instance, the VIVIT peptide inhibits NFAT signaling directly[110, 147], or inhibition can be based on natural calcineurin blockers such as RCAN1, cain/cabin-1 and A-kinase anchor protein 79 (AKAP79) which directly bind calcineurin, or on enzymes that regulate nuclear export of NFAT such as GSK-3β, MAPKs and the dual-specificity tyrosine kinase 1a (Dyrk1a)[56, 117]. Thus, based on current literature, it is not

unlikely that inhibition of calcineurin-NFAT will be a therapeutic option in the future, targeting the pathological hypertrophic response.

Is syndecan-4 a mechanosensor in cardiomyocytes and does it play a role in development of heart failure?

The transmembrane proteoglycans, among them the syndecans, were discovered 30 years ago[148] and it is now evident that all animal cells express one or more of the less than ten known transmembrane proteoglycans[149]. Invertebrates express one syndecan, reflecting a long evolutionary history, and two rounds of duplication produced the four vertebrate isoforms, namely syndecan-1-4. The extracellular cleavage domain and the attachment sites for heparan sulfate glycosaminoglycan (GAG) chains, the transmembrane domain and the cytoplasmic domain are highly conserved among species, reflecting a conserved function. Mice lacking syndecans are viable and fertile, and phenotypes different from wild type appear when subjecting them to postnatal stress or injury. Syndecans have been implicated in a variety of biological functions spanning from regulation of cell adhesion, growth factor signaling and cell migration to metabolism. In particular, they function as co-receptors in these processes.

External load *per se* plays a critical role in determining cardiac muscle mass. Although it is well known that mechanical forces influence on cardiomyocyte function and structure, little is known about how mechanical stimuli are converted into intracellular signals. Syndecan-4 (illustrated in Fig. 7) is expressed in focal adhesions in many cell types, and in cardiomyocytes it has been localized to the Z-disc and costamere[68]. The Z-disc constitutes a physical anchor for myofilaments, the cytoskeleton, receptors and transmittors of mechanical signals[37]. Thus, Z-disc protein complexes have been proposed to be an essential part of the stretch-sensing apparatus in cardiomyocytes[36].

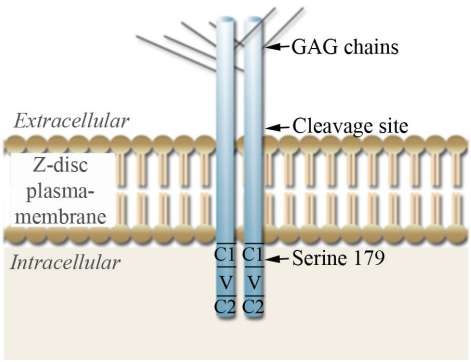

Fig. 7 The proteoglycan syndecan-4.
The transmembrane proteoglycan syndecan-4 is localized to the cardiomyocyte Z-disc, and consists of an extracellular domain with attached glycosaminoglycan (GAG) chains and a cleavage site for shedding of this domain. The cytoplasmic domain of 28 amino acids consists of two conserved regions (C1/C2) and the syndecan-4-specific V-region. Serine 179 is indicated.

Due to its localization and connectivity to both the ECM and the cytoskeleton, we propose a role for syndecan-4 as a transducer of mechanical stimuli in cardiomyocytes. This is based on our findings in Paper III showing 1) no development of concentric hypertrophy nor increase in cardiomyocyte width of pressure-overloaded syndecan-4 KO hearts, 2) a correspondingly reduced NFAT activation, 3) minimal calcineurin-NFAT activation in syndecan-4 KO cardiomyocytes subjected to cyclic mechanical stretch, and 4) increased syndecan-4 levels in pressure-overloaded human hearts. Interestingly, a role for syndecan-4 as a mechanotransducer in fibroblasts has also been hypothesized by others[150].

It has previously been suggested that mechanical stimuli converge on integrins[151, 152], transmembrane receptors that bind to the ECM and activate intracellular signaling pathways instructing cytoskeletal organization and gene expression. As syndecan-4 is a co-receptor for β1 integrin[149, 153], its role as a mechanosensor in cardiomyocytes may be through integrin interaction.

Interestingly, syndecans carry 3-5 heparan sulfate GAG chains extracellularly which are known to bind ligands such as growth factors, the ECM, cytokines, chemokines and pathogens. Heparan sulfate as a GAG is essential for animal life[154], and although this aspect has not been studied in this thesis, syndecan GAG chain function is likely to be relevant also for the heart due to levels of both ligands and syndecan being altered in cardiac disease.

The extracellular core protein also associates with proteases resulting in cleavage and shedding of the extracellular part. Increased shedding has been linked to wound repair, inflammation, tumor progression and cell stress[149, 155]. Indeed, although it was a small study of 76 patients, syndecan-4 in the serum was recently identified as a novel biomarker for patients with chronic heart failure[156], reflecting that yet another aspect of syndecan function is relevant for human heart disease. Interestingly, syndecan-4 serum levels correlated with LV geometry parameters such as mass, and correlated negatively to EF. Thus this aspect of syndecan-4 function may represent a biomarker of ventricular remodeling.

Syndecans have the ability to signal through their small cytoplasmic domain, especially to the cytoskeleton, and they are linked to PDZ-domain protein networks. The 28 amino acid cytoplasmic domain, very similar in zebrafish and up to humans[157], and absolutely conserved between mammals[158], consists of the C1 and C2 domains, similar in syndecan 1-4, with the isoform-specific V-region separating the two conserved domains. Thus, the V-region of syndecan-4 is expected to play a distinct role. Although syndecans lack intrinsic kinase

activity, they are involved in signaling by directly interacting with signaling molecules. In fibroblasts, syndecan-4 regulates localization, activity and stability of PKCα through a direct interaction with the V-region[71, 159]. In Paper III, we identified that the V-region contains a PIxIxIT-similar motif, a motif found in other calcineurin-binding proteins such as NFAT[147], AKAP[160] and RCAN1[101, 103], and that calcineurin bound directly to the V-C2 region. Furthermore, we found that syndecan-4 without its extracellular domain activated NFAT, emphasizing the importance of the cytoplasmic domain in syndecan-mediated signaling. Thus, we identified a molecular mechanism likely to underlie the inhibited development of concentric hypertrophy and reduced NFAT activation in mechanically stretched syndecan-4 KO cardiomyocytes.

Phosphorylation of serine 179 (pS179) within the C1 region of syndecan-4 has been shown to constitute a molecular switch regulating the function of the cytoplasmic part. pS179 negatively regulates higher-order oligomerization, PKCα binding and activity, binding of syntenin, cell mobility and growth by altering the conformation of the cytoplasmic tail[72, 161-164]. In Paper III, we found that pS179 also negatively regulated the interaction with calcineurin, and the level of pS179 was reduced in pressure-overloaded murine and human hearts, favoring binding of calcineurin to syndecan-4. The only known kinase responsible for regulating pS179 is PKCδ[165], and until now, no phosphatase has been shown to regulate this site. We speculate based on our findings that calcineurin itself may dephosphorylate S179 in the heart (Paper III).

As discussed above, syndecan-4 KO mice did not develop concentric hypertrophy following pressure overload, but rather developed cardiac dilatation and failure. Although our data clearly indicate syndecan-4-mediated, pro-hypertrophic NFAT activation in cardiomyocytes, syndecan-4 may also mediate other effects in the heart that contribute to the premature failure compared to wild type. This may be related to other *non*-investigated signaling pathways such as those involved in the inflammatory response as well as *non*-cardiomyocyte effects related to the ECM and development of fibrosis. In a very recent study, syndecan-4 KO mice were shown to have impaired cardiac function and increased mortality due to ruptures following MI[69]. Lack of syndecan-4 was associated with a reduced inflammatory reaction, impaired fibroblast conversion to myofibroblasts and reduced ECM deposition in the infarct region after MI. Thus, this study indicates a protective role of syndecan-4 in remodeling of the infarct region. In a parallel study, however, cardiac function

was found to be improved in mice lacking syndecan-4 following MI[82]. Interestingly, hypertrophy in the infarct border zone with a concomitant increase in NFAT activation was increased in syndecan-4 KO mice after infarction. This suggests that additional layers of complexity are present in syndecan-4-mediated cardiac remodeling and NFAT activation, and that syndecan-4 function in the heart may vary whether it is related to global pressure-overload or regional remodeling following an infarct.

In conclusion, our study and recent literature suggest that syndecan-4 is involved in cardiac remodeling and dysfunction following pressure-overload and MI, regulating central aspects such as hypertrophy, inflammation and fibrosis. Due to its cellular localization and binding of both extra-and intracellular molecules, it is likely that syndecan-4 transmits mechanical stimuli in pressure-overloaded and mechanically stressed cardiomyocytes. Furthermore, it is likely that several aspects of syndecan-4 function such as extracellular GAG chain interactions and shedding, as well as intracellular signaling, are relevant for heart disease.

Does O-GlcNAc play a role in development of heart failure and does it affect calcineurin-NFAT signaling?

Of the annotated human cardiac proteins, 62% have a known PTM, and approximately 25% have more than one type of PTM, according to current literature[166, 167]. PTMs define the functional and structural plasticity of proteins in eukaryotes as well as prokaryotes, they modulate protein activity and interactions in fundamental molecular processes and they represent disease- and tissue-specific modifications. The most studied PTMs are perhaps phosphorylation cascades, the histone code of acetylation and methylation, ubiquitination and glycosylation[168]. Glycosylation can either be N-linked and irreversible as in proteoglycans such as syndecan-4, or O-linked (O-GlcNAc), with dynamics comparable to phosphorylation[169].

Subsequent to the discovery in 1984[76], O-GlcNAc signaling has been implicated in a diverse array of physiological and pathological functions in the cell[77] and although O-GlcNAc alterations are now believed to be involved in cardiovascular pathophysiology[78-81], regulation and function in the heart are currently not well understood. O-GlcNAcylation encompasses nuclear, cytoplasmic and mitochondrial proteins of nearly all functional classes[77, 170-173] and is achieved by the two highly conserved enzymes, OGT and OGA. OGT deletion is

embryonically lethal suggesting O-GlcNAc as a PTM to be essential for life[174] and to date, OGT has been found in all tissues examined[169]. In brain and spinal cord, proteomic analyses have identified nearly a thousand O-GlcNAcylated proteins[175].

In hypertrophic and failing hearts, a shift towards a more glycolytic metabolism occurs[176-180], suggesting that more substrate for glycosylation is available in the failing heart. Accordingly, in Paper V we found upregulated levels of O-GlcNAc signaling and the cycling enzymes in hypertrophic and failing myocardium of various etiologies, i.e. chronic hypertension, aortic constriction and MI. Functionally, the consequences of the observed increase in cardiac O-GlcNAcylation may be of importance to the failure progression. Numerous studies have shown that during acute ischemic and hypoxic stress, O-GlcNAc mediates cardioprotection[78-81, 181-185]. Severe injury such as trauma-hemorrhagic shock induces O-GlcNAc in the heart, and increasing O-GlcNAc levels during resuscitation improve cardiac recovery and reduce inflammation in rodents[173, 186]. Recently, the role of O-GlcNAcylation in post-MI failure was addressed by deletion of OGT in the adult mouse heart[187]. These mice showed exacerbated dysfunction after MI, indicating a cardioprotective effect of protein O-GlcNAcylation following MI. Thus, these studies indicate that O-GlcNAcylation can be cardioprotective against stresses such as ischemia and hypovolemic shock.

Interestingly, O-GlcNAcylation was recently assessed in the physiologically hypertrophied hearts of swim-exercised mice, and found to be down-regulated[188], suggesting that our finding of increased O-cardiac GlcNAcylation in the chronically diseased heart is part of a pathological mechanism. Moreover, our finding of increased O-GlcNAcylation in *non*-failing hypertrophic ventricles suggests that increased O-GlcNAc may be a cellular mechanism preceding development of failure (Paper V).

When chronically increased, O-GlcNAcylation contributes to cardiovascular dysfunction, such as seen in diabetes[79, 169]. In cardiomyocytes from diabetic mice, reducing protein O-GlcNAc levels improve contractile function[189], while hyperglycemia, mimicking diabetes by increasing O-GlcNAc levels, impairs cardiomyocyte Ca^{2+} cycling[190]. Moreover, cardiomyocytes from hyperglycemic, diabetic rats exhibit impaired relaxation[191]. Interestingly, we found that pharmacological inhibition of OGA, increasing O-GlcNAc, decreased the contractility of post-MI failing hearts, suggesting that chronic O-GlcNAcylation promotes cardiac dysfunction (Paper V). As several proteins such as PLN[192], actin, myosin heavy chain, myosin light chain and troponin I[193] are known to be O-GlcNAcylated, it seems

reasonable to assume that the reduced function could be related to altered activity of several of these key contractile proteins.

Another interesting aspect of O-GlcNAc signaling concerns the interplay with protein phosphorylation (Fig. 8). O-GlcNAc cycles on serine/threonine residues of proteins with a timescale, distribution and abundance similar to phosphorylation, and there is an extensive crosstalk with phosphorylation cascades[79, 169, 194-199]. OGT and OGA are activated by both O-GlcNAcylation and phosphorylation and in many cases, OGT, OGA, kinases and phosphatases are found in the same complexes[195, 199]. Actually, all known O-GlcNAcylated proteins are also phospho-proteins. When phosphorylation of 700 sites was determined after increasing O-GlcNAc with an OGA inhibitor, phosphorylation of virtually all actively cycling sites was either increased or decreased[196].

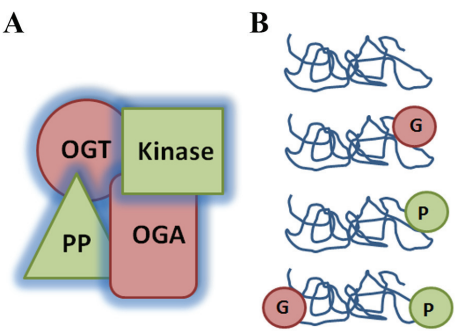

Fig. 8 Crosstalk between protein O-linked phosphorylation and glycosylation.
A multi-enzymatic complex of OGT, OGA, kinases and phosphatases (PP) (A) regulates the dynamic, serine/threonine protein modifications of phosphorylation (P) and O-GlcNAc (G) (B). From the top; a naked protein, an O-GlcNAcylated protein, a phosphorylated protein and a protein modified by both. The figure is modified from[199].

Since we observed a relatively high number of proteins being O-GlcNAcylated in the heart (Paper V), crosstalk with phosphorylation is likely also important in cardiac remodeling. Furthermore, results from studies in which serine/threonine residues have been mutated have been accepted as evidence for the importance of phosphorylation without considering O-GlcNAcylation. In view of this, PTMs might need re-evaluation considering also O-GlcNAcylation of specific residues.

O-GlcNAc signaling has been linked to NFAT activity in immune cells[74, 200]. It was demonstrated that OGT is central in lymphocyte activation and IL-2 production through regulation of NFAT. Interestingly, NFAT was O-GlcNAcylated following direct binding to OGT and O-GlcNAcylation likely regulated nuclear translocation of NFAT. Since OGT and OGA often are found in the same complexes as enzymes regulating phosphorylation, interaction with calcineurin and for instance GSK-3β could be novel mechanisms for NFAT

regulation. Thus, interaction between O-GlcNAc and NFAT signaling is something that probably will be investigated in the heart in the near future, by us or others. Furthermore, the maturing of proteomic analysis will probably reveal more details concerning the role of O-GlcNAc in heart disease and the specific proteins involved[167].

Is pathological cardiac remodeling in fish induced by the cortisol stress response?

The fish heart demonstrates a high degree of anatomical and physiological plasticity in response to stimuli such as sexual maturation and environmental changes such as temperature, hypoxia and food availability[201]. In salmonids, the ventricle consists of an outer compact layer of circumferentially arranged, spindle-like cardiomyocytes and an inner spongy myocardium[202]. Plastic changes involve hyperplasia and hypertrophy of both layers[201]. For example, salmonids undergo cardiac hypertrophy as a routine remodeling mechanism during cold acclimation[203].

Millions of tons of fish are processed every year in the aquaculture industry, and cardiac-related mortality has become a costly problem[20-25, 204, 205]. Wild salmonids are characterized as very athletic and can migrate thousands of kilometers[202], however when farmed, they generally grow faster, are fat and become poor swimmers. The ventricle of farmed salmonids is often more rounded, in contrast to the pyramidal structure of wild fish, with fat depositions, coronary arteriosclerosis and malformations[201]. When raised under identical conditions, cardiac function does not differ between wild and farmed fish[206], indicating that there are other underlying causes of the observed pathological remodeling in farmed fish than genetics.

In humans, severe stress is associated with a poor prognosis for individuals with cardiac disease[207, 208]. High cortisol responsiveness to stress is associated with increased cardiovascular mortality[209] and serum cortisol is a predictor of cardiac events in patients with chronic heart failure[210]. Furthermore, oral administration of glucocorticoids is a risk factor for heart disease[211]. In the aquaculture industry there is an increasing worry about stress-induced mortality[20, 25]. In particular, farmed fish experience social stress as well as stress related to handling etc. Cortisol is the major steroid stress hormone in salmonids as well as in humans, and cortisol receptors are expressed in the myocardium[212] (and Paper IV). When applied to neonatal cardiomyocytes or fetal hearts, cortisol induce hypertrophy[213-215]. In Paper IV we showed that fish responding to stress with high plasma cortisol 1) have bigger hearts, 2) a

thicker compact myocardium, 3) increased expression of hypertrophic markers, 4) increased RCAN1 levels and 5) increased focal fibrosis and collagen expression. Based on these results, we suggest that cortisol-induced cardiac remodeling may be one of the underlying, explanatory factors for increased cardiac-related mortality in farmed salmonids. Interestingly, these results also suggest that NFAT activation in the diseased heart is an evolutionary conserved response.

Future perspectives – calcineurin-NFAT signaling in heart failure

As mentioned, the overall aim of this thesis is to expand the basic knowledge of molecular signaling pathways underlying development of heart failure, particularly the role of calcineurin-NFAT signaling. When so-called nodes that many signals converge on are identified, detailed, reductionist analyses contributing to the big puzzle are needed to understand the mechanisms, with the ultimate goal of developing novel therapeutic molecules. Based on current literature, calcineurin-NFAT is such a central node, and it is not unlikely that future therapeutics one day will target this pathway in the heart.

Main conclusions

The results presented in this thesis show that NFATc isoform-specific mechanisms exist within cardiomyocytes, such that ANGII activates NFATc1 and c4 while NE activates c1, c2 and c3. In the myocardium of AS patients, all four NFATc isoforms are substantially up-regulated, and despite a fraction being phosphorylated, NFATc activation was increased in these hearts at multiple levels. When pressure overload was relieved in mice, NFAT activation was reversed, suggesting that the detrimental effects of this pathway in the diseased heart may be targeted pharmacologically or surgically. Methodologically, measurements of NFATc isoform-specific activation and protein levels were made possible by evaluation of NFATc and phospho-NFATc antibodies using peptide arrays, blocking peptides and isoform-specific overexpression.

Using a syndecan-4 KO model, this thesis shows that syndecan-4 is essential for development of concentric myocardial hypertrophy following pressure overload in mice. In cardiomyocytes, mechanical stress-induced as well as autonomous growth-induced NFAT activation was mediated by syndecan-4, and calcineurin interacted directly with the cytoplasmic part of syndecan-4. When overexpressed, syndecan-4 activated NFAT by dephosphorylation. This effect was regulated by phosphorylation of the cytoplasmic domain of syndecan-4. Collectively, these results imply that syndecan-4 is a mechanosensor in the myocardium, regulating calcineurin-NFAT signaling in a pressure-overloaded heart.

By investigating the myocardium of AS patients and rat models of chronic heart disease; hypertension, MI and AB, we show that the protein PTM called O-GlcNAc, which has been connected to NFAT signaling, is increased in all these chronically diseased hearts. A pharmacological increase in O-GlcNAc following MI suggested that O-GlcNAc promotes cardiac dysfunction after an infarction. Thus, our results suggest that O-GlcNAc is part of a chronic stress response in hypertrophic as well as in failing hearts.

Finally, this thesis shows that in salmonid fish, the cortisol stress response is associated with cardiac hypertrophy, focal fibrosis and NFAT activation, suggesting NFAT activation to be a conserved mechanism in the diseased heart and stress to be an explanatory factor of stress-induced cardiac mortality in the fish farming industry.

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Syndecan-4 Is Essential for Development of Concentric Myocardial Hypertrophy via Stretch-Induced Activation of the Calcineurin-NFAT Pathway

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Abstract

Sustained pressure overload leads to compensatory myocardial hypertrophy and subsequent heart failure, a leading cause of morbidity and mortality. Further unraveling of the cellular processes involved is essential for development of new treatment strategies. We have investigated the hypothesis that the transmembrane Z-disc proteoglycan syndecan-4, a co-receptor for integrins, connecting extracellular matrix proteins to the cytoskeleton, is an important signal transducer in cardiomyocytes during development of concentric myocardial hypertrophy following pressure overload. Echocardiographic, histochemical and cardiomyocyte size measurements showed that syndecan-4$^{-/-}$ mice did not develop concentric myocardial hypertrophy as found in wild-type mice, but rather left ventricular dilatation and dysfunction following pressure overload. Protein and gene expression analyses revealed diminished activation of the central, pro-hypertrophic calcineurin-nuclear factor of activated T-cell (NFAT) signaling pathway. Cardiomyocytes from syndecan-4$^{-/-}$-NFAT-luciferase reporter mice subjected to cyclic mechanical stretch, a hypertrophic stimulus, showed minimal activation of NFAT (1.6-fold) compared to 5.8-fold increase in NFAT-luciferase control cardiomyocytes. Accordingly, overexpression of syndecan-4 or introducing a cell-permeable membrane-targeted syndecan-4 polypeptide (gain of function) activated NFATc4 *in vitro*. Pull-down experiments demonstrated a direct intracellular syndecan-4-calcineurin interaction. This interaction and activation of NFAT were increased by dephosphorylation of serine 179 (pS179) in syndecan-4. During pressure overload, phosphorylation of syndecan-4 was decreased, and association between syndecan-4, calcineurin and its co-activator calmodulin increased. Moreover, calcineurin dephosphorylated pS179, indicating that calcineurin regulates its own binding and activation. Finally, patients with hypertrophic myocardium due to aortic stenosis had increased syndecan-4 levels with decreased pS179 which was associated with increased NFAT activation. In conclusion, our data show that syndecan-4 is essential for compensatory hypertrophy in the pressure overloaded heart. Specifically, syndecan-4 regulates stretch-induced activation of the calcineurin-NFAT pathway in cardiomyocytes. Thus, our data suggest that manipulation of syndecan-4 may provide an option for therapeutic modulation of calcineurin-NFAT signaling.

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Introduction

Sustained pressure overload leads to compensatory myocardial hypertrophy and subsequent decompensation and heart failure, a leading cause of morbidity and mortality. Mechanical stimuli, circulating hormones, as well as autocrine and paracrine factors are involved in initiating myocardial hypertrophy. Transduction of mechanical stimuli may involve molecules that bind the extracellular matrix to the cytoskeleton, but exactly which molecules participate is still unknown. Further unraveling of such signaling pathways is essential for defining novel therapeutic targets.

We have previously shown increased myocardial mRNA expression of all four members of the syndecan family and increased protein levels of syndecan-4 in the hypertrophic non-infarcted region of the left ventricle following myocardial infarction [1]. Syndecan-4 is a transmembrane proteoglycan that connects extracellular matrix proteins to the cardiomyocyte cytoskeleton [2], and has been localized to costameres and Z-discs [3], potentially important sites for signal transduction across the membrane [4,5]. Moreover, syndecan-4 is a co-receptor for several growth factors [6].

Various intracellular signaling pathways have been implicated in transduction of mechanical stimuli during induction of cardiac hypertrophy, including mitogen-activated protein kinase (MAPK) cascades, protein kinase C (PKC)-mediated signaling [7–9] and the calcineurin-nuclear factor of activated T-cell (NFAT) pathway [10]. Interestingly, syndecan-4 has been shown to bind and regulate localization and activity of one of these, namely PKC-α [11].

We hypothesized that syndecan-4 acts as a signal transducer in cardiomyocytes responding to an increase in mechanical stress on the myocardium with subsequent activation of pro-hypertrophic signaling. In this study, we show that syndecan-4 is essential for development of concentric myocardial hypertrophy by acting as a scaffolding protein for mechanical stretch-induced activation of the calcineurin-NFAT pathway.

Results

Lack of syndecan-4 inhibits development of concentric myocardial hypertrophy during pressure overload

Syndecan-4$^{-/-}$ mice had normal cardiac dimensions and function compared to wild-type (WT) mice (Table S1). Three weeks after inducing pressure overload by aortic banding (AB), WT developed concentric left ventricular (LV) hypertrophy manifested as a significant increase in posterior wall thickness (Fig. 1A–C). In contrast, syndecan-4$^{-/-}$ mice displayed no increase in wall thickness following AB (Fig. 1A–C), but a significant increase in LV cross-sectional (Fig. 1D) and longitudinal diameter compared to sham-operated syndecan-4$^{-/-}$ mice (syndecan-4$^{-/-}$-SHAB) and WT-AB (Table S1). LV dilatation in syndecan-4$^{-/-}$-AB was paralleled by reduced cardiac function compared to WT-AB, as indicated by reduced LV fractional shortening (Fig. 1E). Accordingly, syndecan-4$^{-/-}$-AB exhibited increased lung weight and left atrial diameter, as well as increased levels of angiotensin II in serum compared to WT-AB (Tables S1 and S2).

In agreement with the observed lack of concentric hypertrophy, cardiomyocyte width was unchanged in syndecan-4$^{-/-}$-AB compared to syndecan-4$^{-/-}$-SHAB (Fig. 1F), whereas in WT-AB cardiomyocyte width was increased by 25% compared to WT-SHAB. The cardiomyocytes were longer in both WT-AB (8%) and syndecan-4$^{-/-}$-AB (10%) compared to respective controls.

LV histological sections demonstrated less densely packed cardiomyocytes in syndecan-4$^{-/-}$ compared to WT after both SHAB and AB (Fig. 1G). This finding was confirmed by transmission electron microscopy, as syndecan-4$^{-/-}$ hearts exhibited wider, irregular gaps between adjacent cardiomyocytes, in addition to disorganized Z-lines, compared to WT following both SHAB and AB (Fig. 1H). Myocardial wet weight was significantly increased in syndecan-4$^{-/-}$ compared to WT only following AB (Table S2). These data demonstrate increased extracellular space in syndecan-4$^{-/-}$-AB compared to WT-AB, in which concentric myocardial hypertrophy with wider cardiomyocytes dominated. Immunohistochemical examination showed equal staining for collagen type I (Fig. 1G), and immunoblotting showed similar changes in collagen I, III and VIII following AB in both genotypes (Table S2).

The lack of cardiomyocyte hypertrophy in syndecan-4$^{-/-}$ mice was associated with lower expression of cardiac markers of hypertrophy (Fig. 1I–J). Expression of α-skeletal actin, previously shown to correlate with myocardial hypertrophy [12] was lower in syndecan-4$^{-/-}$-AB than in WT-AB (Fig. 1I). Expression of B-type natriuretic peptide (BNP), which has been shown to be regulated by the transcription factor NFAT [13,14] and associated with myocardial hypertrophy, was attenuated in syndecan-4$^{-/-}$-AB compared to WT-AB (Fig. 1J). Expression of atrial natriuretic peptide (ANP) was increased after AB, but there were no differences between the two genotypes (Fig. 1K). Expression of regulator of calcineurin 1–4 (RCAN1-4; also known as MCIP1), a direct target for NFAT [14–16], was significantly lower in syndecan-4$^{-/-}$-AB compared to WT-AB (Fig. 1L), indicating reduced NFAT activity in syndecan-4$^{-/-}$ mice after AB. Both the reduction in RCAN1-4 and in BNP are consistent with inhibition of the calcineurin-NFAT pathway, as they have been shown to be direct targets of NFAT in cardiomyocytes [14].

NFAT signaling is inhibited in syndecan-4$^{-/-}$ mice

To study the role of syndecan-4 in pro-hypertrophic NFAT activation, NFAT phosphorylation (pNFAT) and NFAT luciferase reporter activity were investigated in pressure overloaded hearts and isolated cardiomyocytes subjected to mechanical stress and autonomous hypertrophy. The calcineurin-dependent NFAT isoform c4 (NFATc4) has previously been found to be sufficient for cardiac hypertrophy [13] and to be activated in the hypertrophic human myocardium [17], and thus activation of this isoform was investigated in our study. Syndecan-4$^{-/-}$ mice demonstrated reduced NFATc4 activation compared to WT following AB, shown as increased pNFATc4 levels (Fig. 2A). Increased levels of pNFAT indicate inhibition of the calcineurin-NFAT pathway, since dephosphorylation of these transcription factors by calcineurin is necessary for translocation to the nucleus. Following sham-operation, there was a minimal reduction in NFAT activation in syndecan-4$^{-/-}$ hearts.

To study whether syndecan-4 acts as a mechanotransducer for activation of calcineurin-NFAT signaling in the pressure overloaded heart, cardiomyocytes were subjected to cyclic mechanical stretch. In NFAT-luciferase reporter cardiomyocytes, 24 hrs of mechanical stretch was sufficient to induce significant calcineurin-dependent NFAT activation (Fig. S1A–B). Importantly, syndecan-4$^{-/-}$-NFAT luciferase cardiomyocytes showed minimal activation of NFAT (1.6-fold) compared to a 5.8-fold increase in NFAT-luciferase control cells following mechanical stretch (Fig. 2B). Accordingly, pNFATc4 levels were significantly higher in syndecan-4$^{-/-}$ cardiomyocytes than in WT cells following mechanical stretch (Fig. 2C). Finally, the role of syndecan-4 in calcineurin-NFAT activation in cardiomyocytes subjected to autonomous hypertrophy, was investigated. Syndecan-4$^{-/-}$ cardiomyocytes subjected to autonomous hypertrophy [18] showed significantly higher levels of pNFATc4 compared to WT cardiomyocytes (Fig. 2D), with a corresponding lower level of NFAT activation as measured by luciferase activity in syndecan-4$^{-/-}$-NFAT luciferase cells (Fig. 2E) and a reduced hypertrophic response as measured by radioactive leucine-incorporation after five days in culture (Fig. 2F). Altogether our data indicate that pro-hypertrophic NFAT signalling was inhibited in cardiomyocytes lacking syndecan-4.

Overexpression of membrane-localized syndecan-4 *in vitro* activates NFAT

Increased levels of full-length syndecan-4 in the membrane of HEK-293 cells caused diminished cytoplasmic pNFATc4 levels

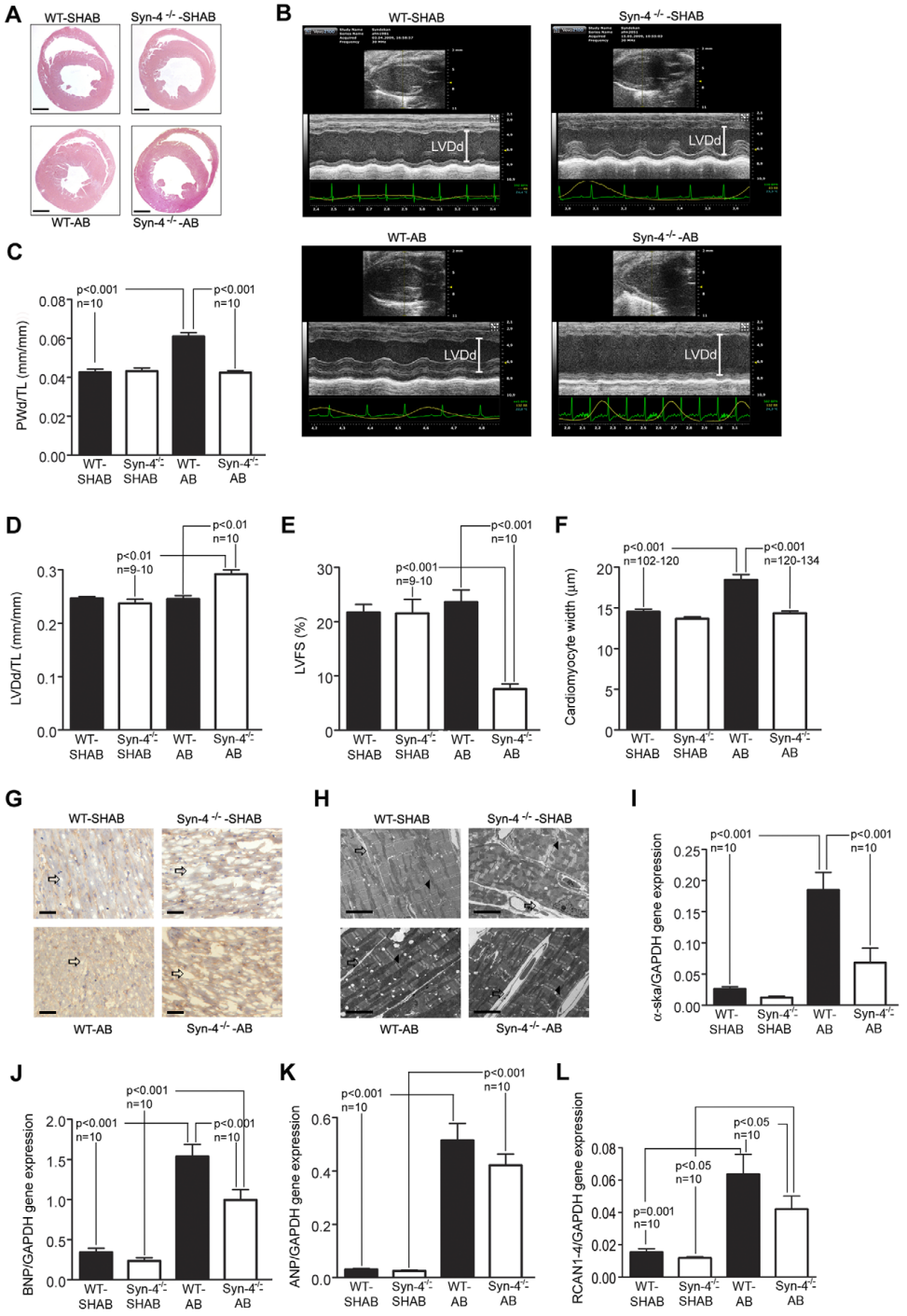
A
WT-SHAB
Syn-4 -/- -SHAB
WT-AB
Syn-4 -/- -AB
B
WT-SHAB
Syn-4 -/- -SHAB
LVDd
WT-AB
Syn-4 -/- -AB
LVDd
C
PWd/TL (mm/mm)
p<0.001 n=10
p<0.001 n=10
WT-SHAB
Syn-4-/- SHAB
WT-AB
Syn-4-/- AB
D
LVDd/TL (mm/mm)
p<0.01 n=9-10
p<0.01 n=10
E
LVFS (%)
p<0.001 n=9-10
p<0.001 n=10
F
Cardiomyocyte width (μm)
p<0.001 n=102-120
p<0.001 n=120-134
G
WT-SHAB
Syn-4 -/- -SHAB
WT-AB
Syn-4 -/- -AB
H
WT-SHAB
Syn-4 -/- -SHAB
WT-AB
Syn-4 -/- -AB
I
α-ska/GAPDH gene expression
p<0.001 n=10
p<0.001 n=10
J
BNP/GAPDH gene expression
p<0.001 n=10
p<0.001 n=10
p<0.001 n=10
K
ANP/GAPDH gene expression
p<0.001 n=10
p<0.001 n=10
L
RCAN1-4/GAPDH gene expression
p=0.001 n=10
p<0.05 n=10
p<0.05 n=10

Figure 1. Lack of syndecan-4 inhibits development of concentric hypertrophy three weeks after induction of pressure overload. (A) Representative cross-sections (HE staining) of the hearts of wild-type (WT) and syndecan-4$^{-/-}$ (Syn-4$^{-/-}$) mice after sham operation (SHAB) or aorta banding (AB) (n = 3). Scale bars, 0.1 mm. (B) Representative 2D and M-mode tracings from the left ventricle (LV) (LVDd, LV diameter in diastole) (n = 9–10). Echocardiographic measurements of (C) posterior wall thickness in diastole (PWd) and (D) LVDd normalized to tibia length (TL), and (E) LV fractional shortening (LVFS) (n = 9–10). (F) Cardiomyocyte width (3 animals in each group). (G) Representative sections of LV myocardium stained for collagen I (brown staining indicates collagen I) (n = 2–3). Scale bars, 100 μm. (H) Representative transmission electron microscopy images of LV myocardium (n = 2). Scale bars, 5 μm. Arrows indicate intercellular spaces and arrowheads indicate Z-lines. Real-time PCR analysis of (I) α-skeletal actin (α-ska), (J) B-type natriuretic peptide (BNP), (K) atrial natriuretic peptide (ANP) and (L) regulator of calcineurin 1–4 (RCAN1-4). mRNA levels were normalized to GAPDH in LV (n = 10). Values are mean ± s.e.m.
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(Fig. 3A, upper panel) and reduced cytoplasmic/increased nuclear NFAT levels (second panel from top), indicating that increased syndecan-4 levels in the membrane induce NFAT activation by dephosphorylation and translocation to the nucleus. To evaluate syndecan-4 as a regulator of intracellular NFAT activation in cardiomyocytes, we generated a cell-permeable peptide consisting of the membrane and cytoplasmic regions of syndecan-4. Cardiomyocytes treated with this peptide *in vitro* showed complete dephosphorylation of NFATc4, while total amount was unaltered (Fig. 3B), consistent with membrane-localized syndecan-4 being important for NFAT activation through its intracellular domain in cardiomyocytes. To evaluate if membrane localization of syndecan-4 is important for NFAT activation, NFAT-luciferase cardiomyocytes were treated with a cell-permeable peptide consisting only of the cytoplasmic region of syndecan-4. This peptide significantly reduced NFAT-luciferase activity (Fig. 3C), indicating that NFAT activation is inhibited when syndecan-4 is soluble, and not localized in the surface membrane.

We also investigated levels of enzymes that have been reported to regulate NFAT phosphorylation in pressure overloaded hearts, and hence may be involved in the syndecan-4-mediated NFAT activation. There was, however, no difference in the level of calcineurin in WT and syndecan-4$^{-/-}$ hearts after AB (Fig. S2 A). Glycogen synthase kinase (GSK) 3-β phosphorylated by Akt is known to antagonize calcineurin action and development of cardiac hypertrophy by phosphorylating NFAT [19–21]. Both GSK3-β and Akt were phosphorylated to the same extent in the two genotypes following AB (Fig. S2 B–D). It has also been shown that each of the three main branches of mitogen-activated kinases (MAPK) directly modulates calcineurin-NFAT signaling and hypertrophic response in the heart [22]. However, activation of extracellular signal regulated protein kinase (ERK) 1/2, c-jun N-terminal kinases (JNK) 2/3 and p38 was comparable in WT and syndecan-4$^{-/-}$ hearts following AB (Fig. S2 E–J). Thus, lack of syndecan-4 did not influence the calcineurin level, GSK-3β or MAPK activation, suggesting that another mechanism regulating NFAT phosphorylation was responsible for the activation of NFAT by syndecan-4. As syndecan-4 is known to directly interact with signaling molecules [23], we hypothesized that syndecan-4 interacts directly with calcineurin.

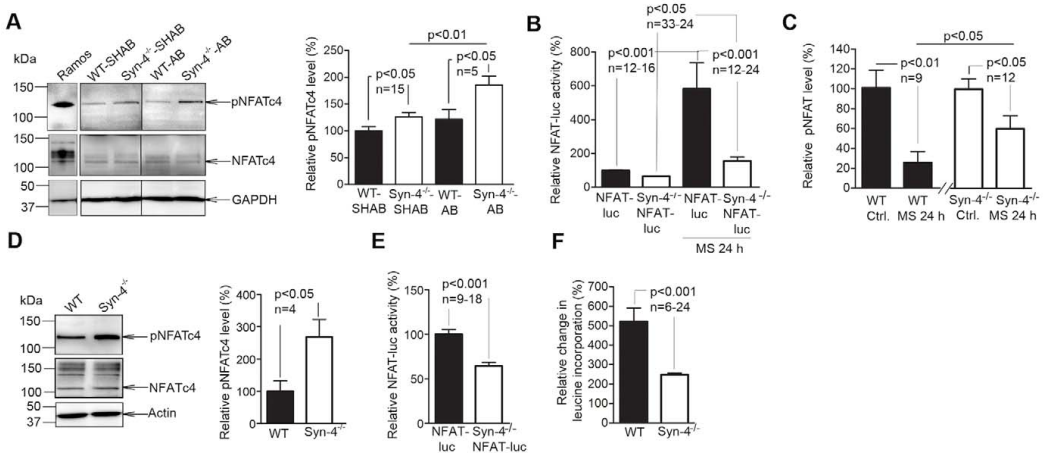

Figure 2. Decreased activation of the calcineurin-nuclear factor of activated T-cell (NFAT) pathway in syndecan-4$^{-/-}$ mice in response to aortic banding. (A) Phosphorylated NFATc4 (pNFATc4) and NFATc4 levels in the left ventricle of wild-type (WT) and syndecan-4$^{-/-}$ (Syn-4$^{-/-}$) mice 24 h after aortic banding (AB) or sham-operation (SHAB) analyzed by immunoblotting. An NFAT-rich cell lysate (Ramos) was used as control for NFAT-positive protein bands (n = 5–15). (B) Luciferase (luc) activity in neonatal cardiomyocytes from NFAT-luciferase and Syn-4$^{-/-}$-NFAT-luciferase mice subjected to 24 h of cyclic mechanical stress (MS) (n = 12–33). (C) pNFATc4 levels in WT and Syn-4$^{-/-}$ neonatal cardiomyocytes subjected to 24 h of cyclic MS (n = 9–12). (D) pNFATc4 and NFATc4 levels in neonatal cardiomyocytes from Syn-4$^{-/-}$ and WT mice subjected to autonomous hypertrophy (n = 4). (E) Luciferase activity in neonatal cardiomyocytes from NFAT-luciferase and Syn-4$^{-/-}$-NFAT-luciferase mice subjected to autonomous hypertrophy (n = 9–18). (F) Radioactive leucine incorporation in Syn-4$^{-/-}$ and WT mice subjected to five days of autonomous hypertrophy (n = 6–24). GAPDH and actin were used for loading control. Values are mean ± s.e.m.
doi:10.1371/journal.pone.0028302.g002

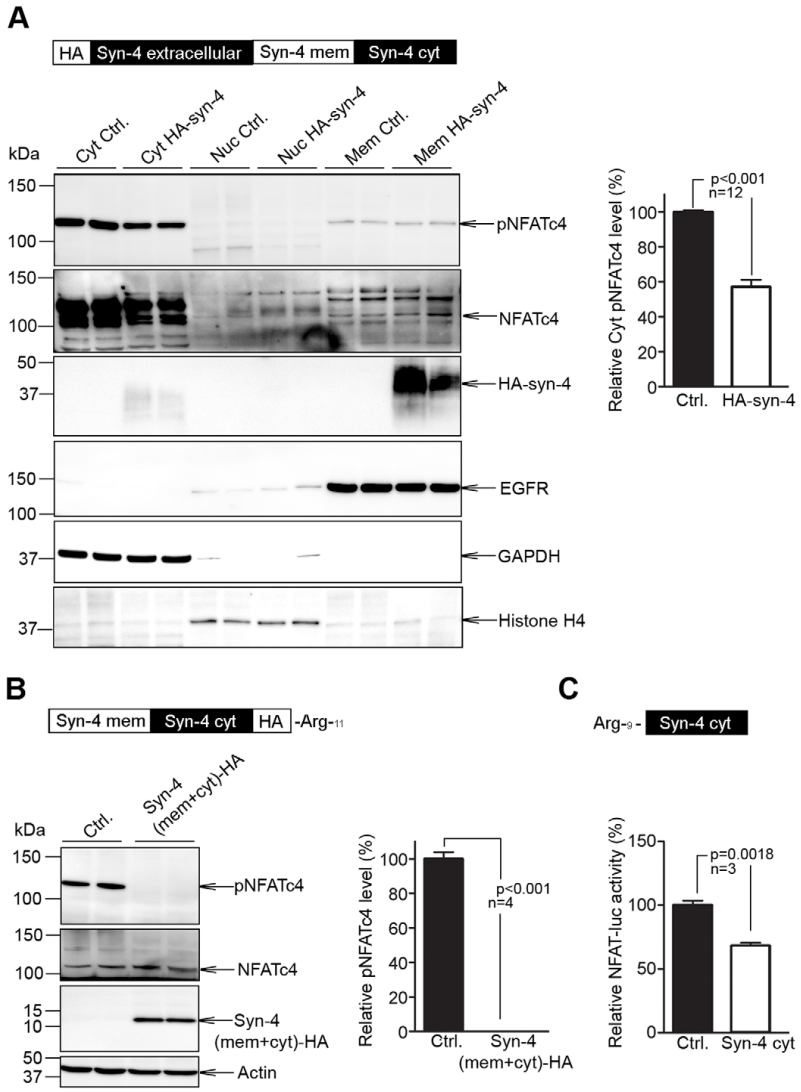

Figure 3. Membrane-localized syndecan-4 activates nuclear factor of activated T-cell (NFAT) *in vitro*. Phosphorylated NFATc4 (pNFATc4), NFATc4 and syndecan-4 levels in (A) HEK-293 cells overexpressing full-length HA-tagged syndecan-4 (HA-syn-4, Cyt = cytoplasmic, Nuc = nuclear, Mem = membrane, EGFR = epidermal growth factor receptor (marker of membrane fraction)) (n = 12). Wild-type (WT) cardiomyocytes treated with a cell-permeable peptide consisting of the membrane and cytoplasmic parts of syndecan-4 (syn-4 (mem+cyt)-HA) (n = 4), analyzed by immunoblotting (B), and NFAT-luciferase reporter cardiomyocytes treated with a cell-permeable peptide consisting of the cytoplasmic part of syndecan-4 only, analyzed by luciferase activity (n = 3) (C). GAPDH, histone H4, EGFR and actin were used as loading controls. Values are mean ± s.e.m.
doi:10.1371/journal.pone.0028302.g003

Calcineurin associates with syndecan-4, and the association is regulated by dephosphorylation of serine 179 in syndecan-4 during pressure overload

To examine whether calcineurin associates with syndecan-4, pull-down experiments using biotinylated peptides spanning the cytoplasmic part of syndecan-4 were performed. Recombinant His-trigger factor (TF)-calcineurin subunit A (CnA) was pulled down with syndecan-4 and to a lesser extent with syndecan-2 (Fig. 4A, alignment of syndecan-2 and 4 shown in Fig. 4B). Then, syndecan-4 and CnA were co-transfected into HEK-293 cells and immunoprecipitated with syndecan-4 (Fig. 4C; epitope mapping of syndecan-4 antibodies shown in Fig. S3) and CnA (Fig. 4D) antibodies.

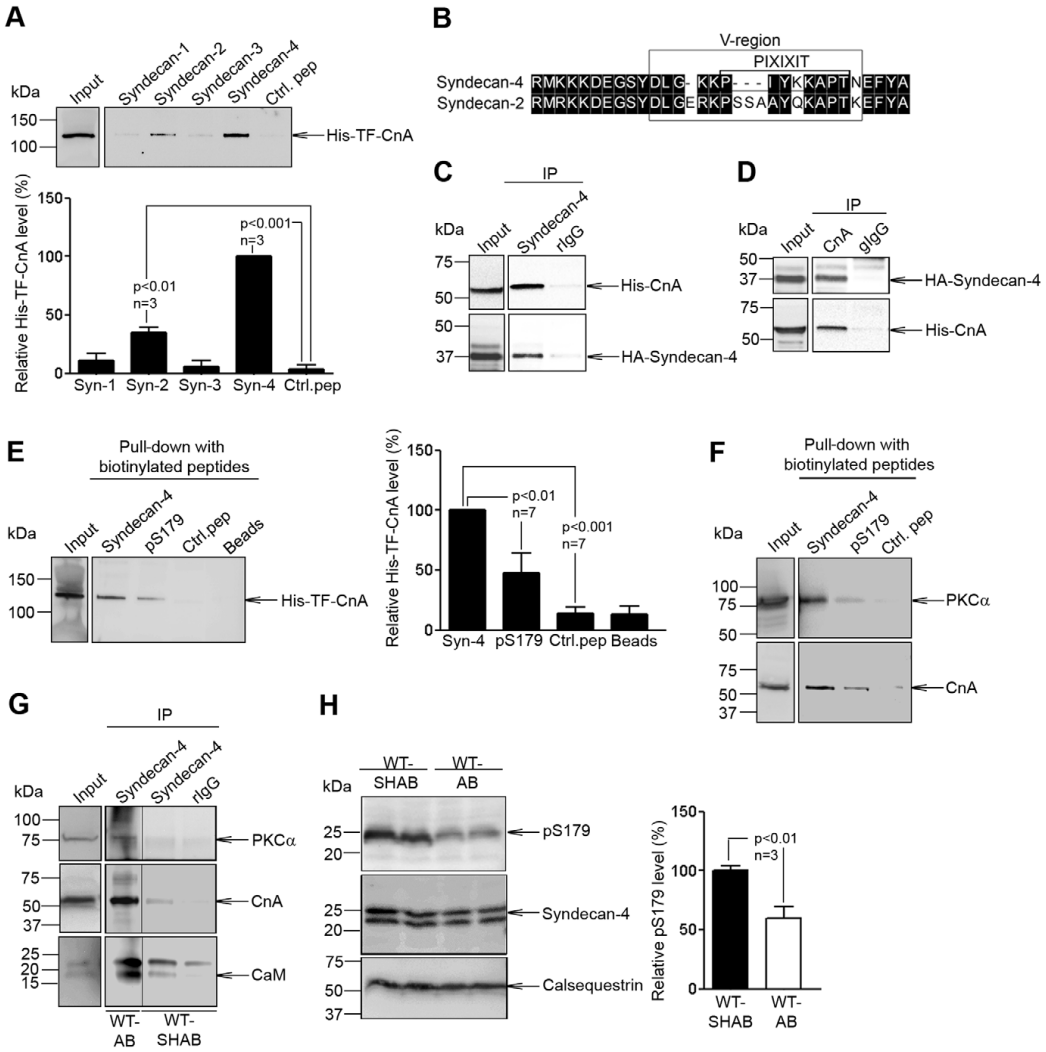

Figure 4. Association of syndecan-4 with calcineurin A (CnA). (A) Pull-down experiments with biotinylated peptides covering cytoplasmic domains of syndecan-1–4 were performed using recombinant His-trigger factor (TF)-CnA, and precipitates immunoblotted for presence of His-TF-CnA (n = 3). (B) Alignment of syndecan-4 and syndecan-2. Black boxes indicate similar amino acids within consensus region. Lysates from HEK-293 cells co-transfected with syndecan-4 and CnA subjected to immunoprecipitation with (C) anti-syndecan-4 (n = 3) or (D) anti-CnA (n = 3) were immunoblotted with anti-CnA or anti-syndecan-4, respectively. Pull-down experiments with biotinylated peptides covering non-phosphorylated or phosphorylated cytoplasmic part of syndecan-4 were performed using recombinant His-TF-CnA (E) or left ventricular (LV) tissue before immunoblotted for presence of endogenous CnA (n = 3) and PKC-α (n = 2) (F). (G) LV tissue extracts from WT-SHAB and WT-AB were immunoprecipitated with anti-syndecan-4 or control IgG and lysates and immunoprecipitates immunoblotted for presence of PKC-α, CnA and calmodulin (CaM, n = 2). (H) Relative pS179-syndecan-4/total syndecan-4 levels in aorta-banded (AB) versus sham-operated (SHAB) WT LV tissue (n = 3). Calsequestrin was used for loading control. Values are mean ± s.e.m.
doi:10.1371/journal.pone.0028302.g004

Immunoblotting revealed the presence of co-immunoprecipitated CnA (Fig. 4C, upper panel) and syndecan-4 (Fig. 4D, upper panel).

Phosphorylation of serine 179 (pS179) in the intracellular domain of syndecan-4 has been shown to regulate protein interactions, such as PKCα association [24]. Pull-down experiments using biotinylated peptides spanning either the non-phosphorylated or phosphorylated cytoplasmic part of syndecan-4 showed that less recombinant His-TF-CnA bound to pS179-syndecan-4 than to non-phosphorylated

syndecan-4 (Fig. 4E). Furthermore, endogenous CnA in LV tissue was pulled down with the non-phosphorylated form, but only minimally with pS179-syndecan-4 (Fig. 4F). As expected, less PKCα bound to pS179-syndecan-4.

In immunoprecipitation experiments using LV tissue, more co-precipitation of CnA with syndecan-4 was observed after AB compared to sham (Fig. 4G). Furthermore, co-immunoprecipitation of the CnA co-activator calmodulin and PKCα were also increased after AB (Fig. 4G). Since pS179 appeared to inhibit syndecan-4-CnA association, pS179-syndecan-4 levels were analyzed following AB using a specific pS179-syndecan-4 antibody. pS179-syndecan-4 levels were found to be reduced by 40% in WT-AB versus WT-SHAB, favoring CnA-syndecan-4 association following AB (Fig. 4H). In the murine heart, syndecan-4 was identified as separate bands at ~20–24 kDa consistent with reported sizes of syndecan-4 protein [25].

Consistent with phosphorylation of syndecan-4 being a regulator of CnA association, expression of S179E-syndecan-4 (mimicking constitutive S179 phosphorylation) increased pNFAT levels compared to WT syndecan-4 and was similar to non-transfected cells (Fig. 5A). Conversely, expression of S179A-syndecan-4 (mimicking no S179 phosphorylation) reduced pNFATc4 levels compared to non-transfected cells and WT syndecan-4 expression (Fig. 5A), indicating that membrane-bound syndecan-4 is an activator of CnA.

Finally, co-transfection of syndecan-4 and CnA into HEK-293 cells resulted in reduced pS179 levels compared to syndecan-4 transfection alone, indicating that calcineurin regulates its own binding and activation by dephosphorylation of syndecan-4 (Fig. 5B).

Intracellular V- and C2-regions in syndecan-4 bind to the autoinhibitory domain of calcineurin

The cytoplasmic region of syndecan-4 consists of three regions; two that are conserved between the syndecans, C1 and C2, and the isoform-specific V-region (Fig. 6A). Alignment of syndecan-4 with other CnA-binding proteins; NFAT [26], AKAP79 [27] and calcineurin inhibitor (cain) [28], revealed a putative CnA-binding motif (PIxIxIT) in the V-region (Fig. 6B). The cytoplasmic region of syndecan-4 was synthesized as overlapping 20-mer peptides on a membrane overlayed with active CnA or endogenous calcineurin in tissue lysate followed by immunoblotting. Strongest CnA-binding was observed in the amino acid sequence SYDLGKK**PIYKKAPT**NEFYA, which contains the PIxIxIT–similar motif and the C2-region (Fig. 6C, amino acids 179–198, underlined).

To identify the syndecan-4 binding region, full-length CnA protein was synthesized as 20-mer peptides and overlayed with a biotin-syndecan-4 (171–198) peptide followed by anti-biotin-peroxidase conjugated immunoblotting. Syndecan-4 binding was located to the autoinhibitory domain in CnA (Fig. 6D, middle panel and Fig. 6E). Consistent with our other data, a biotin-phospho-peptide (pS179-syndecan-4) bound only minimally to CnA (Fig. 6D, right panel).

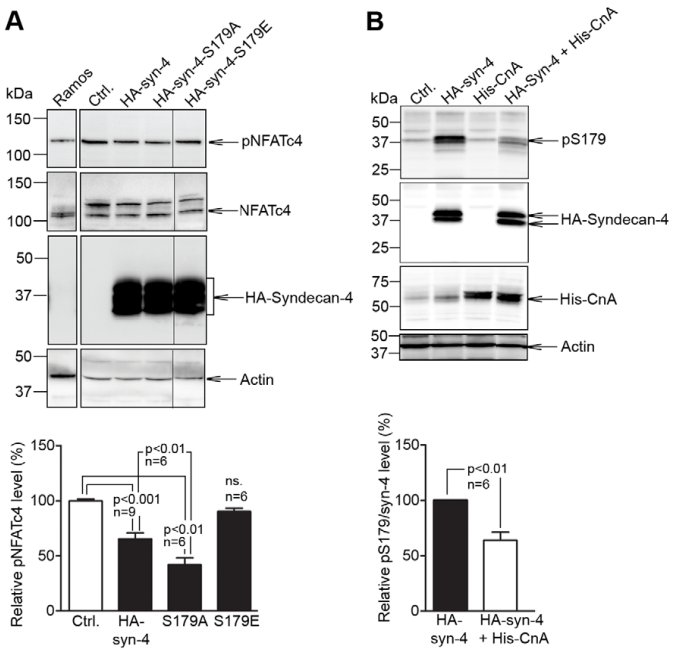

Figure 5. Level of phosphorylated syndecan-4 regulates NFAT activation and is decreased by calcineurin. (A) Phosphorylated NFATc4 (pNFATc4), NFATc4 and syndecan-4 levels in HEK-293 cells transfected with HA-syn-4, HA-syn-4-S179A (mimicking no S179 phosphorylation) or HA-syn-4-S179E (mimicking constitutive S179 phosphorylation) (n = 6–9) and (B) pS179-syndecan-4, syndecan-4 and calcineurin A (CnA) levels in HEK-293 cells co-transfected with CnA and HA-syn-4 or HA-syn-4 alone (n = 6) analyzed by immunoblotting. Actin was used a loading control. Values are mean ± s.e.m.

doi:10.1371/journal.pone.0028302.g005

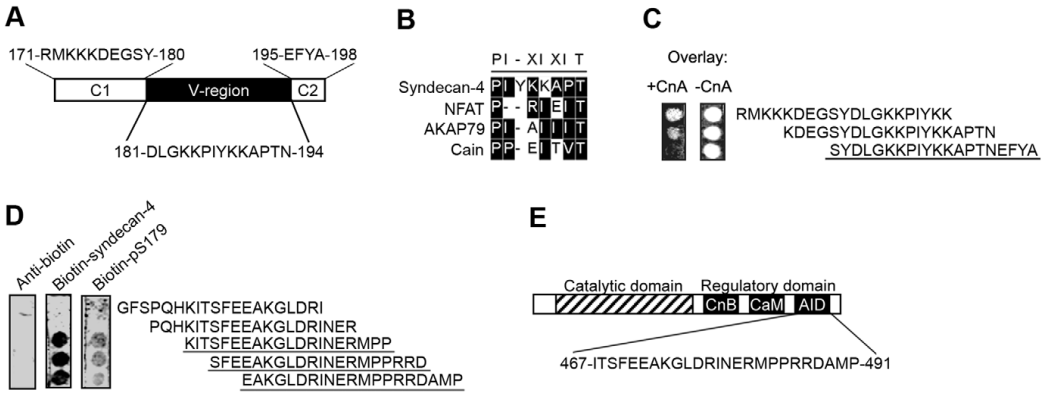

Figure 6. V- and C2-region in syndecan-4 bind to the autoinhibitory domain in calcineurin A (CnA). (A) Schematic illustration of the three cytoplasmic regions in syndecan-4. (B) Alignment of syndecan-4 with other calcineurin binding motifs. Identification of PIxIxIT-similar motif (×represents any amino acid) in V-region. Black boxes indicate similar amino acids within the consensus region. (C) Syndecan-4 residues important for CnA binding were identified by overlaying an array of immobilized syndecan-4 20-mer peptides with 4 amino acids offset with recombinant CnA (left panel). (D) CnA residues important for syndecan-4 binding were identified by overlaying an array of immobilized CnA peptides with 3 amino acids offset with a biotinylated peptide covering the cytoplasmic region of syndecan-4 (middle panel) or the phosphoform (pS179, right panel). Immunoblots without pre-incubation with CnA (C, right panel) or biotinylated peptide (D, left panel) were used as negative controls. Underlined amino acids indicate those relevant for CnA-syndecan-4 binding. Overlay experiments were performed three times with similar results. (E) Schematic illustration of various interaction domains in CnA. Autoinhibitory domain is defined to amino acids 467–491 [53].
doi:10.1371/journal.pone.0028302.g006

Increased myocardial syndecan-4 levels and reduced serine 179 phosphorylation in patients with aortic stenosis

In order to demonstrate a direct relevance to human heart disease, we examined levels of syndecan-4 and syndecan-4 phosphorylation in biopsies taken peroperatively from patients with hypertrophic myocardium due to aortic stenosis and controls. We found a 92 and 72% increase in syndecan-4 mRNA and protein levels, respectively, in patients with aortic stenosis, with a corresponding 21% reduction in pS179-syndecan-4/total syndecan-4 ratio and a 197% increase in RCAN1-4 mRNA (Fig. 7 A–C), indicating a state that favors calcineurin-NFAT activation in the hypertrophic human heart.

Discussion

Our main findings are that syndecan-4$^{-/-}$ mice did not develop concentric hypertrophy in response to chronic pressure overload, and that lack of syndecan-4 specifically inhibited the calcineurin-NFAT pathway *in vivo* and *in vitro*. In particular, NFAT activation was almost totally inhibited following stretch of syndecan-4$^{-/-}$ cardiomyocytes, whereas it was substantially increased in wild-type cardiomyocytes in response to stretch. Syndecan-4 was shown to bind to the autoinhibitory domain in calcineurin, and this binding was enhanced by dephosphorylation of S179 in syndecan-4 (depicted in Fig. 8). Finally, dephosphorylation of syndecan-4 occurred during pressure overload both in mice and man.

Activation of the calcium- and calmodulin-dependent phosphatase calcineurin is necessary and sufficient to induce pathological cardiac hypertrophy [13,29–32]. Calcineurin dephosphorylates the transcription factors belonging to the NFAT family, leading to their activation [13]. Most studies inhibiting calcineurin activity have produced a significant attenuation of pathological hypertrophy [32], which is in accordance with our *in vivo* findings including lack of concentric hypertrophy during pressure overload in syndecan-4$^{-/-}$ mice.

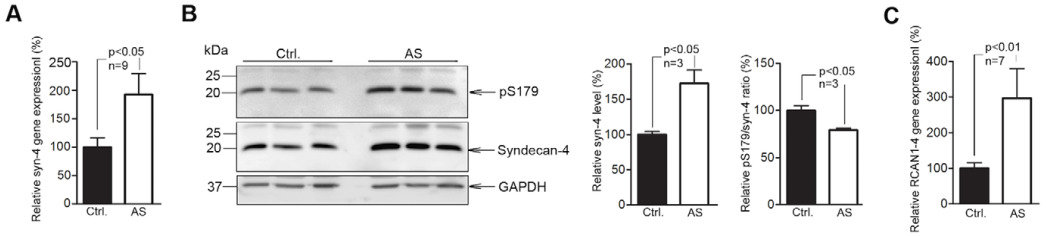

Figure 7. Increased myocardial syndecan-4 levels and reduced serine 179 phosphorylation (pS179) in patients with aortic stenosis. (A) Real-time PCR analysis of syndecan-4 (n = 9), (B) immunoblots of pS179-syndecan-4 (top panel) and syndecan-4 (bottom panel) levels with relative syndecan-4 and pS179-syndecan-4/syndecan-4 ratios (n = 3) and real-time PCR analysis of regulator of calcineurin 1–4 (RCAN1-4) (n = 7) (C) in myocardial biopsies from patients with severe aortic stenosis (AS) versus control patients. GAPDH was used for loading control. Values are mean ± s.e.m.
doi:10.1371/journal.pone.0028302.g007

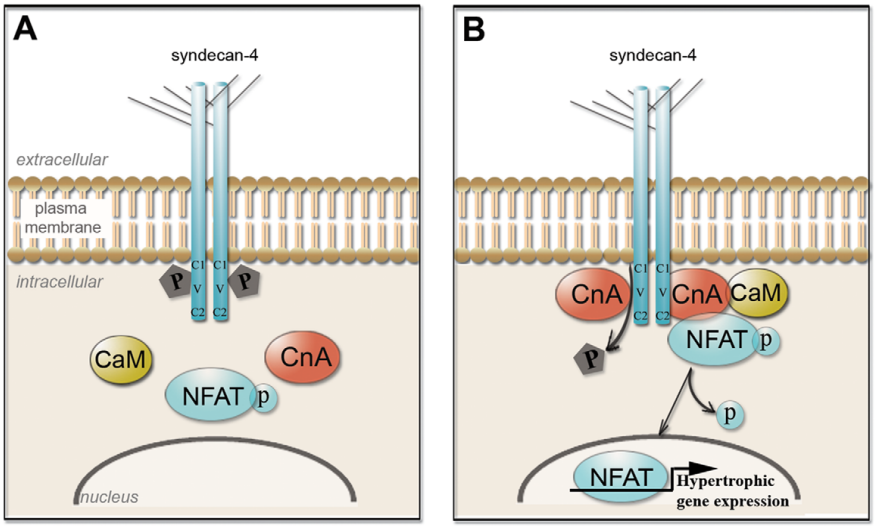

Figure 8. Schematic illustration of proposed syndecan-4-CnA interaction. Phosphorylation of S179 in syndecan-4 renders the CnA-NFAT pathway inactive (left box). Dephosphorylation of syndecan-4 induces binding of CnA and calmodulin (CaM) to syndecan-4 and subsequent activation of the NFAT transcription factor (right box).
doi:10.1371/journal.pone.0028302.g008

Signaling molecules involved in the myocardial hypertrophic response to increased mechanical stress, and regulators of the calcineurin-NFAT pathway are only partially known. It has been suggested that mechanical signals in cardiomyocytes converge on membrane proteins such as integrins [33,34]. In addition, studies indicate a role for mechanotransducer complexes involving the Z-discs and related proteins [35,36]. As syndecan-4 connects the extracellular matrix proteins to the cytoskeleton, is a co-receptor with $\alpha5\beta1$ integrin [37] and is localized to costameres and Z-discs [3], we hypothesized that syndecan-4 senses mechanical stress and activates pro-hypertrophic signaling responses such as the calcineurin-NFAT pathway. A role for syndecan-4 as a mechanotransducer has previously been suggested by us [1] and others [38]. We demonstrate that following stretch, NFAT activation was nearly abolished in cardiomyocytes lacking syndecan-4. We also show that lack of syndecan-4 reduced NFAT activation and expression of the NFAT target gene RCAN1-4 during increased mechanical stress induced by left ventricular pressure overload. These data clearly indicate that syndecan-4 is a regulator of NFAT activity in cardiomyocytes in response to increased mechanical stress.

We then studied the mechanism for activation of calcineurin-NFAT signaling by syndecan-4, and showed that CnA binds directly to the intracellular V- and C2-regions in syndecan-4. The V-region confers binding specificity for each syndecan family member and the syndecan-4 V-region contains a PIxIxIT-similar motif. A loosely conserved PIxIxIT motif has been suggested to represent the CnA binding site in AKAP79 [27], NFATs [26], cain [28,39] and RCAN1-4 [16,40]. The syndecan-4 binding region was located to the autoinhibitory domain of CnA. This domain is thought to block the active site within the catalytic region of CnA, rendering the phosphatase inactive [41–43]. Increased syndecan-4-calmodulin co-precipitation was observed during pressure overload. Calmodulin has been found to increase calcineurin activity more than 20-fold. Interestingly, the PDZ domain of CASK, which is known to bind calmodulin, is also able to bind to the C2 region of syndecans [44]. Thus, a direct interaction between CnA and syndecan-4 might facilitate activation of calcineurin by bringing calmodulin and CnA in close proximity.

We also demonstrated that CnA-syndecan-4 interaction is regulated by phosphorylation of S179 in syndecan-4. During cardiac pressure overload, we found a decrease in phosphorylated S179-syndecan-4 and an increased association between syndecan-4 and calcineurin, suggesting that dephosphorylation of pS179-syndecan-4 causes increased binding of calcineurin. More directly, we showed that expression of a construct mimicking absent S179 phosphorylation (S179A) increased NFAT activity. These results indicate that alterations in syndecan-4 phosphorylation level may have functional significance for development of cardiac hypertrophy. Moreover, the significantly reduced myocardial phosphorylation of S179-syndecan-4/total syndecan-4 ratios combined with increased NFAT activation found in patients with aortic stenosis indicates a direct relevance for this mechanism in human heart disease. Interestingly, a reduction in phosphorylation of S179 in syndecan-4 was induced by overexpression of CnA, suggesting that calcineurin dephosphorylates syndecan-4 *in vivo* and thereby regulates its own activity.

Concentric hypertrophy of the myocardium during pressure overload is considered to be a compensatory response of the heart to deal with increased afterload during such conditions as aortic stenosis and hypertension. Lack of compensatory hypertrophy may thus cause myocardial dilatation and possibly dysfunction. Accordingly, Meguro et al. [45] showed that inhibition of calcineurin-NFAT activation with cyclosporine attenuated pressure overload hypertrophy and enhanced the susceptibility to heart failure. Similarly, we found reduced cardiac function and attenuated concentric hypertrophy in syndecan-$4^{-/-}$-AB. Although our data clearly indicate syndecan-4-mediated, pro-

hypertrophic NFAT activation in cardiomyocytes, syndecan-4 may also mediate other effects in the pressure-overloaded heart. In a recent study, syndecan-$4^{-/-}$ mice were shown to have impaired cardiac function and increased mortality following myocardial infarction [46], while in another study, cardiac function was improved and NFAT activation increased in syndecan-$4^{-/-}$ mice after infarction [47], suggesting additional layers of complexity in syndecan-4-dependent NFAT regulation and remodeling of the heart.

In summary, our data show that syndecan-4 is essential for development of compensatory myocardial hypertrophy in the pressure overloaded myocardium. Specifically, syndecan-4 regulates stretch-induced activation of the pro-hypertrophic calcineurin-NFAT signaling pathway in cardiomyocytes. Syndecan-4 was found to bind to the autoinhibitory domain of calcineurin and this binding was regulated by phosphorylation of syndecan-4. Patients with aortic stenosis exhibited decreased pS179-syndecan-4/syndecan-4 ratios, indicating a direct relevance to human myocardial hypertrophy. In conclusion, our data indicate that syndecan-4 is a scaffolding protein for activation of calcineurin-NFAT signaling and development of concentric myocardial hypertrophy during pressure overload, both in mice and man.

Methods

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996) and was approved by the Norwegian National Animal Research Committee (permit of approval number STFDU1736). The human myocardial biopsy protocol was approved by the Regional Committee for Research.

Ethics in Eastern Norway (permit of approval number 1.2005.316) and conforms to the Declaration of Helsinki.

Animal model and transthoracic echocardiography

Banding of the ascending aorta was carried out in 7 week old WT (C57BL/6, Møllergaard, Denmark) and syndecan-$4^{-/-}$ mice [48]. Anaesthesia was induced with >5% isoflurane gas and <95% O_2 in a gas chamber, followed by tracheostomy and ventilation with 2% isoflurane and 98% O_2, with a tidal volume of 350 µl and a respiratory frequency of 160 min^{-1} on a MiniVent ventilator (Harvard Apparatus, Holliston, MA 01746, USA). A sternal split of the cranial 1/3 of the sternum was performed and an 8-0 silk ligature was tied around the ascending aorta and a 26G blunted needle, which was subsequently removed. Sham-operated animals underwent the same procedure without banding of the aorta. After twenty-one days echocardiography was carried out using a VEVO 2100 (Visualsonics, Toronto, Canada) before the animals were sacrificed. Echocardiographic examinations were performed under standardized conditions with the animals in the supine position, spontaneously breathing 1.5% isoflurane and 98.5% O_2 on a mask. Echocardiographic data were analyzed off-line using VEVO 2100 1.1.0 software from Visualsonics. Three representative cycles were analyzed and averaged. Two-dimensional (2D) images of the LV were obtained both in long and short axes. Short axis recordings were obtained at the level of the papillary muscle. M-mode tracings were recorded at the level of both the papillary muscles and the aortic valves, with 2D guidance. LV wall thickness and cavity dimensions were measured through the largest diameter of the ventricle both in systole and diastole. LV fractional shortening (LVFS) in percent, was calculated using the following formula.

$$LVFS = (LVDd - LVDs)/LVDd \times 100$$

where LVDd is LV diameter in diastole, and LVDs is LV diameter in systole. Doppler recordings were obtained in the left parasternal long axis position. Pulsed wave Doppler was used for measuring flow velocities across the constriction, as well as in the left ventricular outflow tract (LVOT) and in the mitral annulus. Cardiac output (CO) was calculated in LVOT using the following equation

$$CO = LVOT\ VTI \times \pi \times (\text{diameter}/2)^2 \times HR$$

where VTI is the velocity time integral, HR is heart rate and diameter is measured in LVOT.

The LV, right ventricular free wall and lungs were weighed and normalized to tibia length. Only mice with a maximum flow velocity across the stricture >3 m/s and <4 m/s 24 hours after AB and >4 m/s and <6 m/s three weeks after AB were included (mean values, 4.3±0.1 m/s in syndecan-$4^{-/-}$ mice versus 4.3±0.1 m/s in WT).

LV pressure measurements

Immediately after finishing echocardiography, a 1.4-Fr microtip pressure transducer catheter (SPR-671, Millar Instruments, Houston, TX) was introduced into the left ventricle through the right carotid artery for measurements of LV pressures and calculations of its maximal positive and negative first derivatives. Data recorded from 10–15 consecutive beats were analyzed. LV pressure measurements were performed in un-operated WT and syndecan-$4^{-/-}$ mice, and not following AB as the aortic constriction was too tight to allow for retrograde insertion of the catheter.

Estimation of LV fluid content

Three weeks after AB or SHAB LV from WT and syndecan-$4^{-/-}$ mice were weighed immediately upon dissection, cut into pieces and dried for 24 h at 90°C, before weighing again.

Angiotensin II concentration in plasma

Plasma was extracted from full-blood containing 0.44 mM o-phenanthroline, 25 mM EDTA, 1 mM p-hydroxy-mercuribenzoic acid and 0.12 mM pepstatin A and the concentration of angiotensin II was measured using the Max Human Angiotensin 2 ELISA Kit according to the manufacturers protocol (EA3501-1, Assaypro, St. Charles, MO) in WT and syndecan-$4^{-/-}$ mice three weeks after AB or SHAB.

Human myocardial biopsies

LV apical myocardial biopsies (10 mg) were obtained immediately before crossclamping the aorta in twelve patients with severe aortic stenosis and twelve control patients with coronary artery disease during elective aortic valve replacement and coronary artery bypass operations, respectively. Biopsies were taken from a normal appearing and contracting area using a 22G needle (Easy Core Biopsy System, Boston Scientific International S.A., Cedex, France). Patients 40–85 years old with symptomatic aortic stenosis, a mean aortic gradient >50 mmHg or an aortic valve area <0.7 cm^2, and without significant coronary artery disease were included in the aortic stenosis group. Patients 40–85 years old with stable angina pectoris, no known previous myocardial infarction, ejection fraction >50%, and without significant valve disease were included in the control group. The patients had no chest pain or

electrocardiographic signs suggesting ongoing ischemia preoperatively. Informed written consent was obtained from each patient.

NFAT-luciferase mice

The NFAT-luciferase mice have nine copies of an NFAT-binding site from the interleukin (IL)-4 promoter (5′-TGGAAAATT-3′) inserted upstream of the luciferase reporter gene, driven by the α-myosin heavy chain promoter [31], and were a kind gift from Jeffery D. Molkentin (Cincinnati Children's Hospital Medical Center, University of Cincinnati, Cincinnati, OH). Neonatal mice from the progeny were used for isolation of cardiomyocytes.

Syndecan-4$^{-/-}$-NFAT luciferase mice

Syndecan-4$^{-/-}$-NFAT luciferase reporter mice were generated by crossing syndecan-4$^{-/-}$ mice with the NFAT-luciferase mice. Neonatal mice from the progeny were used for isolation of cardiomyocytes.

Quantification of luciferase activity

For measurements of luciferase activity, cardiomyocytes were harvested according to the Luciferase Assay System protocol (E1500, Promega) and luminescence from sample duplicates was quantified on a Victor 3 1420 Multilabel Counter (PerkinElmer, Waltham, MA).

Adult cardiomyocyte isolation and cell size measurements

Cardiomyocytes were enzymatically isolated from WT and syndecan-4$^{-/-}$ mice three weeks after AB or SHAB (n = 3 in each group) by cannulating the aorta and retrogradely perfusing with a Ca^{2+}-free Tyrode's solution containing 0.05 mg/ml collagenase (Yakult, Tokyo, Japan) for 10 min. The left ventricle was then removed and minced, cardiomyocytes precipitated, and extracellular [Ca^{2+}] was gradually increased. Cells were stored at room temperature until use. Cell length and width measurements were determined in a genotype-blinded manner from 2-dimensional images of randomly selected cells.

Electron microscopy

Two hearts from WT and syndecan-4$^{-/-}$ mice, respectively, obtained three weeks after AB or SHAB were perfused with 2.0% glutaraldehyde buffered in 0.2 M cacodylate at pH 7.4 for 15 min. LV blocks (about 1 mm^3) were fixed in 2% glutaraldehyde in cacodylate buffer for 2 h, rinsed overnight and transferred to a 1% OsO_4 solution for 30 min on ice. After rinsing in cacodylate, the specimens were dehydrated in graded ethanols and embedded in Epon. Ultrathin sections (60–100 nm) were stained with uranyl acetate and lead citrate and examined and photographed in a Tecnai G2 spirit BioTWIN 120 kV, LaB6, Transmission Electron Microscope with 4 k Eagle camera (FEI Company, Eindhoven, The Netherlands). All images were examined by two investigators, blinded for mouse identity.

Histology

Hearts fixed in formalin were processed into paraffin wax. Six 4 μm thick slices were cut along the short axis from the mid-ventricular level of each tissue sample. Slices were stained with hematoxylin-eosin for morphology.

Immunohistochemistry

The excised hearts were placed in ice-cold phosphate-buffered saline (PBS, pH 7.4), quickly embedded in Tissue-Tek^{TM} O.C.T. compound (Sakura Finetek, Torrance, CA, USA) and frozen in dry ice-cooled isopropanol before storing at −70°C. Five μm thick sections were cut using the Cryostat 1720 from Leitz, Wetzlar, Germany and collected on SuperFrost®Plus slides (Menzel-Gläser, Braunschweig, Germany). Sections were fixed for 5 minutes in acetone at −20°C and air dried. After rinsing in Tris-buffered saline (TBS, pH 7.6) containing 0.05% Tween 20, sections were incubated with the DAKO Peroxidase Blocking Reagent (DAKO, Glostrup, Denmark) for 15 minutes at 37°C followed by TBS rinsing. Sections were then incubated for 30 minutes at 37°C with affinity-purified antibodies (rabbit) against collagen type I (1:100 dilution) (Rockland, Gilbertsville, PA). After three washes in TBS the primary antibody was labelled for 30 minutes using the DAKO EnVision^{TM} +, Peroxidase, Rabbit K 4010 system and 3,3′-diamino-benzidine tetrahydrochloride as the chromogen. Sections were counterstained with hematoxylin for 1–2 minutes, mounted and examined under a Leitz Aristoplan microscope.

RNA isolation and real time quantitative PCR

Total RNA was isolated from the LV of mice and LV biopsies from patients using RNeasy mini kit (Cat# 74106, Qiagen Nordic, Norway). RNA quality was determined using 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). RNA integrity number (RIN) >7.5 was required for further analysis. Reverse transcription reactions were performed with iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA). Pre-designed TaqMan assays (Applied Biosystems, Foster City, CA) were used to determine gene expression of α-skeletal actin (Mm00808218_g1), ANP (Mm01255748_g1), BNP (Mm00435304_g1), RCAN1-4 (Mm01213406_m1) and GAPDH (Mm03302249_g1) in WT and syndecan-4$^{-/-}$ mice three weeks after AB or SHAB, and expression of syndecan-4 and RCAN1-4 (Hs01120954_m1) in human myocardial biopsies (Hs01120909_m1). The results were detected on an ABI PRISM 7900 Sequence Detection System (Applied Biosystems).

Tissue extracts

LV tissue extract A. Frozen murine LV tissue was pulverized in a mortar with liquid nitrogen before transfer to lysis buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% Triton) with protease (1 mM PMSF and Complete EDTA-free tablets; Roche Diagnostics) and phosphatase inhibitors (1 mM Na_3VO_4, 10 mM NaPP and 50 mM NaF) in a glass tube. The samples were homogenized with a Polytron 1200 and centrifuged at 70 000× g for 60 min at 4°C. Supernatants were collected and stored at −70°C. **LV tissue extract B.** Frozen murine LV tissue or human myocardial LV biopsies were homogenized using a Polytron 1200 in a PBS buffer containing 1% Triton X-100, 0.1% Tween20 and protease (Complete EDTA-free tablets) and phosphatase inhibitors (10 mM NaF and 1 mM Na_3VO_4), incubated on ice for 30 min and centrifuged at 14 000–20 000 g for 10 min. The resulting supernatants collected and stored at −70°C. Protein concentrations were determined by Micro BCA Protein Assay Kit (Pierce, Rockford, IL).

Immunoprecipitation

LV extracts (500 μg tissue extract A) from WT mice 24 h after SHAB or AB were incubated with antibodies (2 μg) and 30 μl protein A/G agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA, sc-2003) overnight at 4°C. Immunocomplexes were washed once for 10 min in immunoprecipitation (IP)-buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton), four times 10 min in IP-buffer with high salt (1 M) and finally once for 10 min in IP-buffer, boiled in SDS loading buffer and analyzed by immunoblotting. Rabbit IgG (sc-2027) and goat

IgG (sc-2028) were used as negative controls. Immunocomplexes from HEK-293 extracts (in presence of 1.5 mM $CaCl_2$) were washed three times in lysis buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% Triton). Immunoprecipitates were analyzed by immunoblotting.

Isolation of neonatal mouse cardiomyocytes

Solutions used; Trypsin solution: 1.11 mg Trypsin powder (Sigma, St.Louis, MO) mixed with 30 ml cold Hanks Balanced Salt Solution (HBSS, Gibco-BRL, Gaithersburg, MD). Collagenase solution: 23.25 mg collagenase Type-2 powder (Worthington Biochemical, Lakewood, NJ) mixed with 100 ml HBSS. Light-medium: 375 ml DMEM (Gibco-BRL), 125 ml M-199 (Gibco-BRL), 5 ml 100× penicillin/streptomycin/glutamine solution (Sigma) and 5 ml 1 M HEPES (Gibco-BRL). Dark medium: Light medium mixed with 25 ml horse serum (Gibco-BRL) and 12.5 ml fetal bovine serum (Gibco-BRL). All solutions were filter sterilized.

The procedure is based on a protocol by Masahiko Hoshijima, University of California, San Diego, CA. In brief, 40–60 neonatal pups were quickly decapitated and their hearts removed and placed in cold HBSS. The LV cut into pieces were digested for 1×6 min and 7×10 min in 18 ml of collagenase solution. Each resulting supernatant was transferred to a 50 ml conical tube with cold dark medium on ice, containing serum to inactivate the collagenase. The pooled supernatants were centrifuged at 124× g for 6 min and the pellet resuspended in warm dark medium. The cells were plated in 150 cm^2 flasks and incubated at 37°C for 2×75 min for differential plating. The resulting suspension with unattached cardiomyocytes was spun at 124×g for 10 min to pellet cells. After resuspension of pellet in warm dark medium, the cells were counted and plated in 6-well dishes or Omni Trays (Omni Tray Cell Culture Treated w/Lid, Nalge Nunc International, Rochester, NY) coated with 0.2% gelatin (Sigma) added 0.1% fibronectin (Sigma) at a density of 2.5×10^5 cells/ml, and incubated at 37°C.

Treating cardiomyocytes with peptides

Isolated neonatal cardiomyocytes were cultured for two days in 6-well plates at a density of 0.6×10^6 cells/ml. Cardiomyocytes were treated with the arginine-coupled (50 μM) peptide (Genscript Corp.) for 90 min and washed twice with RNase-free PBS before being harvested in IP-buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) with protease inhibitors (Complete Mini EDTA-free tablets, Roche) or for quantification of luciferase activity as described above. Harvested protein was analyzed by immunoblotting. Peptide-treatment for longer time-periods have been shown to result in considerable degradation of the peptides [49].

Cyclic mechanical stretch of neonatal cardiomyocytes

Isolated neonatal cardiomyocytes were plated on collagen I coated silicone membranes at a density of 2.5×10^5 cells/ml. Cyclic mechanical stretch was induced by stretching cells for 10 min–24 hours (10%, 1 Hz) using the FlexCell Tension System FX-4000 (Dunn Labortechnik, Germany). Cyclosporin A (CsA) (SandImmun Neoral, 500 ng/ml; 586107, Novartis, Switzerland) was used as a calcineurin inhibitor with vehicle serving as control (Cremophore, C5135, Sigma).

Quantification of 3^H-leucine incorporation

Isolated cardiomyocytes were left in culture for two-five days in order to induce autonomous hypertrophy [18]. Five μCi/ml 3^H-leucine (American Radiolabel Chemicals, MO) was added to the medium at day two in culture. The cells were washed 6 times in 95% EtOH before harvested in 0.2 mol/l NaOH at day five in culture. Serum-stimulated cells served as positive control. 3^H-leucine incorporation was quantified by measuring counts per minute (CPM) in duplicates from each sample at the Wallac Winspectral 1414 Liquid Scintillation Counter (PerkinElmer, MA). Samples were diluted in Pico-Fluor 40 (PerkinElmer).

Transfection of HEK-293 cells

HEK-293 cells were cultured in DMEM supplemented with 10% fetal calf serum, 100 U/ml penicillin, 1% non-essential amino acids, and maintained in a 37°C, 5% CO_2 humidified incubator. The HEK-293 cells were transfected with HA-tagged syndecan-4 full length, syndecan-4 S179A, syndecan-4 S179E, His-tagged CnAα cloned into pcDNA 3.1/myc-HIS-A (custom made, Genscript Corporation, Piscataway, NJ) using Lipofectamine 2000 as instructed by the manufacturer (Invitrogen, Carlsbad, CA). After 24 h total lysate (IP-buffer or lysis buffer), or cytoplasmic, nuclear and membrane protein fractions were harvested according to the protocol of the Compartmental Protein Extraction Kit (2145, Chemicon/Millipore, Billerica, MA), and analyzed by immunoblotting.

Pull-down experiments

Biotinylated peptides (5 μM) were incubated in total heart extract (whole tissue lysate B) from WT mice overnight at 4°C. Monoclonal anti-biotin-conjugated beads were added for 60 min before the bead pellet was washed once for 10 min in IP-buffer, four times 10 min in IP-buffer with high salt (1 M) and finally once for 10 min in IP-buffer. To analyse for a direct interaction, biotinylated peptides (10 μM) were incubated with 10 μg/ml recombinant His-TF-CnA (custom made from Genscript Corporation, Piscataway, NJ) in a 100 μl binding buffer (10 mM NaCl, 20 mM Tris/HCl, pH 8, 6 mM $MgCl_2$, 0,1% Triton X-100) containing 1% BSA with gentle rocking for 3 h at 4°C. Twenty five μl monoclonal anti-biotin-agarose (A-1559, Sigma, Saint Louis, MO) was added and incubated with gentle rocking overnight at 4°C. After collection by centrifugation, the beads were washed three times with 1 ml binding buffer. Bound proteins were extracted and boiled with 50 μl 2× SDS sample buffer for five min before analysis by immunoblotting.

Overlays

CnA overlays were conducted using active recombinant protein expressed in *E. coli* (# 14-446, Upstate, Lake Placid, NY), native CnA purified from bovine (# 14-390, Upstate) or endogenous CnA in heart extract. Syndecan-4 overlays were conducted using a biotin-syndecan-4 (171–198) peptide. Peptide arrays consisting of either the cytoplasmic part of syndecan-4 or the whole of CnA synthesized as partly overlapping 20-mer peptides (Biotechnology Centre, Oslo, Norway) were first blocked in 5% non-fat dry milk or 1% casein in Tris-Buffered Saline Tween 20 (TBST) for 2 h at room temperature. The overlays were performed by incubating overnight at 4°C and then washing the membrane 5 times in cold phosphate-buffered saline with Tween 20 for 5 min at 4°C. These were overlayed with CnA (active recombinant protein (Upstate, Lake Placid, NY), native bovine CnA (Upstate) or endogenous CnA from LV extract) or a biotin-syndecan-4 peptide, respectively. Bound CnA or syndecan-4 was detected by immunoblotting.

Immunoblot analysis

Cell and tissue extracts and immunoprecipitates were analyzed on 6%, 7.5%, 10%, 15% or 4–20% SDS/PAGE and blotted onto

PVDF membranes. The PVDF membranes and peptide arrays were blocked in 5% non-fat dry milk or 1% casein in TBST for 60 min at room temperature, incubated for 1–2 h at room temperature or overnight at 4°C with primary antibodies, washed five times five min in TBST and incubated with a horseradish-peroxidase-conjugated secondary antibody. Blots were developed by using ECL Plus (GE HealthCare, RPN2132). The chemiluminescence signals were detected by Las-1000 or Las-4000 (both Fujifilm, Tokyo, Japan). Actin, GAPDH or Coomassie Brilliant Blue (Bio-Rad, R-250) was used as loading control.

Antibodies

Immunoblotting and immunoprecipitation were carried out using anti-GSK-3β (1:1000 dilution, #9315, 27C10), anti-pGSK-3β (1:1000 dilution, #9336, Ser9), Sapk/JNK (1:500 dilution, #9252), anti-pSAPK/JNK (1:500 dilution, #9251, Thr183/Tyr185), anti-p38 MAPK (1:500 dilution, #9212), anti-pp38 MAPK (1:500 dilution, #9211, Thr180/Tyr182) all from Cell Signaling Technology, Danvers, MA, anti-Δ-Heparan Sulfate (3G10 epitope, 1:1500 dilution, #370260, Seikagaku Corporation, Tokyo, Japan), anti-syndecan-4 (1:200 dilution, sc-15350, used for coimmunoprecipitation as epitope mapping demonstrated recognition of amino acid sequence located to N-terminus of syndecan-4 (Fig. S3A)), anti-pS179-syndecan-4 (1:200 dilution, sc-22252-R), anti-calcineurin A (1:200 dilution, sc-6124) all from Santa Cruz, CA, anti-syndecan-4 (1:2000, #1420, a kind gift from A. Horowitz, Angiogenesis Research Center and Section of Cardiology, Dartmouth Medical School, Lebanon, NH and J. Li, Division of Cardiology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, epitope mapping demonstrated specific recognition of amino acid sequence located to the V-region in syndecan-4 (Fig. S3B)), anti-pS179-syndecan-4-HRP (1:2000, #41144, custom made, Genscript Corporation, Piscataway, NJ), anti-EGFR (1:500, sc-03, Santa Cruz) anti-actin (1:500 dilution, sc-8432, Santa Cruz), anti-calsequestrin (1:2500 dilution, PA1-913, Affinity BioReagents, Golden, CO) anti-PKC-α (1:200 dilution, sc-208, Santa Cruz, CA), anti-CaM (1:200, C7055, Sigma), anti-pNFATc4 (1:200 dilution, sc-32630-R), anti-NFATc4 (1:200 dilution, sc-13036), anti-GAPDH (1:500 dilution, sc-20357), anti-histone H4 (1:500 dilution, sc-8658) all from Santa Cruz, anti-collagen type I (1:750 dilution, #1310-01, Southern Biotech, Birmingham, AL), anti-collagen type III (1:3000 dilution, #600-401-105, Rockland Immunochem., Gilbertsville, PA), anti-collagen type VIII (1:500 dilution, #LC-C46861, LifeSpan Biosciences, Seattle, WA), anti-biotin-HRP (1:2500 dilution, A-0185) and anti-biotin-conjugated beads (A-1559) both from Sigma. Horseradish peroxidase-conjugated anti-mouse (1:2500 dilution, NA931V), donkey anti-rabbit IgG HRP affinity purified polyclonal antibody (1:2500 dilution, NA934V) both from GE HealthCare and anti-goat IgGs (1:2500 dilution, HAF109, R&D Systems, Minneapolis, MI) were used as secondary antibodies.

Peptide synthesis

Peptide arrays were synthesized on cellulose paper using a Multipep automated peptide synthesizer (INTAVIS Bioanalytical Instruments AG, Koeln, Germany) as described [50]. Arginine coupled peptides were synthesized and purified to 95% purity (Genscript Corp, Piscataway, NJ) [51]. Biotinylated peptides were purified to 55–95% purity (Genscript Corporation or Biotechnology Centre, Oslo, Norway).

Syn-4 (mem+cyt)-HA-Arg_{11}: ERTEVLAALIVGGVVGILFAVFLILLLVYRMKKKDEGSYDLGKKPIYKKAPTNEFYAYPYDVPDYA-R_{11}

Arg_9-Syn-4 (cyt): R_9-RMKKKDEGSY DLGKKPIYKKAPTNEFYA

Biotinylated-syndecan-4: Biotin-RMKKKDEGSYDLGKKPIYKKAPTNEFYA (97% purity)

Biotinylated-pS179-syndecan-4: Biotin-RMKKKDEG**pS**YDLGKKPIYKKAPTNEFYA (95% purity)

Biotinylated-scrambled-syndecan-4: Biotin-GTKYPKMDRGKLFKYKAKPEDNESAYIK (70% purity) or Biotin-YYKPYFAGDLKKAKTPSNEI (85% purity)

Biotinylated-syndecan-1: Biotin-RMKKKDEGSYSLEEPKQANGGAYQKPTKQEEFYA (55–65% purity)

Biotinylated-syndecan-2: Biotin-RMRKKDEGSYDLGERKPSSAAYQKAPTKEFYA (85–90% purity)

Biotinylated-syndecan-3: Biotin-RMKKKDEGSYTLEEPKQASVTYQKPDKQEEFYA (60–70% purity)

Densitometric Analysis

Densitometric analysis was performed using Image Gauge 3.46, Science lab 99 (Fujifilm, Tokyo, Japan), Image Quant (Fujifilm), Scion Image (Scion Corporation, Frederick, Maryland) or ImageJ (NIH).

Equipment and settings used for obtaining and processing images

All figure layouts were made using Photoshop CS2 or CS3 (Adobe Systems Inc., San Jose, CA). Cropping of images was carried out, and labeling and molecular size markers were added. When electrophoretic blots were cropped, care was taken to retain important bands, and at least six band widths above and below the band of interest where possible. Occasionally, brightness and contrast was adjusted equally across the entire image, including controls, in Image Gauge or Photoshop CS2 or CS3.

Statistics

Data are expressed as group mean ± s.e.m. and analyzed in Statistica 6.0 or GraphPad Prism 5.0. All data were tested for normality before comparisons between groups were made. All *in vivo* and PCR data were examined using one-way ANOVA with adjustment for multiple comparisons using the method of Hothorn et al. [52]. Western data were analyzed using paired or unpaired two-tailed Students *t*-test, or ANOVA with subsequent Newman-Keuls or Bonferroni post hoc test. Differences were considered significant for $p<0.05$.

Supporting Information

Figure S1 Activation of calcineurin-dependent nuclear factor of activated T-cell (NFAT) signaling in cardiomyocytes in response to cyclic mechanical stretch. (A) Relative NFAT luciferase activity in neonatal cardiomyocytes from NFAT-luciferase reporter mice subjected to cyclic mechanical stretch (MS) (10%, 1 Hz) for 10 min–24 hours (n = 6–19). (B) Relative NFAT luciferase activity in neonatal cardiomyocytes from NFAT-luciferase reporter mice subjected to 24 hrs of cyclic mechanical stretch with and without the calcineurin-inhibitor cyclosporin A (CsA) (n = 8–19).Vehicle (Veh) served as control. Values are mean ± s.e.m.
(PDF)

Figure S2 Activation of nuclear factor of activated T-cell (NFAT)-interacting, pro-hypertrophic signaling pathways in wild type (WT) and syndecan-$4^{-/-}$ (Syn-$4^{-/-}$) mice in response to pressure overload induced by aortic banding (AB). Representative immunoblots and relative quantity of (A) calcineurin (CnA), (B) glycogen synthase kinase (GSK)3-β and phosphorylated GSK3-β

(pGSK3-β), (C–D) Akt and phosphorylated Akt (pAKT), (E–F) c-jun N-terminal kinases (JNK)2/3 and phosphorylated JNK2/3 (pJNK2/3), (G–H) extracellular signal regulated protein kinase (ERK)1/2 and phosphorylated ERK1/2 (p ERK1/2) and (I–J) p38 and phosphorylated p38 (pp38) in left ventricles (LV) from WT and Syn-4$^{-/-}$ mice 24 h after AB or sham operation (SHAB) (n = 5–6). A calcineurin- rich cell lysate (Ramos) was used as control for the calcineurin-positive protein band. Vinculin and GAPDH were used as loading control. Values are mean ± s.e.m.
(PDF)

Figure S3 Epitope mapping of syndecan-4 antibodies. Syndecan-4 residues important for antibody binding were identified by overlaying an array of immobilized syndecan-4 20-mer peptides with (A) anti-syndecan-4, (sc-15350), (B) anti-syndecan-4 (#1420), (C) anti-pS179-syndecan-4 (sc-22252-R) or (D) anti-pS179-syndecan-4-HRP (# 41144). Underlined and bold amino acids indicate the core epitope. (A) Anti-syndecan-4 (sc-15350) recognized the amino acid sequence SIRETEVIDPQDLLEGRYFSGALPDD located to the N-terminus of syndecan-4 consistent with the source information from the producer (Santa Cruz) (n = 1). (B) Anti-syndecan-4 (#1420) strongly and specifically recognized the amino acid sequence KDEGSYDLGKKPIYKKAPTNEFYA located to the cytoplasmic part of syndecan-4. Further mapping revealed that the core epitope was located to the V-region in syndecan-4 (underlined sequence in B) (n = 3). Both pS179-syndecan-4 antibodies (sc-22252-R, (n = 2) and #41144 (n = 3)) recognized pS179-syndecan-4 strongly (right panels in C and D, respectively) compared to the non-phosphorylated syndecan-4 (left panels). Consistent with our findings and according to Genscript, the #41144 antibody is 32 times more specific for pS179-syndecan-4 than non-phosphorylated syndecan-4.
(PDF)

Table S1 Animal characteristics, heart rates, left ventricular pressures and echocardiographic measurements.
(DOC)

Table S2 Myocardial structure and neurohormonal changes.
(DOC)

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Author Contributions

Conceived and designed the experiments: AVF IGL CRC GC. Performed the experiments: AVF IGL CRC IS EKØ ML HOJ AH SD SN TL WEL BS GF TT. Analyzed the data: AVF IGL CRC IS EKØ ML HOJ AH SD SN TL WEL BS GF TT SAW-A PFG GC. Contributed reagents/materials/analysis tools: SAW-A PFG. Wrote the paper: AVF IGL CRC GC.

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Supporting information

Table SI. Animal characteristics, heart rates, left ventricular pressures and echocardiographic measurements

		Un-operated		SHAB		AB	
		WT	Syn-4$^{-/-}$	WT	Syn-4$^{-/-}$	WT	Syn-4$^{-/-}$
Characteristics							
	n	6	6	10	9	10	10
	BW, g	21.8 ± 1.3	21.9 ± 1.1	23.7 ± 0.9	25.0± 0.9	25.7 ± 1.2	21.3 ± 0.9*
	TL, mm	15.9 ± 0.1	17.3 ± 0.1*	16.6 ± 0.2	17.4 ± 0.1*	17.1 ± 0.2	17.5 ± 0.1
	LVW/TL, mg/mm	4.45 ± 0.17	4.55 ± 0.17	4.78 ± 0.15	4.84 ± 0.24	7.77 ± 0.28§	8.19 ± 0.22§
	RVW/TL, mg/mm	1.15± 0.09	1.10 ± 0.07	1.22 ± 0.03	1.19 ± 0.05	1.63 ± 0.12§	1.98 ± 0.12*§
	LW/TL, mg/mm	8.88 ± 0.27	8.49 ± 0.22	8.48 ± 0.12	8.56 ± 0.18	16.03 ± 2.40§	23.98 ± 1.77*§
Echo-2D							
	n			10	9	10	10
	LVILDd, mm/mm			0.479 ± 0.009	0.463 ± 0.008	0.491 ± 0.008	0.532 ± 0.007*§
Echo-M-mode							
	n	6	6	10	9	10	10
	IVSd/TL, mm/mm	0.044 ± 0.002	0.042 ± 0.002	0.046 ± 0.001	0.043 ± 0.001	0.063 ± 0.002§	0.044 ± 0.002*
	IVSs/TL, mm/mm	0.056 ± 0.004	0.050 ± 0.003	0.055 ± 0.001	0.055 ± 0.002	0.079 ± 0.002§	0.049 ± 0.003*
	LVDd/TL, mm/mm	0.250 ± 0.007	0.235 ± 0.006	0.246 ± 0.004	0.240 ± 0.009	0.243 ± 0.005	0.277 ± 0.009*§
	LVDs/TL, mm/mm	0.202 ± 0.009	0.188 ± 0.004	0.193 ± 0.006	0.189 ± 0.011	0.193 ± 0.008	0.262 ± 0.012*§
	LVFS, %	19 ± 3	24 ± 5	22 ± 2	22 ± 3	24 ± 2§	8 ± 1*§
	PWd/TL, mm/mm	0.040 ± 0.001	0.036 ± 0.002	0.043 ± 0.001	0.043 ± 0.001	0.062 ± 0.002§	0.048 ± 0.003*
	PWs/TL, mm/mm	0.052 ± 0.002	0.048 ± 0.002	0.057 ± 0.002	0.057 ± 0.002	0.079 ± 0.003§	0.056 ± 0.002*
	LVEF, %	40 ± 5	41 ± 2	44 ± 3	44 ± 4	44 ± 3	17 ± 2*§
	LAD/TL, mm/mm	0.114 ± 0.006	0.103 ± 0.005	0.096 ± 0.004	0.098 ± 0.003	0.148 ± 0.010§	0.176 ± 0.006*§
	AOD/TL, mm/mm	0.093 ± 0.002	0.083 ± 0.003	0.086 ± 0.002	0.087 ± 0.003	0.089 ± 0.002	0.085 ± 0.002
Echo-Doppler							
	n	6	6	12	9	12	12
	HR, beats/min	436 ± 16	479 ± 36	457 ± 16	460 ± 21	495 ± 14	488 ± 12

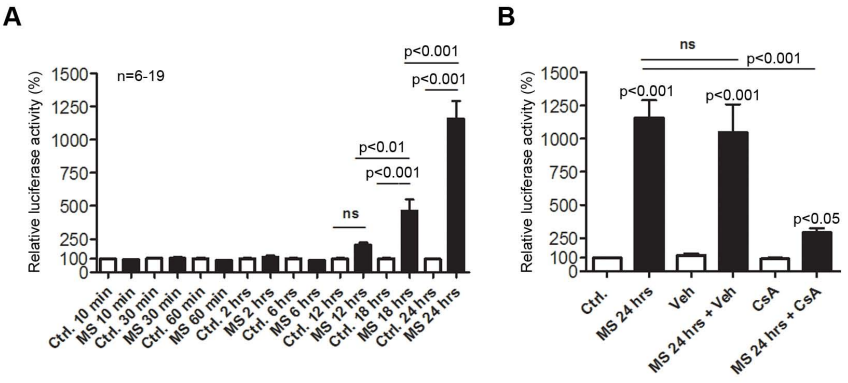
	AB V_{max}, m/s					4.3 ± 0.1	4.4 ± 0.1
	Mit PHT, ms	19.6 ± 2.5	21.3 ± 1.5	20.7 ± 1.3	20.9 ± 0.9	17.2 ± 0.9	16.0 ± 0.7§
	VTI, cm	4.05 ± 0.40	3.71 ± 0.21	3.86 ± 0.28	4.37 ± 0.26	3.81 ± 0.20	2.80 ± 0.22*§
	CO, ml/min	20.87 ± 3.08	20.84 ± 2.47	21.39 ± 1.83	24.69 ± 2.17	24.77 ± 2.26	17.54 ± 1.55*
Catheterization							
	n	9	9				
	LVSP, mmHg	95 ± 2	105 ± 4*				
	LVEDP, mmHg	8.9 ± 2.0	13.1 ± 2.1				
	$LVdP/dt_{max}$, mmHg/s	7818 ± 658	8843 ± 695				
	$LVdP/dt_{min}$, mmHg/s	7346 ± 452	8691 ± 526				

SHAB, 3 week sham-AB operated group; AB, 3 week aorta banded group; WT, wild type mice; Syn-$4^{-/-}$, syndecan-$4^{-/-}$ mice; BW, body weight; TL, tibia length; LVW, left ventricular weight; RVW, right ventricular weight; LW/TL, lung weight; Echo-2D, two-dimentional echocardiography; LVILDd, left ventricular internal longitudinal diameter in diastole; IVSd/s, inter-ventricular septum thickness in diastole respectively systole; LVDd/s, left ventricular diameter in diastole respectively systole; LVFS, left ventricular fractional shortening; PWd/s, posterior wall thickness in diastole respectively systole; LAD, left atrial diameter; AOD, aortic diameter; HR, heart rate; AB Vmax, peak flow over aortic constriction; Mit PHT, mitral pressure half time; LVOT VTI, left ventricular outflow tract velocity time integral; SV, stroke volume; CO, cardiac output; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end diastolic pressure; $LVdP/dt_{max}$, maximum rate of left ventricular pressure rise; $LVdP/dt_{min}$, maximum rate of left ventricular pressure decline; *, Syn-$4^{-/-}$ significantly different from WT in same group ($p < 0.05$); §, AB significantly different from SHAB in respective genotype group ($p < 0.05$);); catheterization following AB was not performed as the aortic constriction was too tight to allow for retrograde insertion of the catheter. Values are mean ± s.e.m.

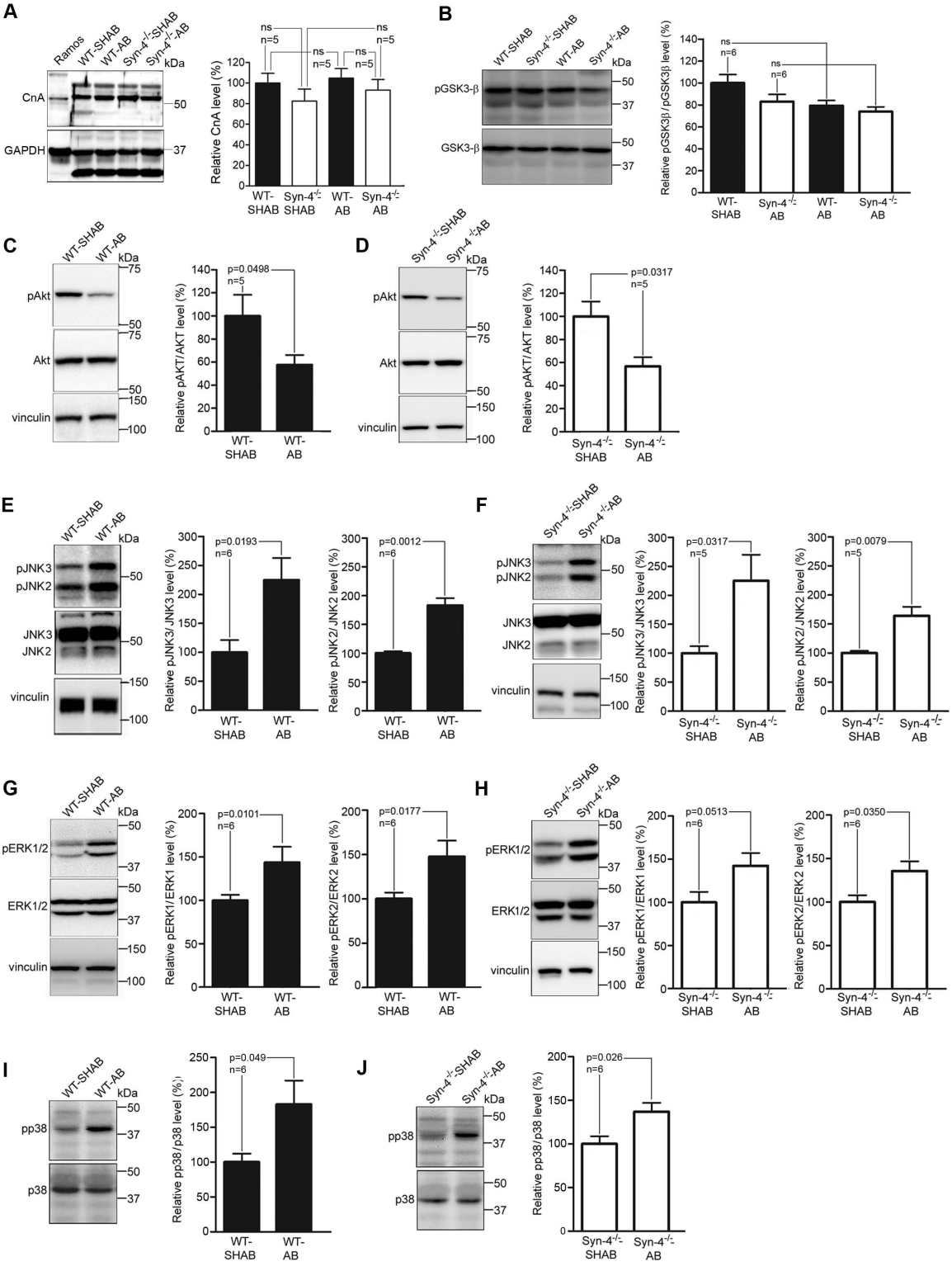
Table SII. Myocardial structure and neurohormonal changes

	SHAB		AB	
	WT	Syn-4$^{-/-}$	WT	Syn-4$^{-/-}$
n	102	112	120	134
Cardiomyocyte width, μm	14.5 ± 0.3	13.7 ± 0.2	18.4 ± 0.7§	14.3 ± 0.3*
Cardiomyocyte length, μm	103.2 ± 1.8	101.6 ± 1.6	111.6 ± 2.0§	111.5 ± 1.8§
n	10	9	10	10
Collagen type I, %	100.0 ± 12.3	112.9 ± 18.2	95.4 ± 9.2	82.5 ± 12.9
Collagen type III, %	100.0 ± 6.6	85.2 ± 9.5	173.0 ± 11.1§	173.7 ± 11.5§
Collagen type VIII, %	100.0 ± 6.7	95.4 ± 5.0	142.1 ± 9.7§	133.4 ± 10.7§
n	7	8	9	12
LV water content, mg	68.6 ± 1.9	64.4 ± 4.8	103.4 ± 6.6§	113.4 ± 3.4§*
n	7	6	7	7
Angiotensin II, pg/ml	700 ± 27	668 ± 36	642 ± 23§	958 ± 19§*

SHAB, 3 week sham-AB operated group; AB, 3 week aorta banded group; WT, wild type mice; Syn-4$^{-/-}$, syndecan-4$^{-/-}$ mice; LV, left ventricle; Collagen assessed by Western blot, values relative to WT-SHAB. *, Syn-4$^{-/-}$ significantly different from WT in same group ($p < 0.05$); §, AB significantly different from SHAB in respective genotype group ($p < 0.05$); Values are mean ± s.e.m.



Suppl. Figure S1
Finsen et al.



Suppl. Figure S2
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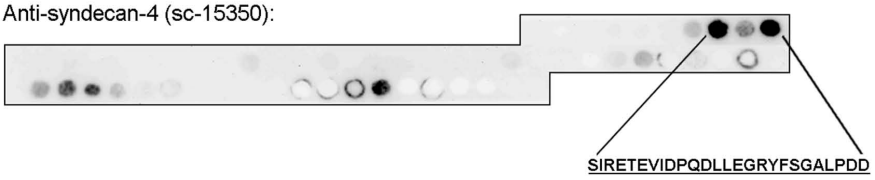
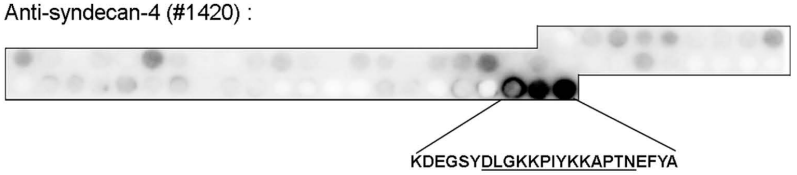
A

Anti-syndecan-4 (sc-15350):



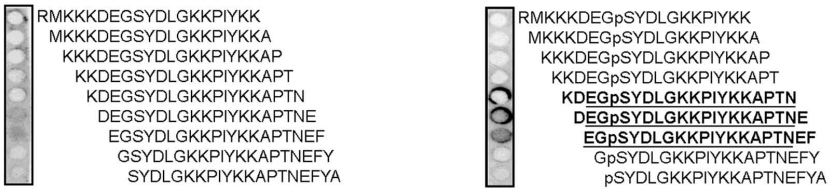
B

Anti-syndecan-4 (#1420) :



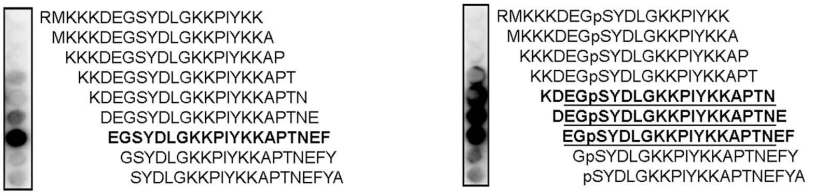
C

Anti-pS179-syndecan-4 (sc-22252-R, discontinued):



D

Anti-pS179-syndecan-4 (#41144):



Suppl. Figure S3
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IV