The AP-1 transcription factor c-jun, but not c-fos, prevents pathologic cardiac remodeling

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for the degree of
Doctor of Sciences

presented by
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2009
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I dedicate my thesis to my beloved husband, Filip.
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II. SUMMARY (IN ENGLISH)

In response to pathologic hypertrophic stimuli, the heart increases its workload, in order to maintain its function. This extra work is initiated through a compensatory hypertrophic response, which primarily aims at promoting cardiomyocyte growth and expansion of the contractile apparatus to enhance and maintain cardiac performance. If these pathologic stimuli are persistent, this response is ultimately followed by activation of stress pathways that in the long term are detrimental for proper heart function as they contribute to pathologic remodelling of the heart resulting in heart dilation and perturbations of contractility.

The AP-1 transcription factors, c-Fos and c-Jun have been implicated in the regulation of this hypertrophic response, as their early-immediate induction was observed upon various hypertrophic stimuli, both in vitro and in vivo. However, the downstream cellular and molecular consequences of induction of these transcription factors in vivo are very poorly understood. Therefore, the goal of my project was to elucidate the exact role of c-Fos and c-Jun in heart function in vivo. For this purpose, I have generated striated muscle-specific c-fos (c-fosΔmu) and c-jun (c-junΔmu) knock-out mice and studied basic heart function as well as cardiac integrity in response to mechanical pressure-overload in these mice.

I now provide genetic evidence in vivo that both transcription factors are not essential for postnatal cardiac growth and function. However, I found that deletion of c-jun, but not of c-fos, resulted in progressive myocardial fibrosis that is accompanied with increased cardiomyocyte apoptosis. Moreover, expression of most of the canonical fetal genes was enhanced in these mice. Pathologic myocardial remodeling was exacerbated when mice were subjected to TAC leading to an apparent reduced cardiac performance. Lack of c-jun was associated with mitigation of transcription of specific cytoskeletal genes that have been recently linked to impaired cardiac remodeling, as well as genetic cardiomyopathies.

Conclusively, while c-Fos is redundant in heart function, c-Jun specifically counteracts pathologic remodeling of the cytoskeleton in cardiomyocytes.
III. SUMMARY (IN GERMAN)

Als Antwort auf pathologische Hypertrophie-Stimuli, kann das Herz seine Kontraktilität und somit seine Kraft erhöhen, damit die Erhaltung der Funktion gewährleistet ist. Diese Extra-Kraft wird durch eine kompensatorische Antwort in der Muskelzelle ausgelöst, die initial Zellwachstum und Expansion der kontraktilen Elemente fördert. Wenn aber diese Stimuli persistieren folgt unmittelbar auf die adaptive zelluläre Antwort die Aktivierung von diversen Stress-Signalwegen, die sich längerfristig negativ auf die Herzfunktion auswirken.


Abschliessend kann gesagt werden, dass c-Fos in der Herzfunktion redundant ist und dass c-Jun dem pathologischen Umbau des Zytoskeletts im Kardiomyozyten entgegenwirkt.
### IV. ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACTA1</td>
<td>Skeletal muscle alpha actin</td>
</tr>
<tr>
<td>ANF</td>
<td>Atrial natriuretic factor</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator Protein 1</td>
</tr>
<tr>
<td>Bex1</td>
<td>Brain expressed gene 1</td>
</tr>
<tr>
<td>BMP1</td>
<td>Bone morphogenetic protein 1</td>
</tr>
<tr>
<td>BNP</td>
<td>Brain natriuretic peptide</td>
</tr>
<tr>
<td>CILP</td>
<td>Cartilage intermediate layer protein</td>
</tr>
<tr>
<td>Col1a1</td>
<td>Collagen type I</td>
</tr>
<tr>
<td>Col3a1</td>
<td>Collagen type III</td>
</tr>
<tr>
<td>DCM</td>
<td>Dilated cardiomyopathy</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated protein kinases</td>
</tr>
<tr>
<td>Fn</td>
<td>Fibronectin</td>
</tr>
<tr>
<td>GPCRs</td>
<td>G-protein coupled receptors</td>
</tr>
<tr>
<td>HCM</td>
<td>Hypertrophic cardiomyopathy</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun NH₂ terminal kinases</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MMPs</td>
<td>Matrix metalloproteinases</td>
</tr>
<tr>
<td>MyBP-C2</td>
<td>Myosin binding protein C, fast-type</td>
</tr>
<tr>
<td>Myot</td>
<td>Myotilin</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T-cells</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinases</td>
</tr>
<tr>
<td>Pn</td>
<td>Periostin</td>
</tr>
<tr>
<td>SERCA2</td>
<td>Sarco-endo-plasmic reticulum Ca²⁺ ATPase</td>
</tr>
<tr>
<td>TAC</td>
<td>Transverse-aortic constriction</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TPM2</td>
<td>Tropomyosin 2, beta</td>
</tr>
<tr>
<td>WISP1</td>
<td>WNT1 inducible signaling pathway protein 1</td>
</tr>
<tr>
<td>α-MHC</td>
<td>Alpha myosin heavy chain 6</td>
</tr>
<tr>
<td>β-MHC</td>
<td>Beta myosin heavy chain 7</td>
</tr>
</tbody>
</table>
V. INTRODUCTION

1. THE HEART

The heart is the first organ to form and function in the embryo, and all subsequent events in the life of higher organisms depend on the heart’s ability to meet the demands of the body for oxygen and nutrients. Inherited mutations in cardiac regulatory genes lead to abnormalities in the heart formation and function, and are the most common form of human birth defects, while the malfunctions of the adult heart represent the most prevalent cause of morbidity and mortality in the industrialized world (1, 2).

1.1. The contractile tissue of heart

The contractile tissue of the heart is composed of cardiac striated muscle, which consists of individual cells, the cardiomyocytes. These cells are the most physically active cells in the body, contracting constantly about 3 billion times or more in an average human lifespan, to pump 7000 liters of blood per day, along 100000 miles of blood vessels (3). As the heart is not a passive, static framework, the contractile apparatus must be maintained with almost crystalline order, to maintain proper heart function. To ensure this, the components of contractile apparatus are under constant coordination of changes in protein synthesis, degradation, assembly, and maintenance.

1.2. The cardiac cytoskeleton

The cytoskeleton is a complex network of filaments and tubules, which transmit mechanical and chemical stimuli within and between cells. It contributes substantially to cell stability by anchoring subcellular structures, such as mitochondria, Golgi apparatus, nuclei, and myofibrils (4). Recently, it was proposed to categorize the cytoskeletal proteins of the cardiomyocyte into 4 different groups (5, 6):
1) Sarcomeric proteins: consisting of the structural proteins: titin, myosin binding protein C, α-actinin, myomesin, and M-protein, and the contractile proteins: myosin, actin, tropomyosin and troponin; 

2) True ‘cytoskeletal’ proteins, forming the intermyofibrillar layer: tubulin, desmin, and actin; 

3) Membrane-associated proteins that form costameres - the subsarcolemmal complexes interconnecting the various intermyofibrillar cytoskeleton networks and linking them to the sarcolemma. There are three types of costameres - the focal adhesion-type complex, composed of proteins such as vinculin, talin, tensin, paxillin, zyxin, and α- and β-integrins; the spectrin-based complex, composed of actin, ankyrin, and desmin; and the dystrophin-associated complex, composed of dystrophin, α- and β-dystroglycans, α-, β-, γ-, and δ-sarcoglycans, dystrobrevin, and syntrophin. 

4) Proteins of the intercalated disc that maintain structural and functional integrity between adjacent myocytes, as well as transmit the force between cells and participate in the signal transduction pathways: desmosomes consisting of desmoplakin, desmocollin, desmoglein and desmin; adherens junctions with N-cadherin, the catenins and vinculin; and gap junctions with connexin. 

While the last three groups of proteins contribute to cell shape, mechanical resistance, signal transduction and morphological integrity of cardiomyocytes, and form the cytoskeleton, the first group of cytoskeletal proteins represents the structural and functional proteins of the sarcomere (4-6). The key elements of the cardiac cytoskeleton are illustrated in Figure 1.1.
1.3. The sarcomere

The basic contractile unit of myofibrils is the sarcomere (Fig 1.2). Due to the precise alignment of the filament systems the characteristic striated appearance of light and dark bands, sarcomere is observable by electron microscopy (Fig 1.2 A). The light
band (isotropic) is termed the I-band; the dark band (anisotropic) is known as the A-band. The principle components of striated muscle sarcomeres include parallel arrays of actin-containing thin filaments that span the I-band and overlap with myosin-containing thick filaments in the A-band. The third and forth filament system is made up of single molecules of titin, which span half of the sarcomere, and the nebulin, which span the length of the actin filaments, respectively. They are anchored in the lateral boundaries of the sarcomere, the Z-discs. Last, in the middle of the sarcomere there is the M-line, in which myosin is anchored (Fig 1.2 B).

**Figure 1.2. A schematic representation of the sarcomeric protein-complex.** (A) Electron micrograph of a skeletal sarcomere. The sarcomeric borders are delineated by the Z-lines (Z) in the middle of the light I-band. The M-band (M) is seen as an electron-dense transverse band in the middle of the dark A-band. (B) A schematic overview of cytoskeletal linkages in striated muscle. The sarcomeres contain four filament systems: actin-thin, myosin-thick, titin, and nebulin filaments. The actin filaments span the I-band (I) and overlap with myosin-thick filaments in the A-band (A). While actin, titin and nebulin are anchored in the Z-lines (Z) and I-band (I), myosin is anchored in the M-line (M). The border Z-lines (Z) precisely align and laterally associate with intermediate filament proteins (such as desmin) and other cytoskeletal proteins (such as plectin). The intermediate filaments and associated proteins link the peripheral myofibrils to costameres at the sarcolemma, to mitochondria, and to the nuclear membrane. This schematic has been adapted from (7) and (4).
Sliding of thin and thick filaments past each other, in the presence of calcium and ATP, results in a shortening of the sarcomere. Simultaneous shortening of many sarcomeres thus leads to the development of a macromolecular force, resulting in muscle contraction (8, 9). Force generated during contraction is transmitted outward to the cytoskeleton through a complex network of proteins that link the sarcomere to the sarcolemma and the extracellular matrix.
2. MYOCARDIAL REMODELING

Cardiovascular diseases such as hypertension, coronary heart disease, heart failure and stroke, are major causes of death in the Western world (2). In order to maintain sufficient cardiac output, the heart must respond to a variety of physiologic and pathophysiologic stimuli. Following a specific cardiovascular stress, a cascade of compensatory structural events occurs within the myocardium resulting in changes in the heart function, geometry and structure. This process is termed myocardial remodeling. Myocardial remodeling is a summation of both cellular and extracellular processes. The cellular processes consist of myocyte growth, initiated primarily through a hypertrophic response, and myocyte death, occurring through apoptosis and/or necrosis. The extracellular processes refer to the remodeling of extracellular matrix, mainly through fibrosis, resulting from an interplay between non-myocyte cells, signaling molecules and matrix proteins.

2.1. Myocyte growth - hypertrophy

2.1.1. Cardiac hypertrophy

Growth of the heart during embryogenesis occurs primarily through proliferation of cardiomyocytes, which is termed hyperplasia. However, soon after birth, cardiomyocytes down-regulate cell-cycle-perpetuating factors, like cyclin A and cdk2, and up-regulate cell cycle inhibitors p21 and p27, and thus withdraw irreversibly from the cell cycle. After that, a subsequent growth of the heart occurs predominantly through hypertrophy (10). Recently however, it was shown that simultaneous p38 MAPK inhibition and fibroblast growth factor 1 (FGF1) stimulation results in cardiomyocyte cytokinesis and proliferation (11), although the exact molecular mechanism is unknown. In general, hypertrophic growth of cardiomyocytes is a cellular response to stress stimuli and mitogenic inputs. The defining features of cardiac hypertrophy are an increase in the heart mass by an increase in cardiomyocyte size, enhanced protein synthesis, and a higher organization of the sarcomere (12-14). Depending on stimuli, cardiac growth can be divided into “physiological” or
“pathological” hypertrophy (Fig. 2.1). The physiological hypertrophy occurs during post-natal development in females during pregnancy and lactation, as well as, and most importantly, in response to chronic exercise. The physiological hypertrophy is associated with proportional increases in the length and width of cardiomyocytes that leads to mild to moderate left ventricular hypertrophy, and is characterized by enhanced cardiac performance via an increase in stroke volume, contractility, and oxygen consumption with preserved relaxation. The pathological hypertrophy arises from common hemodynamic disease states (both pressure- and volume-overload), such as hypertension, valvular abnormalities, and post-myocardial infarction, as well as from cellular stress responses arising from endocrine disorders and contractile dysfunction from inherited mutations in sarcomeric or cytoskeletal proteins. Pathological hypertrophy can be either concentric (functionally adaptive) or eccentric (functionally maladaptive). During concentric hypertrophy, new contractile-protein units are assembled in parallel, resulting in the cardiomyocyte increase in width. Concentric hypertrophy mainly occurs upon pressure-overload during the adaptive phase of cardiac growth. By contrast, the assembly of contractile-protein units in series occurs during eccentric hypertrophy, and leads to increased length of the cardiomyocyte. Eccentric hypertrophy leads to ventricular thinning and heart dilation in the decompensatory phase of cardiac growth, and is one of the causes of dilated cardiomyopathy (DCM). Pathological, but not physiological, hypertrophy triggers a metabolic transition in the heart from an oxidative toward a more glycolitic metabolism characteristic of the fetal stage. This switch is preceded and accompanied by the re-expression of the “fetal” cardiac genes, which encode natriuretic peptides, such as ANF and BNP; proteins involved in contractility, such as α-MHC, β-MHC and ACTA1; calcium handling, such as SERCA2; and metabolism, and are also termed as the “hypertrophic markers” (14-16).
16

Figure 2.1. Cardiac remodeling upon physiologic and pathologic stimuli.
Depending on the circumstances, remodeling can be physiologic or pathologic.
Pathologic remodeling leads to compensatory hypertrophy, which may progress to
decompensation, ventricular dilatation, systolic dysfunction, and ultimately to
electrophysiologic changes leading to malignant ventricular arrhythmia. This
schematic has been adapted from (17).

2.1.2. Cardiac hypertrophy – adaptive or maladaptive?

Cardiac hypertrophy has been considered as an adaptive process. However,
epidemiological studies have demonstrated that a prolonged period of “compensation”
is followed by a “decompensation”, resulting in dilated cardiomyopathy, heart failure
and sudden death (12, 18-21). Recent data suggest that the balance between cell
survival and apoptosis appears to be a major determinant of the transition from
compensated hypertrophy to ventricular dilation (5, 22-24).
2.1.3. Cardiac myopathies

Based on the cause, cardiomyopathies can also be classified as primary or secondary cardiomyopathies. Primary, also called idiopathic, are caused by mutations in genes that encode cytoskeletal proteins, and ventricular remodeling occurs in absence of pressure- or volume-overload. Secondary, pathologic, result from disorders producing pressure- or volume-overload (25). Based on ventricle morphology, cardiomyopathies can be classified as hypertrophic or dilated cardiomyopathies. Hypertrophic cardiomyopathy (HCM) is a relatively common genetic cardiac disease (1:500 in the general population) and the most common cardiovascular disease inherited as an autosomal dominant trait (26). Death may be sudden and unexpected or may occur secondary to stroke or progressive heart failure. In the United States, HCM accounts for 36% of all sudden deaths in competitive athletes under the age of 35 years (27). HCM is characterized by thickening of the left ventricular wall, often asymmetric, and the interventricular septum. Histopathological examination demonstrates hypertrophied and disarrayed myocytes, as well as increased interstitial cardiac fibrosis. Noninvasive analyses by echocardiography often reveal preserved systolic function. Hundreds of mutations in 11 genes that encode protein constituents of the sarcomere have been identified in HCM (Table 2.1), hence, genetic heterogeneity in HCM accounts for some phenotypic diversity observed in patients (25).

Dilated cardiomyopathy (DCM) is characterized by left- or bi-ventricular dilation in association with reduced myocardial contractility, often accompanied by eccentric hypertrophy, myocyte degeneration, and increased interstitial fibrosis (25). In half of all individuals with DCM there is no recognized cause, although 35% of DCM patients might have an inherited disorder (28). Inherited DCM is most commonly transmitted as an autosomal dominant trait, but can also occur as autosomal recessive, X-linked, or matrilinear (i.e. mitochondrial). Mutations in the cytoskeletal proteins are common features leading to DCM (Table 2.1), due to impaired transmission of contractile force generated by the sarcomere. Additionally, mutations in many proteins of the dystrophin-associated complex that cause skeletal muscular dystrophies also frequently cause DCM (25).
Table 2.1. Gene mutations in hypertrophic and dilated cardiomyopathies.

<table>
<thead>
<tr>
<th>Hypertrophic cardiomyopathy (HCM)</th>
<th>Dilated cardiomyopathy (DCM)</th>
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<tr>
<td>Symbol</td>
<td>Disease gene</td>
</tr>
<tr>
<td>TNNT2</td>
<td>Cardiac troponin T2</td>
</tr>
<tr>
<td>TTN</td>
<td>Titin</td>
</tr>
<tr>
<td>MYL3</td>
<td>Essential myosin light chain</td>
</tr>
<tr>
<td>TNNC1</td>
<td>Cardiac troponin C</td>
</tr>
<tr>
<td>MYBPC3</td>
<td>Cardiac myosin binding protein C</td>
</tr>
<tr>
<td>MYL2</td>
<td>Regulatory myosin light chain</td>
</tr>
<tr>
<td>MYH7</td>
<td>Beta myosin heavy chain</td>
</tr>
<tr>
<td>MYH6</td>
<td>Alpha myosin heavy chain</td>
</tr>
<tr>
<td>ACTC</td>
<td>Cardiac actin</td>
</tr>
<tr>
<td>TPM1</td>
<td>Alpha tropomyosin</td>
</tr>
<tr>
<td>TNNI3</td>
<td>Cardiac troponin I</td>
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This table has been adapted from (25).

2.1.4. Heart failure

Heart failure is a chronic and progressive disorder, and is defined as the pathophysiologic state of impaired cardiac function rendering the heart unable to maintain an output sufficient for the metabolic requirements of the body’s tissues and organs (6). Heart failure is a secondary process, which results from a decreased myocardial contraction and/or relaxation (systolic and/or diastolic dysfunction, respectively), or excessive pressure–stroke–volume load. Major causes of heart failure include ischemic heart disease, longstanding valvular defects, hypertension, congenital malformations, cardiomyopathies and arrhythmia (25). In response to these disorders, the intermediate and long-term response of the heart is morphologic remodeling in association with hemodynamic changes. The two major and distinct cardiac remodeling patterns are cardiac hypertrophy or dilation. The clinical diagnosis of cardiac hypertrophy or dilation is based on either post-mortem anatomic histopathologic findings or noninvasive assessment of ventricular dimensions and function by echocardiography or magnetic resonance imaging (25).
Overall, it is still to be answered, if myocardial hypertrophy is only initially beneficial and leads to cardiac demise when sustained (29-31).

2.2. Signaling pathways in cardiac hypertrophy

The hypertrophic growth of cardiomyocytes is initiated by endocrine, paracrine, and autocrine factors that stimulate a wide array of membrane-bound receptors. Their activation results in the triggering of multiple cytoplasmic signal transduction cascades, which ultimately affect nuclear factors and the regulation of gene expression. All of these molecules do not operate in isolation, but participate in a more orchestrated response that generates interdependent and cross-talking networks (Fig. 2.2).

Figure 2.2. Signaling pathways involved in cardiac hypertrophy. Hypertrophic stimuli activate a plethora of intracellular signaling cascades, such as G-proteins, gp130, calcineurin, MAP-kinases, PI3-kinase and others, in a receptor-dependent manner. This activation usually results in phosphorylation, activation, and nuclear translocation of several transcription factors, which then both activate and de-repress the transcription of genes governing cardiac growth and remodeling. Despite the complexity of these pathways, signaling passes through a limited number of common nodal points - some at the cell surface, some in the cytoplasm, and some in the nucleus. This schematic has been adapted from (29).
Cultured neonatal rat cardiomyocytes are widely used as a system for investigating the cardiac growth response, however translation of findings in vitro into an in vivo context is often impossible in particular for heart physiology, for which cell-to-cell communication in organs cannot really be mimicked in culture. Indeed, the hypertrophic response of disassociated neonatal cardiomyocytes in culture may more accurately model a developmental growth program, rather than a pathologic-type program that can occur in vivo. Thus, generation of transgenic mice over-expressing or lacking specific genes and the possibility of carrying out more sophisticated measures of cardiac function are helping in understanding the precise role of each signaling molecule involved in cardiac hypertrophy.

2.2.1. G-protein coupled receptors

G-protein coupled receptors (GPCRs) play an important role in the regulation of cardiac function and adaptation to stress stimuli. The most important myocardial GPCRs include α- and β-adrenergic receptors, which are coupled to GTP-binding proteins, Gs, Gq/G11, and Gi. Upon agonist or antagonist stimulation, they transduce the signal and activate intracellular signaling pathways. Angiotensin II (AngII)-, Endothelin I (ETI)- and α-adrenergic receptors are coupled to Gq/G11, and activate phospholipase C (PLC) and further downstream protein kinase C (PKC), an important step in the development of concentric hypertrophy. Transgenic over-expression of AngII-, ETI- and α-adrenergic receptors as well as their downstream mediator Gq, results in cardiac hypertrophy and subsequently leads to heart failure (32-35). Conversely, combined genetic ablation of G11 null mice and Gq cardiac-specific knock-out mice results in almost complete lack of cardiac hypertrophy or activation of the fetal gene program in response to aortic banding (36), demonstrating a requirement for Gq/G11 for most if not all features of pressure-overload-induced cardiac hypertrophy.

β-Adrenergic receptors are coupled to Gs, inducing adenylyl cyclase activity, accumulation of cAMP and consequently activation of protein kinase A (PKA) (37). PKA phosphorylates several proteins involved in cardiac contraction, including l-type calcium channels, ryanodine receptors, phospholamban and troponin, increasing their
activity and hence cardiac contraction (37). The overt β-adrenergic stimulation is one of the hallmarks of hypertrophy, while its chronic stimulation is thought to be ultimately detrimental. It is supported with studies of transgenic mice over-expressing β₁-ARs or Gs, which show increased cardiac contractility when young, but pronounced cardiac hypertrophy and fibrosis when older, eventually leading to heart failure (38-40). Furthermore, transgenic over-expression of PKA results in dilated cardiomyopathy associated with cardiomyocyte hypertrophy and fibrosis, suggesting that PKA mediates detrimental consequences of chronically elevated β-adrenergic signaling (41). Most importantly, the treatment with β-blockers halts left ventricular remodeling, improves left ventricular ejection fraction and reduces the overall risk of death by over 30% (42, 43).

2.2.2. Calcineurin – NFAT

Calcineurin is a serine/threonine phosphatase expressed in multiple tissues and consists of a catalytic A subunit and a regulatory B subunit. The catalytic subunit is encoded by three genes calcineurin Aα, calcineurin Aβ, and calcineurin Aγ, with CnAα and CnAβ being expressed in adult human, rat and mouse heart (37). A role of calcineurin as a regulator of reactive intracellular signaling through NFAT transcription factors (nuclear factor of activated T-cells) has been firmly established in T cells. Activation of the T-cell receptor increases concentrations of intracellular calcium, which binds calmodulin, resulting in calcineurin activation. Once activated, calcineurin directly dephosphorylates members of the NFAT transcription factor family in the cytoplasm, resulting in their nuclear translocation and the activation of immune response genes (44).

Calcineurin has been implicated as a crucial regulator of the hypertrophic response and its activity increases in response to hypertrophic stimuli, during myocardial infarction and heart failure (45-50). Particularly, transgenic mice that over-express activated forms of calcineurin or NFAT3 in the heart display cardiac hypertrophy that progresses to dilated-heart failure within 2 months, suggesting a maladaptive function (51). Pharmacologic inhibition of calcineurin by cyclosporine A and FK506 attenuated dilated and hypertrophic cardiomyopathy in different mouse models of heart disease, inhibited the development of pressure-overload-induced hypertrophy in
spontaneously hypertensive and aortic-banded rats (52, 53), as well as inhibited cardiac hypertrophy in calcineurin-transgenic mice (51). Furthermore, overexpression of MCIP1 (modulatory calcineurin-interacting protein-1), an endogenous inhibitor of calcineurin, blunted the hypertrophic response and preserved systolic function in mice subjected to chronic pressure-overload (54), as well as blunted cardiac hypertrophy, the progression to dilated cardiomyopathy, and the reduction of ejection fraction that otherwise occurred in mice over-expressing a constitutively active form of calcineurin (55). On the other hand, different studies using cyclosporine A show conflicting results, suggesting that calcineurin signaling could be adaptive (56, 57). However, this pharmacological approach has several limitations as at high doses cyclosporine A not only inhibits calcineurin, but also has toxic consequences for other cells, and nonspecific effects on other regulatory factors that participate in the hypertrophic response, thus confounding the interpretation of results. Genetic studies using either dominant-negative mutants or knock-out mice were speculated to clarify the precise role of calcineurin in ‘adaptive’ or ‘maladaptive’ hypertrophic response. Transgenic mice that over-express the dominant negative mutant of calcineurin in the heart show less prominent hypertrophic response upon pressure-overload than wild-type mice (58). Finally, gene-targeted mice deficient in calcineurin Aβ display a 12% reduction in basal heart size and are largely resistant to diverse hypertrophic stimuli, such as pressure-overload and infusion of AngII or isoproterenol (59). Taken together, these in vivo studies provide strong evidence for a role of calcineurin in cardiac hypertrophy resulting from common causes such as pressure-overload. The next generation of genetic mouse models, which is likely to involve tissue-specific gene ablation, will further refine our knowledge about calcineurin-dependent signaling in the heart.

2.2.3. PI3-Kinase

Phosphoinositide 3-kinases (PI3Ks) comprise a family of enzymes that exhibit both protein and lipid kinase activity and are activated by several receptor tyrosine kinases, such as the IGF-1 receptor, as well as GPCRs, including α- and β2-adrenergic receptors (60). As PI3K has been linked to signaling during cell growth, survival, and proliferation (60), its essential role in hypertrophic growth of heart was assumed. It
has been thus demonstrated that the over-expression of constitutively active PI3K in the hearts of transgenic mice induces an increase in cardiomyocyte size and concentric hypertrophy without fibrosis, and with preserved systolic function, while a dominant-negative form of PI3K led to significantly reduced heart weight/body weight ratio in mice (61). Interestingly, transgenic mice expressing a dominant negative PI3K (p110α) developed hypertrophy upon pressure-overload but not in exercise training, suggesting the specific role of this molecule in the ‘physiological’ hypertrophy (62). In further support, deletion of PTEN, which physiologically counteracts PI3K activity by dephosphorylatingPIP3 (phosphatidylinositol (3,4,5)-trisphosphate), results in compensated cardiac hypertrophy (63). Importantly, the PI3K pathway promotes not only cell growth, but also cell survival, acting as an inhibitor of apoptosis (64).

One of the principal targets of PI3K signaling is the serine/threonine kinase Akt, also known as protein kinase B (PKB). Akt is activated via binding of PI3K-phosphorylated phosphoinositides, which in turn results in its translocation to the membrane. Again, it could be shown that transgenic over-expression of Akt/PKB is sufficient to induce significant cardiac hypertrophy in mice without affecting systolic function (65, 66).

A well-defined direct downstream target of Akt is the glycogen synthase kinase 3β (GSK-3β). Akt directly phosphorylates GSK-3β and thus inhibits its kinase activity. Activated GSK3β is able to phosphorylate and inhibit several molecules involved in hypertrophic signaling, such as the members of the NFAT transcription factor family that are activated by calcineurin (67). Inactivation of GSK3β via phosphorylation by the upstream kinase Akt in vitro is required for cardiomyocytes to undergo hypertrophy (68), while expression of a non-phosphorylatable form of GSK3β prevents cardiac hypertrophy in response to pressure-overload, as well as to other hypertrophic stimuli (69). lpr (lympho-proliferative disease) mice lacking a functional Fas receptor failed to inactivate GSK3β and developed rapid left ventricular dilatation, heart failure, and an increased mortality upon pressure-overload (70). That further supports the importance of GSK3β phosphorylation and inhibition in the development of compensatory hypertrophy.
2.2.4. Gp130

The myocardial growth and survival is also modulated by signals derived from gp130, the receptor for the interleukin 6 family of cytokines. The IL-6-type cytokines, comprising interleukin-6 IL-6, interleukin-11 (IL-11), LIF, oncostatin M (OSM), ciliary neurotrophic factor (CNTF), CT-1 and cardiotrophin-like cytokine (CLC) possess pleiotropic effects in the organism. They bind to their receptor and induce homo- or hetero-dimerization of gp130. Subsequent signal transduction via gp130 involves three major downstream pathways: the JAK-STAT axis, the Ras-Raf-MEK/ERK signaling cascade, and the PI3K/AKT pathway (71).

Members of the IL-6 cytokine family are potent inducers of cardiomyocyte hypertrophy through gp130 activation, suggesting a role of gp130 in the regulation of myocardial growth (72-74). Furthermore, double-transgenic mice for the IL-6 and IL-6 receptors, a combination that leads to constitutive tyrosine phosphorylation and subsequent activation of gp130, display marked cardiac hypertrophy (75). In addition, targeted disruption of gp130 in the mouse results in embryonic lethality associated with hypoplastic ventricular development (76). Cardiac-specific gp130 knockout mice are viable, but they quickly undergo a transition into heart failure after pressure-overload stimulation, which is associated with profound apoptosis (77).

Upon ligand binding, the gp130-receptor complex formation results in phosphorylation of Janus kinase (Jak) and recruitment of signal transducer and activator of transcription (STAT), causing its subsequent phosphorylation and homo- and heterodimerization, and nuclear translocation. STAT3 induces genes involved in hypertrophy and survival pathways (78). Over-expression of STAT3 in transgenic mice is sufficient to induce cardiomyocyte hypertrophy (79), while its deletion in cardiomyocytes results in higher sensitivity to inflammation, cardiac fibrosis, and heart failure with advanced age (80). In addition, the suppressor of cytokine signaling 3 (SOCS3), which acts as an endogenous inhibitor of JAK-mediated gp130 signaling, suppresses CT-1-induced cardiomyocyte hypertrophy (81).

These data demonstrate the requirement of the gp-130 signaling cascade for the development of cardiac hypertrophy, and in general heart integrity.
2.2.5. MAP-Kinases

The MAPK signaling pathway consists of three major branches named for their terminal effector kinase: the extracellular signal-regulated protein kinases (ERK), the c-Jun NH\textsubscript{2} terminal kinases (JNK), and p38 (Fig. 2.3) (82-85). The ERK1/2/5, JNK and p38 are considered as conventional MAP-kinases, while ERK3/4/7/8 and the NEMO-like kinase (NLK) are considered as atypical MAP-kinases (83-85). A multiple agents, such as growth factors, cytokines, and stress, activate the cascade of conventional MAPKs. At first, G-protein coupled receptors activate MAPK kinase kinases (MAPKKKs), which target downstream MAPK kinases (MAPKKs) leading to subsequent activation of MAPKs (83). The activation of atypical MAPKs is generally not mediated by MAPKKs, although the exact mechanisms of activation of these kinases are still not well-understood (85). Through phosphorylation of downstream targets, MAPK regulates a whole plethora of basic cellular processes including proliferation, differentiation, apoptosis, migration and inflammation (82-85).

Figure 2.3. The stress-activated MAP-kinase signaling cascades. MAPK regulates most of the basic cellular processes. MAPK are composed of a three-tier kinase module, in which a MAPK is activated upon phosphorylation by MAPKK, which in turn is activated when phosphorylated by MAPKKK. This schematic has been adapted from (83).
2.2.5.1. JNK

The initiating events that culminate in JNK activation begin at the cell membrane through the activation of Ras/Rac/Cdc42. These low molecular weight GTP-binding proteins directly promote MAPK kinase kinase 1, 2, 3 (MEKK1, 2, 3) and apoptosis signal-regulating kinase-1 (ASK1) activation, which in turn promote phosphorylation and activation of the dual specificity MAPK kinases, MKK4 and MKK7 that in turn directly phosphorylate and activate JNK (86). There are three jnk genes: jnk1 and jnk2 genes are widely expressed in mouse tissues including the heart, while jnk3 has a more limited pattern of expression that is largely restricted to brain, heart, and testis (87). JNKs are activated by various stress and mitogenic stimuli, including growth factors, cytokines, UV irradiation, osmotic shock, toxic compounds, and oncogenes (88). Stimulation of JNK activity results in activation or modulation of several transcription factors such as c-Jun, activating transcription factor-2 (ATF-2), Elk-1, p53, and nuclear factor of activated T cells (NFAT) (86, 88).

Although JNK activation and function in the regulation of hypertrophic response has been studied extensively, its role has not been yet conclusively examined (89, 90). JNK1/2 are rapidly activated in cultured cardiomyocytes and within the adult heart following agonist treatment or stress stimulation, such as angiotensin II, phenylephrine, endothelin, stretch, ischemia and pressure-overload (91-97). Many studies unraveled the role of JNK in hypertrophic response by studying its up-stream regulating kinases. Over-expression of MKK7 in cardiomyocytes induced the characteristic features of hypertrophy in vitro (98). Moreover, expression of a dominant-negative MKK4 mutant abrogated the endothelin-1-induced hypertrophic response of neonatal rat cardiomyocytes in culture and by pressure-overload in vivo (93, 96). Two independent groups have recently reported the phenotype of Mekk1 gene-targeted mice. Mekk1 null mice crossed with a Gaq model of hypertrophic cardiomyopathy, abolished the increase in cardiac mass, myocyte size, hypertrophy-associated ANF induction, and mechanical dysfunction (99). In contrast, pressure-overload-induced hypertrophy in the Mekk1 null mice, was unchanged at 7 d after aortic constriction, but enhanced growth was observed at 14 days (100). However, the assumption that MEKK1 is an exclusive upstream regulator of JNK signaling is unlikely, given its ability to function as an E3 ubiquitin ligase and its ability to regulate MEK1–ERK1/2 signaling in non-myocytes (101, 102). Recent studies have
also employed a loss-of-function approach. Remarkably, dnJNK1/2 transgenic mice and three allele Jnk1/2 gene-targeted mice (Jnk1+/−/Jnk2−/−) each showed an enhanced hypertrophic growth response following pressure-overload induced by aortic banding. Those mice also showed spontaneous cardiac hypertrophy with aging (by 7 months of age), suggesting that JNK signaling normally serves to antagonize cardiac growth in response to both acute and chronic stimulation (103). In another study, pressure-overload in mice with selective deletion of Jnk1, Jnk2 and Jnk3 induced cardiac hypertrophy similar to wild-type mice, but only Jnk1-null mice displayed a significant reduction in fractional shortening after 3 and 7 days of pressure-overload, associated with a significant increase in apoptosis and marked inflammatory infiltrate (104). Collectively, conflicting evidence exists in the literature about the role of JNK in cardiac hypertrophy.

2.2.5.2. ERK

There are currently seven MAP kinases cloned, defined as ERK1–7. ERK1 and ERK2 were the first identified members of ERK MAP kinases and the most widely studied. The ERK cascade in mammalian cells is stimulated via tyrosine kinase receptors (TKRs) and GPCRs. Stimulation of TKRs activates the receptor tyrosine kinase domains, leading to recruitment of several adapter proteins, such as Shc, Grb2, and SOS (Son of sevenless), and transducing the signal to GTP-binding proteins of the Ras family (H-Ras, K-Ras, N-Ras, Rap1). Activated Ras-GTP can activate the Raf family (A-Raf, B-Raf, c-Raf-1) of MAPKKK, which in turn phosphorylates and activates MAPKK MEK1/2 and then in turn dual-phosphorylates MAPK ERK1/2. ERK5 is directly regulated by the dual-specificity MAPK kinase MEK5, while ERK3/ERK4 and ERK6/7 are related family members with uncharacterized upstream activators (82, 88, 105).

In response to agonist stimulation or cell stretching, ERK1 and 2 were activated both in cultured cardiac myocytes and in isolated perfused hearts (106-112), and thus implicate ERK1- and 2-signaling factors as regulators of the hypertrophic response. It was shown that ERK signaling is necessary for PE-induced cardiomyocyte hypertrophy in culture (113). On the other hand, many studies have challenged the view that ERK regulates cardiac hypertrophy. Although Ras-Raf-1-ERK activation
was sufficient to augment c-Fos and ANF promoter activity in cardiomyocytes, inhibition of these signaling factors did not antagonize hypertrophic morphology or cytoskeletal organization in response to the agonists (114, 115). In an \textit{in vivo} study, in \textit{Erk1-/-} and \textit{Erk2+/+} mice, as well as in transgenic mice with inducible expression of an ERK1/2-inactivating phosphatase (DUSP6) in the heart, the hypertrophic response was not diminished after pressure-overload stimulation, neuroendocrine agonist infusion, or exercise (116). Additionally, transgenic over-expression of MEK1 led to concentric hypertrophy without signs of cardiomyopathy or lethality up to 12 months of age, accompanied by a dramatic increase in cardiac function (117). However, \textit{Erk1-/-} and \textit{Erk2+/+} mice showed decompensation and failure after long-term pressure-overload in conjunction with an increase in myocyte apoptosis. Thus, ERK1/2 signaling is not required for mediating physiologic or pathologic cardiac hypertrophy \textit{in vivo}, although it does play a protective role in response to pathologic stimuli (116). Collectively, ERK family members have been implicated in survival signaling in response to stress stimuli, and although there is clearly a lack of consensus regarding the necessity of ERK signaling as a hypertrophic mediator, experimental evidence implicates ERKs as immediate downstream effectors of the hypertrophic response.

2.2.5.3. p38

Four separate p38 MAPK isoforms have been described, p38\(\alpha\), p38\(\beta\), p38\(\gamma\), and p38\(\delta\) (82, 88). The major upstream activators of p38 MAPKs are two MAPKKs, MKK3 and MKK6, which directly phosphorylate the dual site in p38 MAPKs (Thr-Gly-Tyr). Less is known of the MAPKKK factors, which lie upstream of MKK3 and MKK6 in cardiomyocytes, although PAK, TAK1, and MLK3 are potential activators. In cardiac myocytes, mechanical stress, GPCR ligands (angiotensin II, endothelin-1, and PE), and mitogens are potent activators of p38 (118). Activated p38 MAPKs directly phosphorylate serine and threonine residues in a wide array of cytoplasmic proteins and transcription factors to mediate stress-responsive signaling, including MEF2, MAPKAPK2 and 3, ATF-2, ELK-1, Chop, and Max (119). p38\(\alpha\), p38\(\beta\) and p38\(\gamma\) are expressed in the human heart, while p38\(\delta\) is not detectable (11).

Using isolated neonatal cardiomyocytes, it was shown that p38 inhibition by the antagonists SB203580 or SB202190 attenuated cardiomyocyte hypertrophy in culture
in response to endothelin-1, phenylephrine, and leukemia inhibitory factor (120, 121), and transfection with dominant-negative p38β or p38α and blunted the growth response of cardiomyocytes (122). Additionally, over-expression of activated MKK3 or MKK6 in cultured neonatal cardiomyocytes was shown to induce hypertrophy further implicating p38 as a positive regulator of the myocyte growth (120-122). In contrast, other studies have shown that p38 inhibition is not sufficient to attenuate all aspects of agonist-induced cardiomyocyte hypertrophy (93, 123), and that calcineurin-induced cardiac hypertrophy was associated with inhibition of p38 signaling (124). A more uniform data set has emerged in recent years through the use of genetically modified mice. For example, over-expression of activated MKK3 or activated MKK6, as well as TAK1 (MEKK) in the heart, activated p38 and led to development of heart failure (125, 126). These results are consistent with reports that p38 activity is increased in dilated and failed human heart explants (50, 127). Most importantly, cardiomyocyte-restricted p38α knock-out mice developed heart dilation and dysfunction, accompanied by massive fibrosis and apoptosis (128). Recently, transgenic mice expressing dominant-negative mutants of p38α or p38β were generated. Pressure-overload induced by aortic banding induced normal hypertrophic response in dominant-negative p38α transgenic mice, however, induced cardiac hypertrophy in dominant-negative p38β transgenic mice (129). A similar study, using transgenic mice expressing dominant-negative mutants of MKK3, MKK6, and p38α, showed significantly greater cardiac hypertrophy following pressure-overload and progression to cardiomyopathy as they aged, without being stimulated (130). Data obtained in in vivo experiments indicate that p38 probably does not promote hypertrophy when singularly activated, but instead promotes dilated cardiomyopathy. The existing data are discrepant and suggest either promotion of hypertrophic growth in cultured cardiac myocytes, or progression to heart failure in animal models. It is not clear, why under stress conditions cellular response differs in an in vitro and in vivo approach. Potential explanation may be due to the non-specific effects of inhibitors (SB202190 and SB203580), or possibly different functional and overlapping actions of p38 isoforms. Overall, p38 has definitely a role in transducing hypertrophic signals in heart.
In conclusion, the described signaling pathways including G-protein coupled receptors, calcineurin-NFAT signaling, JNK-MAP kinases and p38-MAP kinases participate in cardiac hypertrophic remodeling favoring the transition toward heart failure, and should thus be considered maladaptive. In contrary, PI3K/Akt, gp-130 and ERK-MAP kinases signaling cascades were shown to be necessary for hypertrophic response, and are thus recognized as rather adaptive.

2.3. Myocyte death

2.3.1. Apoptosis versus necrosis

Loss of myocytes due to cell death is an integral part of pathogenesis of heart failure (23, 131, 132). Traditionally, myocyte loss was thought to occur from necrosis, however there has been increasing evidence suggesting that myocyte death can also happen through apoptosis. Apoptosis and necrosis are considered as two independent mechanisms; whereas apoptosis is a genetically controlled process, necrosis is a form of accidental death resulting from cellular injury. Necrosis induces major damage at the cell membrane that results in swelling and lysis, and released intracellular contents provoke an inflammatory response. In contrary, apoptosis is initiated by activation of endogenous proteases that result in the cleavage of chromatin, aggregation of DNA fragments under the nuclear membrane. Finally formation of clusters of membrane-bound cellular organelles referred to as “apoptotic bodies” occurs that are phagocytosed by tissue macrophages and neighbouring cells. Since cell swelling does not occur and cytoplasmic contents are not released into the extracellular space, an inflammatory reaction is not observed (133).

2.3.2. Apoptosis

Myocardial apoptosis is considered to play an integral role in the development of heart pathophysiology, such as ischemia/reperfusion (134, 135), myocardial infarction (136-138) and cardiomyopathies (131, 132, 139, 140), and is an important trigger in the switch from adaptive hypertrophy to heart dilation and failure (24, 141-143).
The cellular processes by which the apoptotic signal is transduced are often divided in two basic pathways, the “extrinsic” and “intrinsic” pathways (Fig. 2.4). In most circumstances, both pathways are executed by caspases, a group of cysteinyl-aspartate-directed proteases. These proteases degrade multiple substrates within the cell including structural proteins, regulatory proteins, and DNA repair enzymes, all of which contribute to cell death progression. As most proteases, caspases are synthesized as enzymatically inert proenzymes, and they usually undergo proteolysis and activation by other caspases in the cascade. Upon activation, initiator caspases (caspase-2, -8, -9 and -10) cleave and activate effector caspases (caspase-3, -6 and -7), thus initiating the proteolytic cascade. This system enables rapid caspase mobilization and cascade amplification, leading to rapid cell death. The extrinsic pathway is a receptor-mediated system that is activated by the binding of extracellular death signal proteins (TNF-α, Fas L, TRAIL and Apo-3L) to their cognate cell surface receptors. Binding of a ligand to these receptors induces their recruitment of the so-called death domain adaptor molecules (e.g. TRADD and FADD), to form the death-inducing complex (DISC). Formation of this complex in turn recruits the initiator caspase-8 and -10, and the further downstream executioner caspase-3. The intrinsic pathway is mediated through the mitochondria and in cardiomyocytes is primarily activated by cellular stimuli, such as hypoxia, ischemia-reperfusion, and oxidative stress. The pro-apoptotic signals induce permeability of outer and inner mitochondrial membranes and lead to formation of mitochondrial permeability transition pore consisting of the voltage-dependent anion channel (VDAC) in the outer membrane, the adenine nucleotide translocase (ANT) in the inner membrane, and cyclophilin D in the matrix. Activation of caspase-8 causes cleavage of Bcl-2 family of proteins, which is known to contain both pro-apoptotic (Bax, Bad, Bid, Bik, Bim, Bak, Bnip3, Bok) and anti-apoptotic (Bcl-2, Bcl-Xl, bcl-W, Bcl-1, Mcf-1) members. Activation of the pro-apoptotic Bcl-2 proteins leads to their translocation to mitochondria, where they interact with the anti-apoptotic Bcl-2 proteins. This interaction depolarizes voltage-dependent mitochondrial channels and releases apoptotic mediators, most notably cytochrome c, Smac/DIABLO, endonuclease G (Endo G), and apoptosis-inducing factor (AIF). Once released, cytochrome c binds to cytosolic adapter molecule apaf1 and caspase-9 facilitating formation of the “apoptosome” complex, which results in activation of caspase-3. Smac/DIABLO indirectly activates caspases by sequestering caspase-inhibitory proteins, while release of Endo G and AIF from mitochondria.
results in their translocation to the nucleus where they either directly or indirectly facilitate DNA fragmentation (90).

2.4. Signaling pathways in cardiac apoptosis

A number of focal signalling pathways have been identified as crucial transducers of both pro- and anti-apoptotic effects in the myocardium. Among these regulators, one of the most studied is the MAP Kinas family.

2.4.1. MAP-Kinases

2.4.1.1. JNK

An emerging area of investigation has demonstrated a critical role for JNK and their upstream activators in apoptosis. For example, jnk null fibroblasts were protected
against UV-stimulated apoptosis and failed to release cytochrome c in mitochondrial-driven apoptosis (144). Expression of JNK or MKK4 inhibitory mutants in cultured cardiac myocytes increased nitric oxide- and ischemia-reperfusion-induced cardiomyocyte apoptosis, suggesting a protective role for JNK signaling (145, 146). In contrast, particularly JNK1 inhibition was recently reported to actually protect cardiac myocytes from ischemia-induced apoptosis, while JNK2 inhibition had no effect (147). Similarly, inhibition of JNK in myocytes blocked apoptosis induced by ischemia, \( \text{H}_2\text{O}_2 \) and norepinephrine (148-150). It was also shown that activated JNK and MKK4 localized to the mitochondria, induced cytochrome c release, and promoted apoptosis of cardiac myocytes (151, 152). This is consistent with an earlier observation made in fibroblasts, where JNK was required for cytochrome c release and mitochondrial-driven cell death (144). JNK signaling can also induce apoptosis through the induction of pro-apoptotic genes, as it directly phosphorylates pro-apoptotic factor Bad (153), Bim and Bmf (154), and activates Bid cleavage (155).

### 2.4.1.2. ERK

Additionally, several studies have shown that the MEK–ERK pathway may protect against apoptosis. ERK signaling was shown to accompany insulin-like growth factor 1 (IGF-1), basic fibroblast growth factor (bFGF), cardiotrophin-1 (CT-1), catecholamines and \( \beta_2 \)-adrenergic induced anti-apoptotic actions (156-160). MEK–ERK inhibition augmented daunomycin- and serum deprivation-induced apoptosis in cultured cardiac myocytes (161-163). *In vivo*, ERK1/2 activation was shown to attenuate the amount of apoptosis in response to ischemia-reperfusion injury of the intact heart (164) while MEK1 transgenic mice are partially resistant to ischemia-reperfusion-induced apoptosis (165). Moreover, \( \text{Erk}2^{+/-} \), but not \( \text{Erk}1^{-/-} \) mice showed a significant increase in myocardial injury and cellular apoptosis following ischemia-reperfusion injury (165), while both \( \text{Erk}1^{-/-} \) and \( \text{Erk}2^{+/-} \) mice showed increased apoptosis after pressure-overload (116). Recent studies have suggested possible molecular mechanisms, by which ERK signaling mediates cellular protection. ERK1/2 might antagonize apoptosis through association with \( \text{PKCe} \) or RSK (p90 ribosomal S6 kinase) facilitating the phosphorylation and inactivation of the pro-apoptotic Bcl-2 family member Bad (152, 166). Furthermore, ERK-dependent
phosphorylation of GATA4 protected cardiomyocytes from daunorubicin-induced apoptosis in association with Bcl-XL upregulation (163). Last, ERK1/2 activation has also been shown to antagonize the extrinsic apoptotic pathway through a mechanism involving inhibition of caspase-8 cleavage (167). Conclusively, it has been demonstrated that MEK–ERK signaling plays a protective role in the myocardium in stress-induced apoptosis.

2.4.1.3. p38

p38 MAPK signaling has also been implicated in stress-induced apoptosis in cardiac myocytes. Numerous studies imply a protective role of p38 signaling upon apoptotic stimulation. For example, over-expression of activated MKK6 antagonized anisomycin-induced apoptosis (168) and consistent with this observation, pharmacologic inhibition of p38 augmented norepinephrine-induced apoptosis (169). In contrast, a number of studies suggested the opposite, a promoting role of p38 MAPK activation in myocyte apoptosis. In those reports, pharmacologic p38 inhibition reduced ischemia-, doxorubicin-, angiotensin II- and norepinephrine-induced cardiac myocyte apoptosis (161, 170-172). In vivo, use of p38 inhibitors has shown protection from ischemia-induced apoptosis (173, 174). Consistent with these data, over-expression of TAK1 in the mouse heart induced p38 activation that was associated with myocardial cell death in vivo (126). Similarly, transgenic mice expressing dominant-negative mutants of MKK6 and p38α within the heart, showed reduced apoptosis following ischemia-reperfusion injury (175). Similarly to studies examining the role of p38 in cardiac hypertrophy, conflicting data were obtained regarding its function in cardiac apoptosis. On one hand, several in vivo studies support the hypothesis that p38 MAPK signaling exerts a pro-apoptotic role heart, but on the other hand, studies in vitro rather speak for a cytoprotective role of p38 in heart.

Conclusively, data obtained in genetically modified animal models suggest a pro-apoptotic role of p38 and JNK and a rather anti-apoptotic role of ERK in heart (90).
2.5. Myocardial extracellular matrix remodeling – fibrosis

Myocyte death triggers an increase in the number of cardiac fibroblasts as well as accelerated and aberrant remodeling of extracellular matrix (ECM) and net accumulation of ECM, collectively termed cardiac fibrosis. This fibrosis may be reparative or reactive. Reparative fibrosis occurs as a reaction to a loss of myocardial material (due to necrosis or apoptosis, after myocardial ischemia or senescence) with structural scar, and it is mainly interstitial. In contrast, reactive fibrosis is observed in the absence of cell loss as a reaction to inflammation, involving diffuse increases in ECM deposition at sites unrelated to focal injury, and is primarily perivascular. During cardiac remodeling, reactive and reparative fibrosis usually coexist (176, 177).

Myocardial fibrosis accompanies different cardiac disease states, including cardiomyopathies, hypertensive heart disease, heart failure, myocardial infarction and sudden death (178-186) and has important functional consequences for the heart. First, increased ECM content results in exaggerated mechanical stiffness and contributes to diastolic dysfunction. Progressive increases in fibrosis can cause systolic dysfunction and left ventricular hypertrophy (LVH). Second, increased collagen content disrupts electrotonic connectivity between cardiac myocytes and provides an electrical substrate for reentrant arrhythmogenesis. Third, perivascular fibrosis surrounding intracoronary arterioles impairs myocyte oxygen availability, reduces coronary reserve, and exacerbates myocyte ischemia (177). Thus, cardiac fibrosis is probably one of the major biological determinant with fatal consequences in cardiac function.

Cardiac ECM is a highly differentiated structure and is composed of many different proteins (Fig 2.5). Myocytes are surrounded by a basement membrane composed primarily of nonfilamentous type IV collagen. Collagen fibrils are composed primarily of collagen I (~80%) and collagen III (~10%), and with smaller amounts of collagen IV, V, VI, elastin, laminin, fibronectin, proteoglycans, glycosaminoglycans. Additionally, ECM contains inactive forms of proteases and growth factors, which regulate cell function upon disruption of ECM. Myocardial ECM is normally subdivided into three components: the epimysium, which surrounds epicardium and endocardium; the perimysium, which groups myofibers into bundles; and the endomysium, which surrounds individual myocytes and connects them to capillaries (176, 177).
2.6. Signaling pathways in myocardial fibrosis

ECM remodeling comprises changes in fibroblast proliferation, as well as collagen synthesis, deposition and degradation, and they all must be tightly regulated to keep proper matrix homeostasis. Fibrillar collagen is synthesized as a precursor polypeptide, exported from the cell, and proteolytically processed to monomers, which are then cross-linked to produce the mature structure. Collagen synthesis is regulated transcriptionally by fibrogenic growth factors, particularly TGFβ, and posttranscriptionally by the rate-limiting enzyme prolyl-4-hydroxylase. Collagen degradation is accomplished by the matrix metalloproteinases (MMP), which proteolytically cleave fibrils to produce collagen remnants (177).

2.6.1. Matrix metalloproteinases

Matrix metalloproteinases are a major group of endogenous enzymes that regulate ECM composition, and thus play an important role in normal tissue remodeling, which affects many important biological processes such as cell migration, invasion, proliferation, apoptosis as well as in developmental processes i.e. morphogenesis, angiogenesis and wound healing (187). As they are capable of degrading all the...
matrices of different organs, including heart, they are also the driving force behind myocardial matrix remodeling (188-191).

MMPs are a family of zinc-containing endoproteinases that currently number 25 species that share structural domains but differ in substrate specificity, cellular sources and inducibility (192, 193). There are two principal types of MMPs: those that are secreted into the extracellular space and those that are membrane bound. However, based on their substrate specificity and primary structure, the MMP family can be subdivided into four groups: collagenases, gelatinases, stromelysins, and membrane-type MMPs (191, 193). The initial studies identified a number of MMPs that are expressed in normal mammalian myocardium (Table 2.2).

**Table 2.2. Representative MMP classes and their members identified in the mammalian myocardium.**

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Symbol</th>
<th>Substrate/Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagenases</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interstitial collagenase</td>
<td>MMP-1</td>
<td>Collagens I, II, III, IV, and basement membrane components</td>
</tr>
<tr>
<td>Neutrophil collagenase</td>
<td>MMP-8</td>
<td>Collagens I, II, III, gelatins</td>
</tr>
<tr>
<td>Collagenase 3</td>
<td>MMP-13</td>
<td>Collagens I, II, III,</td>
</tr>
<tr>
<td>Gelatinases</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gelatinase A</td>
<td>MMP-2</td>
<td>Gelatins, collagens I, IV, V, VII, and basement membrane components</td>
</tr>
<tr>
<td>Gelatinase B</td>
<td>MMP-9</td>
<td>Gelatins, collagens IV, V, XIV, and basement membrane components</td>
</tr>
<tr>
<td>Stromelysins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stromelysin 1</td>
<td>MMP-3</td>
<td>Fibronectin, laminin, collagens III, IV, IX, and MMP activation</td>
</tr>
<tr>
<td>Matrilysin</td>
<td>MMP-7</td>
<td>Fibronectin, gelatins, and basement membrane components</td>
</tr>
<tr>
<td>Membrane-type MMPs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MT1-MMP</td>
<td>MMP-14</td>
<td>Collagens I, II, III, fibronectin, laminin-1; activates pro-MMP-2 and pro-MMP13</td>
</tr>
</tbody>
</table>

This table has been adapted from (189) and (188).

The secreted MMPs are synthesized as inactive (latent) proenzymes (pro-MMP) which bind specific ECM proteins and remain proteolytically quiescent until activation by enzymatic cleavage (194-199). MMPs modulate progression of fibrosis by degradation of matrix components and modulation of collagen synthesis. MMPs are digesting matrix components in a substrate specific manner (Table 2.2). They
initiate the digestion of the collagen molecules, resulting in the release of fragmented matrix peptides, such as matrikines that have biological activity, and thus stimulate new connective tissue formation. Additionally, MMPs may release or activate additional biologically active factors with potent effects on matrix synthesis, such as growth factors PDGF, TGF-β and IGF (190). Collectively, MMPs, once activated, are capable of degrading the ECM. It is thus of great importance that their activity is tightly regulated.

In fact, increased MMPs expression and activation have been shown in various forms of failing heart in patients, as well as through the use of animal models and pharmacological in vitro studies. Increased activation of MMPs, have been shown in LV dysfunction, particularly in dilated cardiomyopathy and congestive heart failure in patients (200-204). Correlation between increased MMPs levels and LV hypertrophy progressing to LV-dilation and heart failure were demonstrated using different animal models, such as pacing LV-failure (205-207), pressure- and volume-overload (208), spontaneously hypertensive heart failure rats (SHHFR) (209-211), and myocardial infarction (MI) (212, 213). However, a direct evidence for a role of MMPs in pathological cardiac remodeling came from genetically manipulated animals. Mice with cardiac-specific over-expression of MMP-1 displayed compensatory myocyte hypertrophy with an increase in the cardiac collagen progressing with age to loss of cardiac interstitial collagen and marked deterioration of systolic and diastolic function (214). Cardiac-restricted over-expression of MMP-2 caused marked LV remodeling and dysfunction, progressing with age to phenotype consistent with DCM (215). It has been demonstrated that deletion of MMP-9 in mice led to attenuated LV-dilation, decreased collagen accumulation and reduced early cardiac rapture upon MI (216, 217) while deletion of MMP-9 activator, Urokinase-like plasminogen activator (uPA), protects against cardiac rupture post-MI (217). MMP-2 null mice show a reduction in the rupture rate after MI (218) and blunted the hypertrophic response after pressure-overload (219). MMP inhibition, either by substrate interaction or by their endogenous physiological inhibitors, the tissue inhibitors of MMPs (TIMPs) (190), has been shown to limit ECM destruction and improve myocardial structure and function in animal models (207, 219). Additionally, studies with genetic ablation of MMP inhibitors have also an important impact on understanding the MMP activity. In fact, deletion of TIMP-1 in mice, led to loss of MMP inhibition and caused LV dilation with ageing (220) and after MI (221). Loss of TIMP-3 function triggered
spontaneous LV dilatation, cardiomyocyte hypertrophy, and contractile dysfunction consistent with human dilated cardiomyopathy (222).

Targeting of MMP species that contribute to pathological myocardial remodeling in developing congestive heart failure, by pharmacological inhibitors, will likely hold a great therapeutic potential. Thus, it is of great importance to define the portfolio of myocardial-specific MMPs and to develop selective inhibition of these MMP species.

2.6.2. Transforming growth factor-β

Another protein that actively regulates ECM homeostasis is transforming growth factor-β (TGF-β), a multifunctional cytokine/growth factor, which initiates and terminates tissue repair (223), and whose sustained production underlies the development of myocardial fibrosis (224, 225).

The transforming growth factor-β family comprises four major subfamilies the Mullerian inhibitory substance (MIS) family, the inhibin/activin family, the bone morphogenic protein (BMP) family, and last, the TGF-β family. They all contain structurally related polypeptide growth factors, each capable of regulating an array of cellular processes including cell proliferation, lineage determination, differentiation, motility, adhesion, and death (224-227).

TGF-β factors signal via transmembrane receptors, the TGF-β receptor family, to intracellular mediators, the Smad family of ‘latent’ transcription factors. Smad 2 and 3 are receptor-specific Smads (R-Smads) that are phosphorylated on serine residues by the type I TGF-β receptor. Upon phosphorylation, the Smads form heteromeric complexes with a common-mediator Smad 4 and can then be translocated to the nucleus, where they regulate gene expression. Smads themselves are weak transcriptional activators and they require association with a number of cofactors. Inhibitory Smads (I-Smads) Smad6 or Smad7, can prevent R-Smad activation, by competing for binding for Smad2 and Smad3 to the TGF-β receptors and by enhancing receptor degradation. Additionally, Smads are known to interact with other transcription factors, which add complexity to the system (223, 226, 228).

In the heart, TGF-β is induced by injury (229, 230), pressure-overload (231, 232), and infusion of hypertrophic agents (233, 234), and its elevated levels are found during
cardiac hypertrophy, dilated cardiomyopathy and myocardial infarction (225, 235-237). Additionally, high levels of this growth factor correlate with cardiac fibrosis (231, 238, 239). TGF-β has numerous actions on the ECM homeostasis. It strongly and ubiquitously stimulate the production and deposition of ECM, by stimulating cells to increase several fold the synthesis of most matrix proteins, such as fibronectin, collagen, and proteoglycans (240, 241). With support to “synthetic phenotype” TGF-β inhibits most of the MMPs and induces TIMPs to prevent EMC degradation (242, 243). Last, it also modulates the expression of integrins and cellular adhesion to the matrix (224).

Mice with genetic disruption of three different isoforms of TGF-β have been generated (244-246) and revealed crucial role of TGF-β isoforms in embryogenesis and immune regulation, as the null mice show embryonic or postnatal lethality. Interestingly, TGF-β1 heterozygous mice exhibit a decrease in myocardial fibrosis and stiffness, improved LV compliance and increased survival over the life span (247), while TGF-β1 over-expression induces cardiac hypertrophy, by increasing interstitial fibrosis and hypertrophic growth of cardiomyocytes and enhance β-adrenergic signaling in vivo (248).

In conclusions, the TGF-β pathway appears to be an integral component of fibrosis within heart and storage of latent TGF-β, which can be activated following injury, may play important role in tissue repair by promoting new synthesis and deposition of extracellular matrix. Thus, TGF-β seems to be a promising target in early phase of cardiac injury. Unfortunately, excessive deposition of fibrotic tissue in the heart results in cardiac pathology, by reducing ventricular compliance, thus usage of TGF-β antagonists may be important in preventing progression of cardiac remodeling into heart failure. Thus, it has to be still elucidated if pro- or anti- TGF-β therapy would be more beneficial in treating pathological cardiac remodeling.
3. ROLE OF TRANSCRIPTION FACTORS IN CARDIAC HYPERTROPHY

Activation of intracellular signaling cascades, including G-proteins, gp130, calcineurin, and MAP-kinases as described above, usually results in phosphorylation, and activation/deactivation of several transcription factors, which are responsible for a shift in expression of plethora of genes involved in hypertrophic response.

3.1. Transcription factors in cardiac hypertrophy

Perhaps the best characterized is zinc-finger-containing transcription GATA4, which regulates cardiac development and a differentiation–specific gene program (249, 250). The over-expression of GATA4 in the heart resulted in cardiac hypertrophy (251), whereas its cardiac-specific deletion attenuated hypertrophy following both pressure-overload and exercise stimulation (252). The potential importance of another transcription factor, nuclear factor (NF)-κB, as a hypertrophic mediator in the adult heart was only recently shown. Mice lacking p50 protein showed reduced heart growth in response to chronic angiotensin II infusion (253) while cardiomyocyte-specific deletion of NF-κB attenuated hypertrophy after angiotensin II or isoproterenol infusion (254). Myocyte enhancer factor 2 (MEF2), a MAD-box-containing transcription factor that binds to the promoters of most skeletal and cardiac muscle-expressed differentiation-specific genes, has also been implicated in regulating hypertrophic gene expression (255). Particularly, over-expression of MEF2A and MEF2C in the heart induced dilated cardiomyopathy with a progressive loss of ventricular performance, and increased hypertrophic response following stress stimulation (256). Similarly, transgenic over-expression of MEF2D was sufficient to drive the fetal gene program and pathological remodeling of the heart (257). Furthermore, MEF2D-null mice were resistant to cardiac hypertrophy, fetal gene activation, and fibrosis in response to pressure-overload and beta-chronic adrenergic stimulation (257). A related MAD-box-containing transcription factor, serum response factor (SRF), also regulates the expression of numerous cardiac muscle-
specific genes (258). The over-expression of SRF in the heart resulted in hypertrophic cardiomyopathy (259), whereas the cardiac specific deletion of SRF caused dilated cardiomyopathy that was associated with significant defects in the expression of cardiac structural genes (260).

Several lines of evidence suggest that the transcription factors belonging to the activator protein-1 (AP-1) family, which is particularly activated by MAPK signaling (see above), plays a role in myocardial hypertrophy. During my thesis work, I further corroborated this assumption in vivo.

3.2. The AP-1 transcription factor family

The mammalian AP-1 transcription factor complex is a dimeric complex, composed of basic leucine zipper (bZIP) proteins. AP-1 proteins are subdivided into families of the JUN (c-Jun, JunB and JunD), FOS (c-Fos, FosB, Fra-1 and Fra-2), Jun dimerizations partners (JDP1 and JDP2) and the closely related activating transcription factor ATF (ATFa, ATF2, LRF1/ATF3, ATF4 and B-ATF) proteins (Fig. 3.1 A). Upon dimerization, different AP-1 complexes recognize different DNA-response elements in the promoter and enhancers of various genes and thus bind to the DNA backbone (Fig. 3.1 B, D) (261, 262).

While Fos proteins can only heterodimerize with other AP-1 proteins, Jun proteins can both homo- and hetero-dimerize. Some of the Maf proteins (v-Maf, c-Maf and Nrl) can also heterodimerize with c-Jun or c-Fos, whereas other Maf related proteins, including MafB, MafF, MafG and MafK, heterodimerize with c-Fos but not with c-Jun. In addition, other transcription factors, such as NFIL-6 (nuclear factor interleukin-6), NRF (nuclear respiratory factor) and NRL (neural retina leucine zipper), can dimerize with c-Jun or c-Fos (Fig. 3.1 C) (262).

AP-1 transcription factors participate in the regulation of a variety of cellular processes including cell proliferation, differentiation, apoptosis and oncogenesis. However, their specific functions and target gene regulation depend on the specific cell type in which they are expressed, as well as on specific stress stimuli (263, 264).
Figure 3.1. The AP-1 transcription factors. (A) AP-1 (activator protein 1) proteins include the JUN, FOS, ATF (activating transcription factor) protein families, with its respective members. (B) The different dimer combinations recognize different sequence elements in the promoters and enhancers of target genes. The leucine-zipper domain and the adjacent basic domain form a X-shaped -helical structure in the AP-1 complex, which binds to the DNA backbone. (C) c-JUN and c-FOS dimerization partners and the response elements that the dimers bind. (D) The main DNA response-element that are recognized by the AP-1 transcription factors: the TPA-response element (TRE), the cAMP-response element (CRE), the MAF-recognition elements (MAREs) and the antioxidant-response elements (AREs). This schematic has been adapted from (262).

3.3. The AP-1 transcription factors in cardiac hypertrophy

Several models in vitro and in vivo have shown that hypertrophic stimuli led to an early-immediate up-regulation of AP-1 transcription factors, particularly c-Jun, c-Fos, JunB, Fra-1 and Fra-2 (15, 265-267).
The AP-1 member JunD is specifically expressed in the developing heart and cardiovascular system, and although its function in development is dispensable (268), it has been shown to be involved in muscle cell differentiation (269). Additionally, reduced JunD expression has been reported in human failing hearts (270). It was recently reported that mice lacking junD develop less adaptive hypertrophy in heart after mechanical pressure-overload, while cardiomyocyte-specific over-expression of junD in mice resulted in spontaneous ventricular dilation and decreased contractility (267). The data regarding Fra-1 expression upon cardiac hypertrophic stimuli are discrepant. One study demonstrated that Fra-1 is not induced upon aortic banding (271), while the other showed a slight induction using the same stimulus (267). Similarly, in plated cardiomyocytes both repression and induction of Fra-1 expression was shown, depending on the stimulation (272). In vivo, a recent study has demonstrated that conditional fra-1 deletion leads to normal hypertrophic response, whereas fra-1 over-expression causes premature heart failure upon pressure-overload (267). Moreover, fra-1 transgenic mice simultaneously lacking junD revealed a spontaneous congestive heart failure associated with increased cardiomyocyte apoptosis and a primary mitochondrial defect. These data suggest that junD promotes both adaptive-protective and maladaptive hypertrophy in heart depending on its expression levels.

Particularly, c-Jun and c-Fos were shown to be induced in both plated neonatal cardiomyocytes and animal models, by hypertrophic stimuli, such as phenylephrine, isoproterenol, mechanical stretch and pressure-overload (267, 271, 273-278). Several approaches in vitro tried to verify the role of c-Jun and c-Fos in the development of hypertrophy. Transfections of cultured cardiomyocytes or rat hearts with a dominant negative form of c-Jun were shown to inhibit cardiac myocyte hypertrophy in response to phenyephrine, endothelin and angiotensin II (279, 280). Another study using a dominant negative mutant of c-Fos confirmed a contribution of c-Fos/AP-1 for induction of the pathological/fetal gene program, however its role in cardiac hypertrophy does not appear to be critical (281). Studies on isolated adult ventricular myocytes have shown that both α- and β-adrenergic stimulation are associated with an increase in c-Jun and c-Fos. Blockage of these pathway by CRE decoy oligonucleotides was shown to prevent α- but not β-adrenergic-mediated hypertrophy (282). Up to date, there is no genetic evidence in vivo, unravelling the role of c-Jun
and c-Fos in controlling the transcriptional networks in the myocardium. However, interesting results were obtained by targeted disruption of c-Jun. c-Jun knock-out mice are embryonic lethal and c-Jun<sup>−/−</sup> fetuses show malformations in the outflow track of the heart, suggesting that this factor may also have an important developmental role (283). It was shown that JunB can largely substitute for c-Jun in vivo, as homozygous knock-in mice with JunB expressed from the c-Jun locus develop to birth and show normal liver morphogenesis. Still, those mice showed a malformed cardiac outflow tract suggesting that JunB can compensate for c-Jun in the liver but not in the heart during development (284). In contrast, although c-Fos was shown to be involved in the development and function of many distinct tissues, as the c-fos null mice suffer from developmental malformations, i.e. osteopetrosis, lymphopenia and delayed or absent gametogenesis, these mice did not display any perturbations in heart development (285).

Yet there is no evidence in vivo that c-Jun and c-Fos are causally involved in triggering hypertrophy and remodeling of the adult heart in response to stress.
VI. AIM OF THE PROJECT

Upon various stress stimuli, the heart adapts with a hypertrophic response with the aim to maintain its function. However, epidemiological studies have demonstrated that a prolonged period of “compensation” is followed by a “decompensation”, resulting in heart failure and dilated cardiomyopathy, all together being the major cause of death in the Western world (2).

The hypertrophic response results from activation of multiple cytoplasmic signal transduction cascades, which ultimately affect the activation of transcription factors and subsequently the regulation of gene expression. Several studies have shown an early-immediate induction of AP-1 transcription factors, particularly c-Jun and c-Fos, upon hypertrophic stimuli such as pressure-overload (267, 271, 277, 278). However, none of these reports has employed a genetic approach to study in detail the involvement of these factors in transducing the hypertrophic signal in vivo.

Therefore, the aim of my project was to elucidate the in vivo functions of c-Jun and c-Fos in the heart, specifically in the development of cardiac hypertrophy and transition to heart failure.
VII. EXPERIMENTAL PROCEDURES

1. Generation of \(c-jun^{\Delta mu}\) and \(c-fos^{\Delta mu}\) mice

c-\(jun^{f/f}\) and c-\(fos^{f/f}\) mice (kind gift from Prof Erwin F. Wagner, CNIO, Madrid, Spain) were generated as described (286, 287). Mice harbouring deletion of c-\(jun\) or c-\(fos\) in skeletal muscle and cardiomyocytes (c-\(jun^{\Delta mu}\) or c-\(fos^{\Delta mu}\)) were obtained by crossing c-\(jun^{f/f}\) or c-\(fos^{f/f}\) mice with muscle creatin kinase transgenic line (MCK-cre) (288) (kind gift from Prof Joseph M. Penninger, IMBA, Vienna, Austria).

2. Southern blotting

Genomic DNA was extracted from heart, skeletal muscle and liver of c-\(fos^{f/f}\) and c-\(fos^{\Delta mu}\) mice, and from heart, skeletal muscle and kidney of c-\(jun^{f/f}\) and c-\(jun^{\Delta mu}\) mice, according to standard protocol. Then DNA (20 \(\mu g\)) from organs of c-\(fos^{f/f}\) and c-\(fos^{\Delta mu}\) mice or c-\(jun^{f/f}\) and c-\(jun^{\Delta mu}\) mice was digested with BamHI or XbaI restriction enzymes, respectively. Next, DNA was separated on 1.2% agarose gels at 4V/cm. Gels were de-purinated by incubation in 0.5 M HCl for 10 minutes, denatured in 0.5M NaOH, 1.5M NaCl for 40 minutes, and neutralised in 0.5M Tris HCl pH 7.5, 1.5M NaCl for 40 minutes. DNA was blotted capillarly on the nylon membrane (ZetaProbe), and then UV-cross-linked to the membrane (Stratagene). Probes hybridization was performed as described (286, 287).

3. Mouse experiments

Age- (8- to 12-week-old) and weight- (22-26 g) matched mice were subjected to transverse-aortic constriction (TAC) through constriction of the descending aorta as described (289), using 27G needle. Mice were monitored for 6 weeks after surgery, then sacrificed and heart and body weight measurements were taken. Heart functions were determined by echocardiography 5.5 weeks after surgery. Number of
experimental animals is indicated in respective figure legends. All procedures involving animals were approved by the "Veterinäramt des Kantons Zürich" and conform to the relevant regulatory standards.

4. Echocardiography

Echocardiography was carried out in collaboration with Prof T. Pedrazzini and A. Felley, University of Lausanne Medical School, and performed as described before (63). Briefly, echocardiographic measurements of mice were carried out using an ATL HDI 5000 ultrasound device (Philips Medical Systems) equipped with 12 Mhz phase array linear transducer (L-12-5). M-mode images were used for measurements of IVSd, IVSs, LVIDd, LVIDs, LVPWd and LVPWs. Formulas used for other parameters, as well as table of abbreviations, are described in Table 1.

Table 1. Echocardiographic measurements.

<table>
<thead>
<tr>
<th>Annotation</th>
<th>Full name</th>
<th>Mode</th>
<th>Units</th>
<th>Formula</th>
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<td>HR</td>
<td>Heart rate</td>
<td>B</td>
<td>bmp</td>
<td>-</td>
</tr>
<tr>
<td>IVSd</td>
<td>Interventricular septum in diastole</td>
<td>M</td>
<td>mm</td>
<td>-</td>
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<td>IVSs</td>
<td>Interventricular septum in systole</td>
<td>M</td>
<td>mm</td>
<td>-</td>
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<tr>
<td>LVIDd</td>
<td>Left ventricular internal diameter in diastole</td>
<td>M</td>
<td>mm</td>
<td>-</td>
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<tr>
<td>LVIDs</td>
<td>Left ventricular internal diameter in systole</td>
<td>M</td>
<td>mm</td>
<td>-</td>
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<tr>
<td>LVPWd</td>
<td>Left ventricular free wall in diastole</td>
<td>M</td>
<td>mm</td>
<td>-</td>
</tr>
<tr>
<td>LVPWs</td>
<td>Left ventricular free wall in systole</td>
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<td>mm</td>
<td>-</td>
</tr>
<tr>
<td>LV Mass uncorrected</td>
<td>Left ventricular mass uncorrected</td>
<td>M</td>
<td>mg</td>
<td>1.05 LVIDd + LVPWd + IVSd^3 - LVIDd^3</td>
</tr>
<tr>
<td>LV Vold</td>
<td>Left ventricular internal volume in diastole</td>
<td>M</td>
<td>ul</td>
<td>[\frac{[7.0 \times (2.4 + LVIDd)]}{LVIDd^3} \times 1000 ]</td>
</tr>
<tr>
<td>LV Vols</td>
<td>Left ventricular internal volume in systole</td>
<td>M</td>
<td>ul</td>
<td>[\frac{[7.0 \times (2.4 + LVIDs)]}{LVIDs^3} \times 1000 ]</td>
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<td>% FS</td>
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<td>%</td>
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<tr>
<td>% EF</td>
<td>Ejection Fraction</td>
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<td>%</td>
<td>[\frac{(LV Vold - LV Vols)}{LV Vold} \times 100 ]</td>
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<td>ul/min</td>
<td>Heart rate \times\ stroke volume</td>
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<tr>
<td>LVID Trace (SV)</td>
<td>Stroke volume</td>
<td>M</td>
<td>ul</td>
<td>LV Vold - LV Vols</td>
</tr>
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</table>
5. Histology

After extraction, hearts were collected for paraffin-sections. Organs were fixed in 4% formalin (Medite) for 24h at +4°C. Before embedding in paraffin blocks, organs were brought through solutions: PBS (phosphate-buffered saline) for 30 minutes at +4°C, 0.85% NaCl in water for 30 minutes at room temperature, two changes of 50% ethanol in 0.85% NaCl for 20 minutes each at room temperature, two changes of 70% ethanol in PBS for 20 minutes each at room temperature. Then, organs were brought to liquid paraffin using Medite Robot TPC 15, through solutions: 70% ethanol for 45 minutes, 80% ethanol for 45 minutes, 90% ethanol for 30 minutes, three changes of 100% ethanol for 30, 60 and 60 minutes each, three changes of xylene for 30, 45 and 60 minutes each, all at 37°C, and finally two changes of paraffin at 62°C, and finally were embedded in paraffin blocks using Medite Robot (TES 99). Last, hearts were sectioned using microtom (Microm, HM 355 S) at 5μm intervals.

6. Histological stainings

For histological stainings, sections were deparaffinised and hydrated through incubation in two changes of xylene, two changes of 100% ethanol, two changes of 95% ethanol, two changes of 70% ethanol, two changes of water, for 5 minutes each, at room temperature, and then stored in PBS until use. H&E staining was performed using Medite Robot COT 20, according to standard protocol. Elastin van Gieson (EvG) staining was performed as combinational staining of Van Gieson’s staining for collagen fibres and Verhoeff’s staining for elastic fibres and nuclei. After histological staining, sections were dehydrated in single changes of 70% ethanol, 95% ethanol, 100% ethanol, and two changes of xylene, and then embedded with Entellan (Merck), and dried for 24 hours at room temperature.

7. Western blotting
Western blotting using total heart extracts was performed according to standard procedures. Antibodies used in the study: α-c-Fos (Santa Cruz), α-c-Jun (Santa Cruz), α-GFP (Santa Cruz), α-phospho-Smad2 (Cell Signaling), α-Smad2 (Cell Signaling), α-Tubulin (Sigma), α-GAPDH (Santa Cruz), α-Actin (Santa Cruz).

8. Gelatin zymography

Gelatin zymography of total heart proteins was performed as described previously (203) with minor changes. Briefly, samples (100 µg of proteins) were mixed with Laemmli sample loading buffer without β-mercaptoethanol and without boiling were loaded on 10% SDS-polyacrylamide gels containing 2mg/ml gelatin type A from porcine skin (Sigma). After electrophoresis, gels were washed 2 times for 30 minutes in 2.5% Triton X-100 to allow proteins to renature, and then for 10 minutes in 100 mM Tris-HCl pH 7.4. Gels were then incubated at 37°C overnight in developing buffer (50 mM Tris-HCl, pH 7.5, 10 mM CaCl2, 1 µmol/L ZnCl2). Last, to reveal zones of lysis, gels were stained for 30 minutes with 0.5% Coomassie blue R250 and destained for 4 hours with 40%:10% v:v methanol:acetic acid, and then with 5%:7.5% v:v methanol:acetic acid until stacking gel became colorless.

9. Quantitative RT-PCR

RNA was purified from total hearts using TRIzol Reagent (Invitrogen) according to manufacturer’s instructions. 5µg of RNA was used as a template to synthesize cDNA, using Ready-To-Go You-Prime First-Strand Beads (Amersham). Qantitative RT-PCR reactions were set up as recommended by the manufacturer (Roche) and were run an analyzed on the Roche LightCycler 480. Primer sequences are indicated in Table 2.
<table>
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<th>Name</th>
<th>Accession</th>
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<td>SERCA2 (sarco-endo-plasmic reticulum Ca2+ ATPase 2)</td>
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<td>ATGCGAAGACTACAGAAGTTG                  GGCTATTCTGACTTCTCAGTA</td>
</tr>
<tr>
<td>Myot (myotilin)</td>
<td>NM_0010336 21</td>
<td>F</td>
<td>ATGCGAAGACTACAGAAGTTG                  GGCTATTCTGACTTCTCAGTA</td>
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<tr>
<td>MyBP-C2 (myosin binding protein C, fast-type)</td>
<td>NM_146189</td>
<td>F</td>
<td>ATGCGAAGACTACAGAAGTTG                  GGCTATTCTGACTTCTCAGTA</td>
</tr>
<tr>
<td>TPM2 (tropomyosin 2, beta)</td>
<td>NM_009416</td>
<td>F</td>
<td>ATGCGAAGACTACAGAAGTTG                  GGCTATTCTGACTTCTCAGTA</td>
</tr>
<tr>
<td>Ribosomal S18</td>
<td>NM_009416</td>
<td>F</td>
<td>ATGCGAAGACTACAGAAGTTG                  GGCTATTCTGACTTCTCAGTA</td>
</tr>
</tbody>
</table>
10. TUNEL Assay

TUNEL Assay (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) (Roche) was used to assess number of apoptotic cells. Staining was performed according to the manufacture instruction. Apoptotic cells were quantified within the heart are on the section. Number of apoptotic cells on the particular section was related to total heart area on the respective section. Three independent sections per mouse were analyzed.

11. Affymetrix gene expression profiling

Affimetrix gene expression profiling was performed in collaboration with Functional Genomic Center Zürich. Total RNA was extracted from the hearts of 10 weeks old c-jun<sup>Δμ</sup> (n=2) and c-jun<sup>exnu</sup> (n=2) mice using TRizol Reagent (Invitrogen). The quality of the RNA was determined with a NanoDrop ND 1000 (NanoDrop Technologies) and a Bioanalyzer 2100 (Agilent). 1 µg of RNA was reverse-transcribed into double-stranded cDNA with One-Cycle cDNA Synthesis Kit (Affymetrix), purified using a Sample Cleanup Module (Affymetrix) and then in vitro transcribed in presence of biotinylated nucleotides using IVT Labeling Kit (Affymetrix). Then, biotinylated cRNA was purified and its quality and quantity was determined, as described above. 5 µg of biotin-labeled cRNA was fragmented randomly to 35-200 bp and hybridized to GeneChip® GeneChip Mouse Genome 430 2.0 Arrays. The fluorescent intensity emitted by the labeled target was measure in Affymetrix GeneChip Scanner 3000 (Affymetrix). Raw data processing was performed using the Affymetrix AGCC software. Genes with significant expression difference between 2 knock-out mice and 2 wt were selected, based on the average knock-out versus wild-type value greater than 1.3 fold change with a p-value cutoff of 0.05 (using Student’s t-test).

12. Statistical analysis

Statistical significance was calculated using an ANOVA with post-hoc Tukey’s test and Student unpaired t test. Significance was accepted at the level of p < 0.05.
VIII. RESULTS

1. Cardiomyocyte-specific c-fos knock-out mice do not show any spontaneous heart phenotype

It has been shown that c-Fos is involved in the development and function of many distinct tissues, as the c-fos null mice suffer from developmental malformations, i.e. osteopetrosis, lymphopenia and delayed or absent gametogenesis (285). To overcome this phenotype, mice with cardiomyocyte-specific deletion of c-fos (c-fos<sup>Δmu</sup>) were generated, using Cre-loxP recombination system (recombination under control of muscle creatin kinase (MCK) promoter). c-fos cardiomyocyte-specific knock-out mice (c-fos<sup>Δmu</sup>) were born with Mendelian frequencies and did not show any obvious gross developmental abnormalities, as compared to wild-type mice (c-fos<sup>f/f</sup>). The correct targeting was confirmed by Southern and Western blotting (Fig 1). As recombination under the control of MCK-cre promoter leads to gene deletion in skeletal muscle and cardiomyocytes (288), a deleted band (Δ band) can be observed in MCK-cre positive hearts and skeletal muscles but not in liver (Fig 1A). Analysis of c-Fos protein abundance in total heart extracts showed significant decrease of c-Fos protein levels in conditional knock-out mice, as compared to wild-type mice (Fig 1B). Tissue-specific expression of Cre-recombinase leads to excision of exons 2-4 and subsequent expression of green fluorescent protein (GFP) (287) (Fig 1B).

To investigate the role of c-Fos in heart function upon basal (physiological) conditions, mice were aged for 6 months and then cardiac integrity was assessed (Fig 2). c-fos<sup>Δmu</sup> mice did not differ from c-fos<sup>f/f</sup> mice as demonstrated by heart-to-body weight (H/BW) ratios (Fig 2A), and H&E staining of heart cross-sections (Fig 2B).
Fig 1. Generation of c-fos<sup>Δmu</sup> mice. (A) Southern blot analysis of genomic DNA from total heart, skeletal muscle and liver extracts. Deleted band (Δ) occurs only in MCK-cre positive samples from heart and skeletal muscle, while floxed band (flox) is present in all samples as recombination is not complete due to the fact that only 30 to 40% of cells in heart and skeletal muscle are muscle cells. (B) Western blot analysis of c-Fos protein levels in total heart extracts. Significant decrease of c-Fos is seen in hearts from c-fos<sup>Δmu</sup> mice as compared to c-fos<sup>fl</sup> mice. Expression of Cre-recombinase in the heart leads to expression of GFP. Actin was used as a loading control.
Fig 2. c-fos<sup>Δmu</sup> mice do not display any spontaneous alterations in the heart. (A) H/BW ratios are not altered in c-fos<sup>Δmu</sup> mice, as compared to c-fos<sup>+/+</sup> mice. Data are presented as values ± SEM. ns = not significant, p = 0.65; WT n=9, KO n=8. (B) Histological analyses. H&E staining (upper panel) and EvG staining (lower panels) of heart sections display normal heart integrity in c-fos knock-out mice.
2. Deletion of \( c-fos \) in cardiomyocytes does not alter heart function and physiology upon mechanical pressure-overload

Next, I challenged \( c-fos^{\Delta \mu} \) mice with transverse-aortic constriction (TAC) a well-known model to study left ventricular hypertrophy (Table 1, Fig 3-5). First, the response of \( c-fos^{\Delta \mu} \) mice to cardiac stress was measured by echocardiography (Table 1). At baseline, there were no significant differences between \( c-fos^{f/f} \) and \( c-fos^{\Delta \mu} \) mice. 6 weeks after TAC, both \( c-fos^{f/f} \) and \( c-fos^{\Delta \mu} \) mice showed enhanced growth of the left ventricle, when compared to sham-operated mice, as indicated by an increase in interventricular septum (IVS) thickness, increase in left ventricular free wall (LVPW) thickness, and decrease in left ventricular internal diameter (LVID), and in general by increased left ventricle mass (LV mass). Additionally, left ventricle volume (LV Vol) was decreased in both TAC-operated groups, when compared to sham-operated mice. Last, cardiac performance was assessed by calculating functional parameters, such as fractional shortening (FS), ejection fraction (EF), cardiac output (LVID CO), and stroke volume (LVID SV). Both wild type and knock-out mice showed enhanced cardiac work in response to TAC, when compared to unchallenged mice, as an increase in all mentioned parameters was observed. These data suggest that \( c-fos^{f/f} \) and \( c-fos^{\Delta \mu} \) mice developed adaptive cardiac hypertrophy upon TAC.

I then assessed the integrity of the hearts post-mortem (Fig 3-5). First, concentric growth of hearts from TAC operated animals was confirmed, as significant increases in H/BW ratios after TAC in both \( c-fos^{f/f} \) and \( c-fos^{\Delta \mu} \) mice were observed, when compared to sham-operated mice \((p < 0.05)\) (Fig 3A). No significant changes in H/BW ratios were noticed between sham-operated \( c-fos^{f/f} \) and \( c-fos^{\Delta \mu} \) mice \((p = 0.45)\), as well as TAC-operated \( c-fos^{f/f} \) and \( c-fos^{\Delta \mu} \) mice \((p = 0.84)\). Secondly, histological analysis of cross-sections of hearts displayed a slight increase in left ventricular thickness in TAC-operated \( c-fos^{f/f} \) and \( c-fos^{\Delta \mu} \) mice (Fig 3B, upper panels). Additionally, no signs of pathological remodeling were noticed, as Elastin van Gieson (EvG) staining did not reveal any cardiac fibrosis in all analyzed groups (Fig 3B, lower panels). Thirdly, I analyzed mRNA levels of canonical hypertrophic markers (Fig 4). Natriuretic peptides, atrial natriuretic peptide (ANF) and brain natriuretic peptide (BNP), were increased in TAC-stimulated hearts from \( c-fos^{f/f} \) and
c-fos^αnu mice (p < 0.05), as compared to sham-operated hearts. Similarly, I observed up-regulation of contractile proteins, skeletal muscle alpha-actin (ACTA1) and beta-myosin heavy chain (β-MHC) in TAC-induced hearts of c-fos^+/+ and c-fos^αnu mice (p < 0.05), while alpha-myosin heavy chain (α-MHC) was expressed at similar levels in all groups. Last, sarco(endo)plasmic reticulum Ca2+ -ATPase 2 (Serca-2) showed a similar pattern of induction in TAC-operated hearts of wild-type and knock-out animals (p < 0.05). Conclusively, no difference in hypertrophic marker expression was observed between c-fos^+/+ and c-fos^αnu mice upon basal conditions as well as upon TAC. Finally, I performed TUNEL staining, to assess the apoptotic rate in response to TAC, as it was shown that prolonged hypertrophic stimuli cause cardiomyocyte cell death (Fig 5). However, TUNEL staining displayed a similar degree of apoptosis in sham- and TAC-operated wild-type and knock-out hearts (Fig 5A). That was further confirmed by quantification of TUNEL positive nuclei, as similar numbers of apoptotic nuclei were counted in all experimental groups (Fig 5B). These data demonstrate that in my experimental approach, 6 weeks of left-ventricular pressure-overload is not sufficient to induce cell death, neither in c-fos^+/+ nor in c-fos^αnu mice. Overall, these results demonstrate that expression of c-Fos in cardiomyocytes is not required for postnatal cardiac growth as well as for cardiac hypertrophy in response to TAC. Moreover, they demonstrate that deletion of c-Fos does not lead to premature pathologic remodeling of the myocardium and heart failure.
Table 1. Echocardiographic analyses in c-foxΔμ μ and corresponding control mice at baseline and after TAC.

<table>
<thead>
<tr>
<th>Data measure</th>
<th>c-fox+/f sham</th>
<th>c-fox+/f TAC</th>
<th>c-foxΔμ μ sham</th>
<th>c-foxΔμ μ TAC</th>
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<tr>
<td>HR, bpm</td>
<td>492 ± 23</td>
<td>525 ± 38</td>
<td>456 ± 14</td>
<td>525 ± 32</td>
</tr>
<tr>
<td>IVSd, mm</td>
<td>0.75 ± 0.015</td>
<td>0.86 ± 0.028 #</td>
<td>0.79 ± 0.013</td>
<td>0.89 ± 0.013 †</td>
</tr>
<tr>
<td>IVSs, mm</td>
<td>0.97 ± 0.008</td>
<td>1.14 ± 0.031 #</td>
<td>1.00 ± 0.010 ¶</td>
<td>1.18 ± 0.026 †</td>
</tr>
<tr>
<td>LVPWd, mm</td>
<td>0.79 ± 0.019</td>
<td>0.92 ± 0.024 #</td>
<td>0.79 ± 0.109</td>
<td>0.92 ± 0.011 †</td>
</tr>
<tr>
<td>LVPWs, mm</td>
<td>0.99 ± 0.011</td>
<td>1.15 ± 0.020 #</td>
<td>1.00 ± 0.021</td>
<td>1.14 ± 0.016 †</td>
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<tr>
<td>LVIDd, mm</td>
<td>3.84 ± 0.075</td>
<td>3.84 ± 0.112</td>
<td>3.97 ± 0.056</td>
<td>3.73 ± 0.144</td>
</tr>
<tr>
<td>LVIDs, mm</td>
<td>2.78 ± 0.068</td>
<td>2.62 ± 0.107</td>
<td>2.88 ± 0.047</td>
<td>2.48 ± 0.141 †</td>
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<td>LV Mass, mg</td>
<td>102.72 ± 3.78</td>
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<td>112.99 ± 2.82</td>
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<td>LV Vold, ml</td>
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<td>59.78 ± 5.56</td>
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<td>LV Vols, ml</td>
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<td>22.38 ± 3.27 †</td>
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<tr>
<td>FS, %</td>
<td>27.52 ± 0.63</td>
<td>31.79 ± 0.94 #</td>
<td>27.47 ± 0.68</td>
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<td>EF, %</td>
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<td>53.93 ± 1.04</td>
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<td>LVID Trace (CO), ml/min</td>
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<td>17.31 ± 1.47</td>
<td>20.53 ± 1.46</td>
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<tr>
<td>LVID Trace (SV), ml</td>
<td>33.38 ± 1.73</td>
<td>39.70 ± 2.42 #</td>
<td>36.10 ± 1.26</td>
<td>31.11 ± 3.27</td>
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</tbody>
</table>

All values are shown as mean ± SEM. n = 4-6 per group. p < 0.05 is indicated as: # WT TAC vs WT sham; † KO TAC vs KO sham; ¶ KO sham vs WT sham; § KO TAC vs WT TAC. HR, Heart rate; IVSd, Interventricular septum in diastole; IVSs, Interventricular septum in systole; LVPWd, Left ventricular free wall in diastole; LVPWs, Left ventricular free wall in systole; LVIDd, Left ventricular internal diameter in diastole; LVIDs, Left ventricular internal diameter in systole; LV Mass, Left ventricular Mass; LV Vold, Left ventricular Volume in diastole; LV Vols, Left ventricular Volume in systole; FS, Fractional Shortening; EF, Ejection Fraction; LVID Trace (CO), LVID Trace (Cardiac output); LVID Trace (SV), LVID Trace (Stroke volume).
**Fig 3.** *c-fos*Δ*μ* mice show similar concentric heart hypertrophy upon TAC as control mice. (A) H/BW ratios increased significantly in both *c-fos*Δ*μ* and *c-fos*Δ*μ* mice upon TAC. Data are presented as values ± SEM. (*) p < 0.05; n = 4-6 per group. (B) Histological analyses. H&E staining of heart cross-sections showed a slight increase in left-ventricle wall thickness in both TAC-operated groups (upper panels). EvG staining (lower panels) does not display any signs of cardiac fibrosis in all analysed groups.
Fig 4. Expression of hypertrophic markers in hearts of c-fosΔmu and control mice subjected to TAC. ANF, BNP, ACTA1, β-MHC and Serca-2 are induced in hypertrophied hearts of c-fosΔ and c-fosΔmu mice, while α-MHC is expressed at steady-state levels. (*) p < 0.05; (ns) = not significant, p = 0.062; n = 4-6 per group. Data are presented as values ± SEM.
Fig 5. Assessment of apoptosis in hearts of c-fosΔmut and corresponding control mice subjected to TAC. (A) A similar degree of apoptosis is observed in all experimental groups. (B) Quantification of TUNEL positive nuclei does not show any difference between hearts of c-fosff and c-fosΔmut mice neither at baseline nor upon TAC. Data are presented as values ± SEM.
3. Deletion of c-jun in cardiomyocytes causes mild cardiac fibrosis without affecting cardiac function

It has been shown that c-Jun is essential for normal mouse development and organogenesis, as embryos lacking c-jun die at mid-gestation with defects in heart morphogenesis and increased apoptosis in liver (283, 290, 291). To investigate the role of c-Jun in cardiac function, c-Jun was inactivated specifically in cardiomyocytes (c-jun\textsuperscript{Δmu}) using again the MCK-cre transgenic line. c-jun knock-out mice (c-jun\textsuperscript{Δmu}) were born at Mendelian frequencies and did not show any obvious gross developmental abnormalities, as compared to wild-type mice (c-jun\textsuperscript{f/f}). The deletion of c-Jun was confirmed at the DNA and at the protein level (Fig 6). Using Southern blotting, the deleted band (Δ band) could be observed in MCK-cre positive hearts and skeletal muscles but not in kidney (Fig 6A). By Western blotting, a significant decrease of c-Jun protein levels in total heart extracts from knock-out mice could be demonstrated, as compared to wild-type mice (Fig 6B).

I next investigated the role of c-Jun in heart function in aged 6 months old mice. H/BW ratios were unaltered in c-jun\textsuperscript{Δmu} mice, as compared to c-jun\textsuperscript{f/f} mice (Fig 7A). Also the analysis of heart cross-sections by H&E did not show any differences in heart morphology between wild-type an knock-out mice (Fig 7B, upper panel). However, EvG staining unravelled mild fibrosis in hearts of c-jun\textsuperscript{Δmu} mice (Fig 7B, lower panel). Observed fibrosis, however, did not have any functional consequences, as echocardiographic parameters in c-jun knock-out mice did not differ from those measured in wild-type mice (Table 2).
Fig 6. Generation of \textit{c-jun}^{Δmu} mice. (A) Southern blot analysis of genomic DNA from total heart, skeletal muscle and kidney extracts. Deleted band (Δ band) occurs only in samples from MCK-cre positive heart and skeletal muscle, while floxed band (flox) is present in all samples as recombination is not complete due to the fact that only 30 to 40\% of cells in heart and skeletal muscle are muscle cells. (B) Western blot analysis of c-Jun protein levels in total heart extracts. A significant decrease of c-Jun can be observed in hearts from \textit{c-jun}^{Δmu} mice as compared to hearts from \textit{c-jun}^{+/}\textit{f} mice. Actin was used as a loading control.
Fig 7. *c-Jun<sup>Δmu</sup>* mice show no gross spontaneous structural defects in the heart. (A) H/BW ratios are not altered in *c-Jun<sup>Δmu</sup>* mice as compared to *c-Jun<sup>f/f</sup>* mice. Data are presented as values ± SEM. ns = not significant, p = 0.81; WT n=6, KO n=7. (B) Histological analyses. H&E staining of heart sections displays normal heart structure in *c-Jun<sup>Δmu</sup>* mice (upper panel), but EvG staining displays mild fibrosis in *c-Jun<sup>Δmu</sup>* mice (lower panel, violet staining).
Table 2. Echocardiographic assessment of heart function in c-jun<sup>Δmu</sup> and corresponding control mice.

<table>
<thead>
<tr>
<th>Data measure</th>
<th>c-jun&lt;sup&gt;ff&lt;/sup&gt; (mm)</th>
<th>c-jun&lt;sup&gt;Δmu&lt;/sup&gt; (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVSd, mm</td>
<td>0.57 ± 0.016</td>
<td>0.58 ± 0.014</td>
</tr>
<tr>
<td>IVSs, mm</td>
<td>0.66 ± 0.019</td>
<td>0.66 ± 0.021</td>
</tr>
<tr>
<td>LVPWd, mm</td>
<td>0.59 ± 0.023</td>
<td>0.56 ± 0.021</td>
</tr>
<tr>
<td>LVPWs, mm</td>
<td>0.76 ± 0.016</td>
<td>0.73 ± 0.039</td>
</tr>
<tr>
<td>LVIDd, mm</td>
<td>4.42 ± 0.076</td>
<td>4.38 ± 0.088</td>
</tr>
<tr>
<td>LVIDs, mm</td>
<td>3.46 ± 0.070</td>
<td>3.43 ± 0.106</td>
</tr>
<tr>
<td>FS, %</td>
<td>21.87 ± 0.41</td>
<td>21.74 ± 1.10</td>
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<tr>
<td>EF, %</td>
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<td>44.19 ± 1.95</td>
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<td>LV Mass, mg</td>
<td>91.1 ± 5.15</td>
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<td>LV VOLd, ml</td>
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<td>LV VOLs, ml</td>
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<td>19.34 ± 0.67</td>
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<tr>
<td>LVID Trace (SV), ml</td>
<td>39.6 ± 1.69</td>
<td>38.9 ± 0.94</td>
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</table>

All values are shown as mean ± SEM. WT n=6, KO sham n=5. HR, Heart rate; IVSd, Interventricular septum in diastole; IVSs, Interventricular septum in systole; LVPWd, Left ventricular free wall in diastole; LVPWs, Left ventricular free wall in systole; LVIDd, Left ventricular internal diameter in diastole; LVIDs, Left ventricular free wall in systole; LV Mass, Left ventricular Mass; LV VOLd, Left ventricular Volume in diastole; LV VOLs, Left ventricular Volume in systole; FS, Fractional Shortening; EF, Ejection Fraction; LVID Trace (CO), LVID Trace (Cardiac output); LVID Trace (SV), LVID Trace (Stroke volume).
4. Deletion of c-jun in cardiomyocytes leads to heart dilation and impaired cardiac function upon hypertrophic stimulus

In order to examine functions of c-Jun in the heart physiology and adaptation to hypertrophic stress, I subjected c-jun\textsuperscript{Δmu} mice to TAC (Table 3, Fig 8-10). First, hypertrophic response was assessed by echocardiography (Table 3). After 6 weeks, wild-type mice subjected to TAC showed hypertrophic growth of left ventricle, when compared to sham-operated mice, as an increase in interventricular septum (IVS) thickness, increase in left ventricular free wall (LVPW) thickness, and decrease in left-ventricular internal diameters (LVID) were measured. Furthermore, c-jun\textsuperscript{ff} mice showed increased left ventricle mass (LV mass) and decreased left ventricle volume (LV Vol) after TAC, when compared to sham-operated mice. Last, assessment of cardiac performance by calculating functional parameters, such as fractional shortening (FS), ejection fraction (EF), cardiac output (LVID CO), and stroke volume (LVID SV), confirmed concentric hypertrophy that was developed in wild-type mice upon TAC. At baseline, there were no significant differences between c-jun\textsuperscript{ff} and c-jun\textsuperscript{Δmu} mice, however, TAC induced different response in c-jun knock-out mice than in wild-type mice. Primarily, c-jun\textsuperscript{Δmu} mice displayed decreased IVS, increased LVPW and LVID, when compared to sham-operated mice, suggesting eccentric growth of left-ventricle. Although TAC increased LV mass in knock-out mice, as it did in wild-type mice, LV volume was increased, suggesting left-ventricle dilation. Finally, cardiac performance was greatly impaired in TAC-operated c-jun\textsuperscript{Δmu} mice, as a decrease in FS, EF, CO and SV was observed, in comparison to sham-operated c-jun\textsuperscript{Δmu} mice and TAC-operated c-jun\textsuperscript{ff} mice. Thus, those data suggest that in contrast to wild-type mice, c-jun knock-out mice developed eccentric hypertrophy and heart dilation upon left-ventricle pressure-overload.

A post-mortem analysis of hearts revealed more differences between wild-type and knock-out mice in response to TAC (Fig 8-10). Although both c-jun\textsuperscript{ff} and c-jun\textsuperscript{Δmu} mice showed significantly increased H/BW ratios upon TAC (p < 0.05) when compared with sham-treated animals, no difference between unchallenged c-jun\textsuperscript{ff} and c-jun\textsuperscript{Δmu} mice as well as challenged c-jun\textsuperscript{ff} and c-jun\textsuperscript{Δmu} mice were observed (Fig 8A).
hearts in \(c\text{-}jun^{f/f}\) mice, but most importantly heart dilation in \(c\text{-}jun^{amu}\) mice, in response to mechanical pressure-overload (Fig 8B, upper panels). EvG staining did not reveal any fibrosis in the hearts of both sham- and TAC-operated wild-type animals, while in both groups of knock-out mice, collagen deposition was evident (Fig 8B, lower panels). Deletion of c-Jun led to mild heart fibrosis without functional consequences, however, the hypertrophic stimulus greatly enhanced collagen fibre deposition and most likely contributed to decreased cardiac performance. Thirdly, I analyzed mRNA levels of hypertrophic markers by quantitative RT-PCR (Fig 9A). Both natriuretic peptides, ANF and BNP, were significantly enhanced in TAC-operated wild-type and knock-out hearts (\(p < 0.05\)). TAC-operated \(c\text{-}jun^{amu}\) mice showed greater expression of ANF and BNP in hearts than TAC-operated \(c\text{-}jun^{f/f}\) mice (\(p < 0.05\)). Interestingly, both ANF and BNP, were significantly enhanced also at baseline in hearts of \(c\text{-}jun^{amu}\) mice, as compared to hearts of \(c\text{-}jun^{f/f}\) mice (\(p < 0.05\)). ACTA1, a structural protein, was expressed at significantly lower levels in hearts of \(c\text{-}jun^{amu}\) mice at baseline, and was not efficiently induced in hearts of \(c\text{-}jun^{amu}\) mice after pressure-overload, as compared to hearts of \(c\text{-}jun^{f/f}\) mice (\(p < 0.05\)). \(\beta\)-MHC was expressed at significantly lower levels in native hearts of \(c\text{-}jun^{amu}\) mice (\(p < 0.05\)), but was significantly and similarly induced in hearts of both \(c\text{-}jun^{amu}\) and \(c\text{-}jun^{f/f}\) mice after TAC. mRNA levels of \(\alpha\)-MHC and Serca-2 were unchanged in all experimental groups. Interstitial fibrosis is associated with raised amounts of collagen type I (Col1a1), collagen type III (Col3a1) and fibronectin (Fn), and they are commonly recognized as fibrotic markers in heart (231, 247, 292, 293). Indeed, at baseline \(c\text{-}jun^{amu}\) mice showed significant up-regulation of Col1a1, Col3a1 and Fn mRNA levels in native hearts, when compared to \(c\text{-}jun^{f/f}\) mice (\(p < 0.05\)) (Fig 9B). TAC further induced the expression of these genes in hearts of knock-out mice, but not in hearts of wild-type mice confirming histological findings with molecular markers of fibrosis (\(p < 0.05\)). Last, I performed TUNEL staining to assess whether fibrosis and TAC-induced heart dilation in \(c\text{-}jun^{amu}\) mice was associated with increased cardiomyocyte apoptosis (Fig 10). Indeed, \(c\text{-}jun\) knock-out mice displayed significantly increased numbers of apoptotic cardiomyocytes upon TAC, when compared to sham-operated mice and TAC-operated wild-type mice (\(p < 0.05\)). Interestingly, already at baseline, \(c\text{-}jun^{amu}\) mice showed significantly more TUNEL positive nuclei in the hearts than \(c\text{-}jun^{f/f}\) mice
(p < 0.05). The numbers of apoptotic nuclei in the hearts of wild-type mice did not change upon TAC.

Taken together, these data demonstrate that c-Jun is necessary for preventing heart from pathologic remodeling and thus from heart dilation and heart failure, in particular under stress conditions.
Table 3. Echocardiographic analyses in c-junΔmut and corresponding control mice at baseline and after TAC.

<table>
<thead>
<tr>
<th>Data measure</th>
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<th>TAC</th>
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<td>HR, bpm</td>
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<td>467 ± 22</td>
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<td>IVSD, mm</td>
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<td>0.84 ± 0.030</td>
<td>0.74 ± 0.01</td>
<td>0.78 ± 0.014†</td>
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<td>IVSS, mm</td>
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<td>0.99 ± 0.013</td>
<td>0.97 ± 0.028§</td>
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<td>LVIDD, mm</td>
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<td>LVIDs, mm</td>
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<td>LVPWS, mm</td>
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<td>LV Mass, mg</td>
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<td>107.48 ± 3.08</td>
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<td>75.51 ± 2.80</td>
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<td>38.57 ± 2.60†</td>
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All values are shown as mean ± SEM. n= 5-6 per group. p < 0.05 is indicated as: # WT TAC vs WT sham; † KO TAC vs KO sham; ¶ KO sham vs WT sham; § KO TAC vs WT TAC. HR, Heart rate; IVSD, Interventricular septum in diastole; IVSS, Interventricular septum in systole; LVPWD, Left ventricular free wall in diastole; LVPWS, Left ventricular free wall in systole; LVIDD, Left ventricular internal diameter in diastole; LVIDs, Left ventricular internal diameter in systole; LV Mass, Left ventricular Mass; LV VOLD, Left ventricular Volume in diastole; LV VOLS, Left ventricular Volume in systole; FS, Fractional Shortening; EF, Ejection Fraction; LVID Trace (CO), LVID Trace (Cardiac output); LVID Trace (SV), LVID Trace (Stroke volume).
Fig 8. *c-jun*Δmu mice display extensive heart fibrosis and dilation upon TAC. (A) H/BW ratios increase significantly in both *c-jun*Δmu and *c-jun*ff mice after TAC, however the hypertrophic response is similar in both groups. Data are presented as values ± SEM. (*) p < 0.05; n = 11-12 per group. (B) Histological analyses. H&E staining shows TAC-induced concentric growth of the heart in *c-jun*ff mice and the heart dilation in *c-jun*Δmu mice (upper panel). EvG staining revealed mild spontaneous fibrosis in hearts of *c-jun*Δmu mice, being markedly enhanced after TAC (lower panel).
Fig 9. *c-jun*Δmu mice show different pattern of hypertrophic and fibrotic genes expression upon TAC. (A) Relative expression of hypertrophic markers by quantitative RT-PCR. ANF and BNP are expressed at higher levels in *c-jun*Δmu mice at baseline and after TAC. ACTA1 is expressed at lower levels in unchallenged hearts of *c-jun*Δmu mice and is not efficiently induced by TAC in *c-jun*Δmu mice. No difference in β-MHC expression in hearts can be observed between wild-type and knock-out mice, however there is significant decrease in β-MHC levels in hearts of *c-jun*Δmu mice at baseline. mRNA levels of α-MHC and Serca-2 are unchanged in all experimental groups. (B) Relative expression of fibrotic markers. *c-jun*Δmu mice showed spontaneous significant up-regulation of Col1a1, Col3a1 and Fn mRNA levels in hearts, further enhanced with TAC, as compared to control hearts. TAC did not induce expression of those genes in hearts of *c-jun*fl mice. Data are presented as values ± SEM. (*) p < 0.05; n = 5 per group.
**Fig 10. Enhanced apoptotic rate in hearts of c-jun<sup>Δmu</sup> mice at baseline, as well as upon TAC.**

(A) TUNEL staining of heart cross sections. TAC does not induce apoptosis in hearts of c-jun<sup>f/f</sup> mice. c-jun<sup>Δmu</sup> mice show slightly more apoptotic cardiomyocytes already at baseline, while the apoptotic rate markedly increased in hearts of knock-out animals upon TAC. (B) Quantification of TUNEL positive nuclei does not show any difference in number of apoptotic nuclei between sham- and TAC-operated c-jun<sup>f/f</sup> mice. A significant increase in TUNEL positive nuclei is observed in hearts of c-jun<sup>Δmu</sup> mice at baseline and upon TAC. Data are presented as values ± SEM.
5. TAC-induced cardiac fibrosis in *c-jun*Δ*μμ* mice is associated with increased MMP-2 activity and TGF-β/Smad signaling

Cardiac fibrosis, caused by maladaptive extracellular matrix (ECM) remodeling in the myocardium, is an important pathophysiological process implicated in the transition from compensated heart hypertrophy to failure (177, 294). Since deletion of *c-jun* in cardiomyocytes resulted in mild spontaneous fibrosis, which was dramatically enhanced with TAC ultimately leading to LV dilation and progression into heart failure, I first decided to further characterize pathways involved in ECM remodeling in *c-jun*Δ*μμ* mice (Fig 11).

TGF-β is a key player in ECM remodeling, and its elevated levels lead to increased myocardial fibrosis, due to stimulatory effects on fibrillar collagens production (240, 241). Thus, I evaluated if TGF-β signaling is increased in both unchallenged and challenged hearts of *c-jun* knock-out mice. First, I analyzed mRNA levels of TGF-β by quantitative RT-PCR (Fig 11A). At baseline, there were no apparent differences between knock-out and wildtype mice (*p* = 0.14). Upon TAC however, *c-jun* knock-out mice displayed significantly increased in TGF-β expression in hearts (*p* < 0.05), which could not be observed in wild-type counterparts (*p* = 0.33). Next, I assessed activation of Smad2, a well-known TGF-β downstream effector (Fig 11B). In agreement with enhanced TGF-β expression, levels of phosphorylated Smad2 were markedly increased in hearts of *c-jun*Δ*μμ* mice stimulated with TAC, but not in hearts of sham-treated mice, as compared to hearts of *c-jun*Δ*f/f* mice. Total Smad2 protein levels were not altered in any of the experimental groups.

Myocardial remodeling is also associated with increased expression and activity of matrix metalloproteinases (MMPs), proteolitic enzymes, which regulate the ECM composition, by denaturation, degradation and deposition of collagen fibres, which is at least partially regulated through TGF-β signaling (190). I thus evaluated the mRNA expression levels of myocardial MMPs, gealtinases A and B (MMP-2 and -9 respectively), stromelysin 1 (MMP-3), matrilysin (MMP-7), collagenenase 3 (MMP-13), neutrophil collagenase (MMP-8) and membrane-type-1 MMP (MMP-14) (Fig 12A and data not shown). None of the MMPs was differentially expressed at baseline in hearts of *c-jun*Δ*μμ* mice, when compared to hearts of *c-jun*Δ*f/f* mice. TAC-stimulated *c-jun*Δ*f/f* mice did not show up-regulation of any of the MMPs in hearts, when
compared to sham-operated mice. Interestingly, hearts of c-jun knock-out mice stimulated with TAC showed significantly increased mRNA expression of MMP-2 and MMP-14, when compared to hearts of sham-treated animals and wild-type TAC-treated controls (Fig 12A). Furthermore, MMP-9 levels were decreased in hearts of TAC-treated c-junΔmu mice, but this change was significant only in comparison to sham-treated c-junΔmu mice (p < 0.05), but not in comparison to TAC-treated c-junff mice (Fig 12A). MMP-3, MMP-7, MMP-8 and MMP-13 was expressed at steady-state levels in all groups, irrespectively of genotype and stimulus (data not shown).

As quantitative RT-PCR uncovered transcriptional deregulation of MMP-2 and MMP-9 in hearts of c-junΔmu mice subjected to TAC, I next tested whether these findings are accompanied by an enhanced enzymatic activity of MMP-2 and MMP-9 using gelatin zymography (Fig 12B). In all samples zymographic bands of pro-MMP-9 (92 kDa form), pro-MMP-2 (72 kDa from) and activated MMP-2 (66 kDa form) could be detected. However, extracts from hearts of TAC-operated c-jun knock-out mice showed a mild decrease in the activity of pro-MMP-9 and an apparent increase in activity of MMP-2, thus confirming the mRNA expression data. No difference in MMP-2 and MMP-9 activity was observed in sham-operated c-jun knock-out mice and TAC-operated wildtype mice, when compared to sham-operated wild-type mice.

In conclusion, I showed that TAC-induced progressive fibrosis in hearts of c-jun knock-out is associated with activation of the TGF-β/Smad2 signaling pathway, as well as increased MMP-14 and MMP-2 expression, and MMP-2 activity. Basal fibrosis in knock-out hearts is likely to be mediated by distinct mechanisms, as these pathways are not activated in unchallenged c-jun-deficient hearts.
Fig 11. TAC-induced cardiac fibrosis in c-jun<sup>3mu</sup> mice is associated with enhanced TGF-β/Smad signalling. (A) Relative expression of TGF-β assessed by quantitative RT-PCR. No differences in TGF-β expression in hearts between c-jun<sup>f/f</sup> and c-jun<sup>3mu</sup> mice at baseline were observed. TAC significantly induced TGF-β expression only in hearts of c-jun<sup>3mu</sup> mice. Data are presented as values ± SEM. (*) p < 0.05; n = 5 per group. (B) WB analysis of Smad2 proteins. TAC induced phosphorylation of Smad2 in hearts of c-jun<sup>3mu</sup> mice but not in hearts of c-jun<sup>f/f</sup> mice. Levels of total Smad2 were unaltered in all experimental groups. GAPDH was used as a loading control.
Fig 12. TAC-induced cardiac fibrosis in \textit{c-jun}^{\Delta \text{mu}} mice is associated with increased MMP-2 activity. (A) Relative expression of myocardial MMPs assessed by RT-PCR. None of the MMPs were differentially expressed in hearts of \textit{c-jun}^{\Delta \text{mu}} mice at baseline when compared to hearts of \textit{c-jun}^{\text{fl}} mice. TAC significantly increased expression of MMP-2 and MMP-14 in hearts of \textit{c-jun}^{\Delta \text{mu}} mice, and decreased levels of MMP-9. Data are presented as values $\pm$ SEM. (*) $p < 0.05$; $n = 5$ per group. (B) Gelatin zymography on total heart protein extracts. TAC-operated \textit{c-jun}^{\Delta \text{mu}} mice showed greatly increased activity of MMP-2 (arrow at 66 kDa), and slightly decreased activity of pro-MMP-9 (arrow at 92 kDa) in hearts as compared to sham-operated mice and TAC-operated \textit{c-jun}^{\text{fl}} mice. No difference in MMP-2 and MMP-9 activity in hearts was observed between TAC operated \textit{c-jun}^{\text{fl}} mice, when compared to sham-operated controls, as well as sham-operated \textit{c-jun}^{\text{fl}} and \textit{c-jun}^{\Delta \text{mu}} mice.
6. A global expression analysis of hearts reveals up-regulation of fibrotic genes and down-regulation of specific cytoskeletal genes in the absence of c-Jun

To identify possible targets of c-Jun, a global gene expression analysis of unstimulated hearts from c-jun⁰/⁰ and c-junΔmu mice using Affymetrix cDNA microarray chips has been performed. I obtained a list of 146 genes, with 49 genes up-regulated and 97 genes down-regulated, for which differential expression was greater than ±1.3 fold in c-jun deficient hearts compared to c-jun⁰/⁰ hearts (Table 4 and 5).

Next, I performed functional clustering of identified genes. Interestingly, among the genes up-regulated, 14 were associated with a biological cluster of genes that are involved in ECM remodeling and development of fibrosis in heart (Table 4). Surprisingly, 17 of the genes down-regulated were associated with functional clusters of importance for of genes involved in cytoskeletal and muscle organization/function, (Table 5).

Then, I validated several interesting deregulated genes by quantitative RT-PCR (Fig 13 and 14). Since hypertrophic stimulus TAC greatly enhances fibrosis in hearts of c-junΔmu mice, I included hearts of TAC operated mice in this validation. I confirmed up-regulation of genes involved in ECM remodeling, namely cartilage intermediate layer protein (CILP), periostin (Pn, Postn), bone morphogenic protein 1 (BMP1), and WNT1 inducible signaling pathway protein 1 (WISP1) (Fig 13). They were all up-regulated in native hearts of c-junΔmu mice, when compared to hearts of c-jun⁰/⁰ mice (p < 0.05). Importantly, TAC further induced expression of BMP1 and WISP1 in hearts of c-junΔmu mice (p < 0.05), but not in hearts of c-jun⁰/⁰ mice. Although periostin and CILP were up-regulated significantly in TAC-operated c-jun⁰/⁰ hearts, when compared to sham-operated controls (p < 0.05), their expression upon TAC was much higher in hearts of c-junΔmu mice, when compared to hearts of c-jun⁰/⁰ mice (p < 0.05). I then analyzed the expression of the most markedly down-regulated genes involved in cytoskeleton and muscle organization and function, namely brain expressed X-linked gene (Bex1), myosin binding protein C fast-type (MyBP-C2), myotilin, and tropomyosin 2 (TPM2) (Fig 15), as well as skeletal muscle alpha actin (ACTA1) and beta myosin heavy chain 7 (β-MHC, MyH7) (Fig 10). Down-regulation of ACTA1, Bex1, MyBP-C2, myotilin, β-MHC, and TPM2 was confirmed in non-stimulated hearts of c-junΔmu mice, when compared to hearts of c-jun⁰/⁰ mice (p < 0.05).
Expression of ACTA1, Bex1, MyBP-C2, myotilin, β-MHC, and Tpm2 significantly increased upon TAC in hearts of c-jun^{f/f} mice (p < 0.05). But most importantly, c-jun^{mus} mice stimulated with TAC showed no induction of myotilin in hearts, and significantly lower upregulation of ACTA1, Bex1, MyBP-C2, and TPM2 in hearts, when compared to TAC-operated c-jun^{f/f} mice (p < 0.05).

These results indicate that c-Jun acts to prevent pathologic remodeling of the myocardium at baseline, as well as upon mechanical pressure-overload. Our results also indicate that c-Jun inhibits cardiac fibrosis of the myocardium at baseline and in response to stress through regulation of distinct sets of genes. These data also indicate that c-Jun might be particularly important in remodeling the cytoskeleton of cardiomyocytes, which appears to be important in native hearts, as well as hearts subjected to hypertrophic stimuli.
Table 4. Functional clustering of genes up-regulated more than 1.3 times in *c-jun*Δμμ mice versus *c-jun*Δμ mice.

<table>
<thead>
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<th>Functional Clusters</th>
<th>Name</th>
<th>Symbol</th>
<th>Fold change</th>
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SD = Standard deviation;
Table 5. Functional clustering of genes down-regulated more than 1.3 times in c-jun<sup>Δmu</sup> mice versus c-jun<sup>f/f</sup> mice.

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SD = Standard deviation;
Fig 13. Lack of c-jun results in induction of pro-fibrotic genes in heart. CILP, periostin, BMP1 and WISP1 are up-regulated in native hearts of c-jun<sup>mmu</sup> mice. CILP and periostin, but not BMP1 and WISP1, are increased in hearts of c-jun<sup>f/f</sup> mice subjected to TAC. In TAC-stimulated c-jun<sup>mmu</sup> mice, all genes are expressed at higher levels when compared to TAC-operated c-jun<sup>f/f</sup> mice. Data are presented as values ± SEM. (*) p < 0.05; n = 5 per group.
Fig 14. c-Jun is required for the expression of distinct sarcomeric gene transcripts. Decreased expression of Bex1, MyBP-C2, myotilin, and TPM2, was observed in non-stimulated hearts of c-Jun	extsuperscript{Δmu} mice. Bex1, MyBP-C2, myotilin, and Tpm2 expression levels were induced upon TAC in c-Jun	extsuperscript{eff} mice, while they stayed at the same levels in TAC-operated c-Jun	extsuperscript{Δmu} mice. Data are presented as values ± SEM. (*) p < 0.05; n = 5 per group.
IX. DISCUSSION

So far, there are no reports that investigated the \textit{in vivo} role of c-Fos and c-Jun transcription factors in development of cardiac hypertrophy using a genetic approach in mice. The goal of my study was to elucidate the role of c-Fos and c-Jun in mediating the hypertrophic response using striated muscle-specific \textit{c-fos} (\textit{c-fos}\textsuperscript{amu}) and \textit{c-jun} (\textit{c-jun}\textsuperscript{amu}) knock-out mice. First, I analyzed both strains for a spontaneous heart phenotype but did not observe any gross structural defects. However, conditional \textit{c-jun} knock-out mice displayed a slight interstitial fibrosis in the hearts. In agreement with histological data, neither \textit{c-fos}\textsuperscript{amu} nor \textit{c-jun}\textsuperscript{amu} mice showed any alterations in heart function, as examined by echocardiography. Therefore, the next step was challenge both strains with a hypertrophic stress stimulus. I have chosen transverse-aortic constriction (TAC), which is a widely used \textit{in vivo} model to assess left ventricular hypertrophy (271). \textit{c-fos} knock-out mice did not differ from wild-type mice, and showed normal hypertrophic response, demonstrated by a concentric growth of the heart without premature dilation of cardiac chambers. In contrary, \textit{c-jun} knock-out mice displayed an eccentric growth of the heart leading to heart dilation, and a decrease in cardiac performance, features that can also be observed in human failing hearts (12, 18-21). These maladaptive changes were associated with increased cardiac apoptosis and fibrosis. Fibrosis upon TAC could be explained by increased TGF-\(\beta\)/Smad2 signalling and MMP-2 activity. Finally, as c-Jun seemed to be critical for maintenance of cardiac function and protection against heart failure, I performed a cDNA microarray expression analysis of native hearts lacking \textit{c-jun}. Interestingly, two functional clusters were found. The first group of genes was found up-regulated and belongs to molecules that have been mainly discussed in the context of EMC-remodelling and fibrosis in heart. The second group of genes was found down-regulated and consists of members of proteins that play a role in structural and functional organization of the sarcomere.

This study is a first attempt \textit{in vivo} addressing the consequences of lack of c-Jun and c-Fos in native and stressed hearts. In the following, I aim at discussing my results presented herein in comparison to existing reports.
1. **c-Jun, but not c-Fos, is necessary for prevention of stress-induced maladaptive cardiac hypertrophy**

Several studies investigated a role of c-Jun and c-Fos in the development of cardiac hypertrophy using plated neonatal cardiomyocytes as a model system (279-282). Transfection of cultured cardiomyocytes with dominant-negative mutant of c-Fos led to normal hypertrophic myocyte growth in response to phenylephrine (PE) (281). Although I did not stimulate hearts with PE *in vivo*, TAC is known to involve α-adrenergic signalling (295). Therefore, this *in vitro* study is in line with my findings, as I could also not see a difference in the hypertrophic growth upon TAC. In two other reports, authors transfected plated neonatal cardiomyocytes and adult rat heart with dominant-negative (DN) mutant of c-Jun, respectively (279, 280). In both studies, DN-c-Jun inhibited the hypertrophic response induced by endothelin (ET) and PE (280), as well as hypertension, or angiotensin II (AngII) infusion, respectively (279). In my studies I showed that c-Jun is not necessary for normal heart function in the unstressed heart. However under stress conditions (TAC), c-Jun seems to be crucial, as *c-jun* knock-out mice display eccentric hypertrophy and LV dilation, as well as impaired cardiac performance. Concentric and eccentric hypertrophy cannot be mimicked in plated cardiomyocytes, as isolated cells respond to hypertrophic stimuli by proportional increase in cell size. Thus it is difficult to assess whether inhibited growth of cardiomyocytes transfected with DN-c-Jun corresponds to eccentric growth of cardiomyocytes and LV dilation in *c-jun* knock-out mice. On the other hand, transfection of DN-c-Jun into adult rat hearts decreases heart weight and LV thickness in response to AngII, or in presence of hypertension (279), suggesting inhibition of hypertrophic response and not an eccentric hypertrophy. These data are therefore not in line with my observations. It is, however, important to mention that in this study the authors inactivated c-Jun two days before AngII infusion, or introduced DN-c-Jun to already hypertensive hearts; consequently there is a clear difference in the timing of inactivation. More importantly, the approach chosen by these investigators is likely to be not cardiomyocyte-specific as adenoviral delivery will occur in other cell types including fibroblasts, vascular cells etc. Finally and probably most importantly, a dominant negative construct of c-Jun probably acts through very different mechanisms than specific deletion of this gene, as c-Jun is known to heterodimerize with different AP-1 members and even genes from other transcription
factor families. In fact, such an approach is likely to affect transcriptional activity of AP-1 transcription factors in an almost unpredictable way. I therefore conclude that our approach is more likely to provide us with clear insights as to how c-Jun specifically regulates heart integrity and function.

2. c-Jun, but not c-Fos, is regulating some, but not all, canonical cardiac hypertrophy genes

It is well known that cardiac hypertrophy is associated with the re-expression of “fetal” cardiac genes, which are also widely used to monitor the hypertrophic response. I showed that deletion of c-Fos does not alter the expression of hypertrophic markers, such as ANF, BNP, α-Sk. actin, β-MHC and Serca-2, which are induced to similar levels in hearts of both wild-type and knock-out mice upon TAC. This clearly suggests that c-Fos is redundant in regulating these genes. Similarly, deletion of c-jun in cardiomyocytes did not affect induction of ANF, BNP and β-MHC. In contrast, their expression was even enhanced in hearts of c-junΔmu mice compared to wild-type control hearts subjected to TAC, suggesting that c-Jun rather inhibits their transcriptional activation at least under stress conditions. Interestingly, ACTA1 was not significantly induced after TAC in conditional c-jun knock-out mice, as in wild-type mice, suggesting that c-Jun is required for TAC-induced ACTA1 transcriptional activity. It was shown that the proximal regulatory elements in ANF, BNP and ACTA1 genes contain an AP-1 binding sites (278, 296-298). Several studies tried to unravel the transcriptional regulation of hypertrophic markers by AP-1 overall demonstrating rather discrepant results. For example, in one study transfections with c-Fos or c-Jun repressed ANF promoter activity (299), while in another report c-Jun was shown to induce it (300). Omura et al. showed that in presence of DN-c-Jun, ANF and BNP were down-regulated upon agonist stimulation, suggesting that c-Jun acts rather as an activator of ANF and BNP transcription (280). Furthermore, it has been demonstrated that transfection of c-Jun, both c-Jun and c-Fos, but not c-Fos alone, activated the ACTA1 promoter activity (298, 301). It was also shown that low concentrations of c-Fos promoted, but higher concentrations of c-Fos suppressed activation of ANF promoter (300). In contrast, in another study it has been shown that
a dominant-negative mutant of c-Fos decreased β-MHC, ACTA1, ANF and BNP levels, when compared to non-transfected cardiomyocytes (281). My study thus only partially confirms the findings described above. My in vivo results clearly demonstrate that c-Fos is not required to regulate this canonical gene program in the heart. c-Jun appears to inhibit some of these genes, but is required for transactivation of ACTA1, a cytoskeletal protein. It is currently unclear whether c-Jun directly represses transcription as several AP-1 binding sites have been found on the promoter of these genes. It is however not excluded that other AP-1 members might be more crucial to regulate this subset of genes.

3. c-Jun, but not c-Fos, prevents cardiomyocytes from apoptosis in vivo

In my study, I demonstrated that lack of c-Jun caused apoptosis in native hearts, which was further enhanced by stimulation by TAC, while lack of c-Fos did not cause increased apoptosis in hearts neither basally nor upon TAC. AP-1 transcription factors are involved in both induction and prevention of apoptosis, depending on tissue, developmental stage, and stress stimuli. In most reports c-Jun and c-Fos were demonstrated to promote cell death as their up-regulation correlated with conditions promoting apoptosis, such as for example UV radiation (302, 303). These studies, however, do not demonstrate, whether AP-1 is actually causally involved. Some direct evidence was derived from transient over-expression experiments, in which c-Jun or c-Fos were found to induce apoptosis in various cell lines (304, 305). However, the expression levels achieved in such experiments are unphysiologically high, and could therefore result in non-specific effects. A more accurate way to study the function of c-Jun and c-Fos in apoptosis is to examine response of the animals lacking those genes to different pro-apoptotic stimuli. In fact, it was shown that light-induced apoptosis of retinal photoreceptors is decreased in c-fos-deficient mice (306), further supporting its pro-apoptotic role. In my studies, conditional c-fos knock-out mice showed normal concentric hypertrophy. Programmed cell death of cardiomyocytes in response to cardiac stress stimuli is at least partially responsible for transition of compensated heart function to heart failure. Thus lack of c-fos does not increase apoptosis in cardiomyocytes supporting tissue-specific roles of different AP-1 family members.
A pro-apoptotic function of c-Jun was shown in c-jun−/− fibroblasts, which were less sensitive or resistant to UV- and alkylating agents-induced cell death (307, 308). In contrast, two examples of anti-apoptotic functions of c-Jun are provided by genetic experiments in vivo. First, c-jun knock-out mice die during embryonic development and display extensive apoptosis in hematopoietic cells and hepatoblasts (283). Secondly, conditional deletion of c-jun in liver during tumor progression resulted in reduction of tumor size due to increased apoptosis (309). In my study, c-jun knock-out mice display increased basal apoptosis, confirming an anti-apoptotic role of c-Jun also in heart. Consistently, when subjected to TAC, c-jun knock-out mice showed an even more obvious increase in cell death, which might contribute to progression to heart failure. Therefore, c-Jun might have potential role in preventing apoptosis-induced heart failure.

4. c-Jun prevents myocardial ECM remodeling and fibrosis by suppressing expression of pro-fibrotic genes

Myocardial fibrosis is known to accompany maladaptive hypertrophy and progression to heart failure, by contributing to diastolic and systolic dysfunction. I showed that deletion of c-jun in cardiomyocytes leads to mild spontaneous fibrosis without affecting heart function, however, TAC further induced collagen deposition, which resulted in impaired cardiac performance. Several pathways have been involved in regulation of myocardial fibrosis.

Fibrosis can result from increased collagen production and deposition, and is characterized by increased mRNA levels of collagen I and III (Col1a1 and Col 3a1), and fibronectin (Fn). I observed basal up-regulation of Col1a1, Col3a1 and Fn mRNA levels in hearts of c-jun knock-out mice, all of which further increased upon TAC. AP-1 binding sites were identified in the promoters of Col1a1 (310, 311), Col3a1 (312) and Fn (313, 314), and it has been shown to be a positive regulation, as for example a mutation in AP-1 binding site in the Col1a1 gene decreased its promoter activity (315). Since in the absence of c-jun Col1a1, Col3a1 and Fn are expressed at higher levels, I conclude that c-Jun rather inhibits their transcription in cardiomyocytes.
It was also shown that TGF-β stimulates cells to increase the synthesis of matrix proteins, such as fibronectin and collagens, during ECM remodeling (240, 241). In this manner, increased TAC-induced Col1a1, Col3a1 and Fn expression in hearts of c-jun knock-out mice could at least partially be the result of increased TGF-β expression and signalling (through Smad2). On the other hand, Col1a1, Col3a1 and Fn expression is also enhanced in unstimulated hearts of c-jun knock-out mice, whereas TGF-β levels, as well as phosphorylation of Smad2, are not altered at baseline. Conclusively, c-Jun appears to mitigate TGF-β signaling specifically upon cardiac stress conditions.

In addition, fibrosis results from increased ECM degradation, which occurs via proteolytical degradation of ECM components by MMPs. Consistently, mRNA levels of MMP-2 and MMP-14, as well as MMP-2 proteolytic activity were increased in fibrotic hearts of c-jun<sup>−/−</sup> mice subjected to TAC. Although TGF-β acts to inhibit most of the MMPs and in the same time induces TIMPs to prevent ECM degradation (242, 243), it is interesting to mention that it actually increases activity of MMP-2 (316, 317). Additionally, MMP-14 was also shown to activate MMP-2, by proteolytical digestion of its latent form (318). It is thus plausible to assume that increased MMP-2 expression and activity is positively regulated by TGF-β and MMP-14.

Overall, it seems that severe myocardial fibrosis stimulated by TAC in hearts of c-jun knock-out mice results from enhanced synthesis and degradation of ECM components, through activation of TGF-β/Smad2 signaling that might specifically enhance MMP-2 activity.

More insights into c-Jun-dependent transcriptional regulation of pro-fibrotic genes can be derived from my Affymetrix array analyses. 14 out of 49 up-regulated genes in native hearts lacking c-jun clustered around processes associated with ECM remodeling and fibrosis. Four of them, namely cartilage intermediate layer protein (CILP), periostin, bone morphogenic protein 1 (BMP1), and WNT1 inducible signaling pathway protein 1 (WISP1), seem to be particularly interesting, as they are expressed at significantly higher levels in hearts of TAC-stimulted c-jun knock-out mice than in wild-type mice.
CILP is an extracellular matrix protein that is abundant in cartilaginous tissues. Although CILP was not yet directly associated with heart disease, its increased expression is implicated in common musculoskeletal disorders, resulting from maladaptive ECM remodeling of cartilage, including osteoarthritis and lumbar disc disease (319).

Periostin (Pn), is a pro-fibrogenic extracellular protein secreted by fibroblasts, and is strongly up-regulated before and during cardiac remodeling, such as myocardial fibrosis, cardiac hypertrophy, LV dilation, and myocardial infarction (320, 321). Periostin was also shown to promote differentiation of progenitor cells into cardiomyocytes (322). As periostin can directly interact with other ECM proteins, such as fibronectin and collagen I (323, 324), it is possible that up-regulation of Fn, Col1a1 and Col3a1 genes in hearts of c-jun<sup>−/−</sup> mice primarily results from increased expression of periostin.

BMP1, is the prototype of a subgroup of structurally similar secreted metalloproteinases that play key roles in formation of the ECM via biosynthetic processing of its components, such as collagens, laminin and proteoglycans (325, 326). In addition, BMP1-like proteinases were shown to activate TGF-β family members (327). So far, there are no studies showing pro-fibrotic functions of BMP1 particularly in heart. It is thus very interesting that increased BMP1 expression occurs during both spontaneous and TAC-induced cardiac fibrosis in c-jun<sup>−/−</sup> mice, but not during TAC-induced hypertrophic response in c-jun<sup>+/−</sup> mice.

Finally, WISP1 is a member of the cysteine-rich 61, connective tissue growth factor, and nephroblastoma overexpressed (CCN) family of growth factors that exert potent and overlapping effects on ECM remodeling (328). WISP1 is expressed in the heart at low basal levels, but interestingly, WISP-1 stimulated cardiomyocyte hypertrophy, fibroblast proliferation, and ECM expression in vitro (329). Thus, WISP1 might be another factor responsible for fibrotic remodeling in hearts of c-jun knock-out mice.

Conclusively, my results suggest that c-Jun prevents myocardial ECM remodeling and fibrosis, possibly by suppressing the transcription of several pro-fibrotic genes. However, a direct transcriptional repression of these genes by c-Jun remains to be confirmed.
5. c-Jun regulates important sarcomeric genes

Among 97 genes down-regulated in unchallenged c-jun deficient hearts identified by microarray analysis, 17 clustered around biological groups of genes involved in cytoskeletal and muscle organization and function. Of particular interest are ACTA1, Bex1, MyBP-C2, Myotilin, and TPM2, since they were not induced upon TAC in hearts of conditional c-jun knockout mice, as in wild type control hearts. They are all abundantly expressed in skeletal muscle. ACTA1, MyBP-C2, Myotilin, β-MHC and TPM2 are highly expressed in cardiac muscle, and they play an important role in the organization and function of the sarcomere.

Bex1 (Brain Expressed X-linked gene) expression is transiently elevated at the end of the proliferation state of skeletal muscle regeneration (330). Bex1 knock-out mice display a functional deficit in exercise performance, as well as altered muscle regeneration upon cardiotoxin-induced injury (331). Interestingly, Bex1 has been mapped close to the dystrophin gene, and since absence of dystrophin is the underlying cause of Duchenne’s muscular dystrophy (DMD), the most common form of inherited neuromuscular disorder, Bex1 knock-out mice may provide an important mouse model to study DMD.

ACTA1 belongs to the family of actin proteins that are highly conserved proteins of the contractile apparatus that possess functions in cell integrity, structure and motility (4). The two skeletal muscle and cardiac muscle isoforms of actin are encoded by the ACTA1 and ACTC gene, respectively. Particularly, ACTA1 forms the backbone to the sarcomeric thin filament in skeletal muscle and plays a central role in muscle contraction. More than 20 different mutations in the ACTA1 gene are associated with two muscle diseases: actin myopathy and nemaline myopathy (NM) (332, 333). Interestingly, mutations in the regions of actin involved in attachment to Z-lines and intercalated discs led to familial DCM and heart failure, likely because of impaired force transmission. Additionally, other ACTA1 mutations result in different myopathies, including hypertrophic cardiomyopathy (HCM), dilated cardiomyopathy (DCM) and nemaline myopathy (NM) (334-336).

MyBP-C is a sarcomeric protein associated with the thick filament of striated muscle. MyBP-C has been assigned in both the structural assembly and stability of the sarcomeres, as well as in the modulation of contraction, as it exhibits strong affinity for myosin and titin (4). To date nearly 150 mutations in MyBP-C have been
published, making it the second most common HCM disease gene (337). Recent animal studies have confirmed the important role of MyBP-C. Homozygous knock-in mice containing shortened MyBP-C myosin and titin-binding domains exhibit progressive DCM and disarrayed myofibrils (338), while mice expressing MyBP-C that totally lacked the myosin- and titin-binding domains had mild cardiac hypertrophy, with some animals experiencing sudden death upon stress (339). Furthermore, mice lacking MyBP-C in heart exhibited profound cardiac hypertrophy and impaired cardiac function (340).

The tropomyosin (TM) family consists of four genes TPM 1-4, where α-TM is encoded by TPM1 gene, while β-TM by TPM2 gene. TMs are fibrous proteins that bind actin filaments, providing structural stability of the thin filaments. Together with the troponins, TMs regulate the interaction of the thin and thick filaments, whereas together with tropomodulin, TMs regulate thin filament length (4). Mutations in TM have been linked to distinct inherited diseases of cardiac and skeletal muscle, including HCM, DCM and NM (341, 342). Interestingly, over-expression of TPM2 leads to postnatal death between day 10–14, and the hearts of these mice exhibit pathological abnormalities (343).

Finally, myotilin, a sarcomeric protein that localizes to Z-discs in mature sarcomere and interacts with alpha-actinin, actin, F-actin and filamin C, also possess an important role in sarcomere organization (344). Myotilin is mutated in two forms of muscle disease, limb-girdle muscular dystrophy type 1A (LGMD1A), and myofibrillar myopathy. Surprisingly, myotilin knock-out mice maintain normal muscle and heart sarcomeric and sarcolemmal integrity (345), while mice over-expressing myotilin in an LGMD1A mouse model showed severe muscle degeneration (346).

Overall, described genes are important regulators of proper muscle and heart integrity and function, as their mutated forms have causative effects in various forms of cardiac and muscle disorders, including dystrophies and myopathies. It is plausible that maladaptive remodeling in mice lacking c-jun in cardiomyocytes is caused directly by decreased expression of these proteins, particularly ACTA1, Bex1, MyBP-C2, β-MHC, Myotilin, and TPM2.
X. OUTLOOK

Microarray gene expression analysis unravelled a plethora of genes that are differentially expressed in hearts in the absence of c-jun. While several of the up-regulated genes were shown to have pro-fibrotic functions during cardiac remodeling, some of the down-regulated genes possess an important role in the structural and functional organization of the sarcomere. These data provide new insights about c-Jun in the prevention of pathological cardiac remodeling. However, additional experiments need to be performed, to fully clarify c-Jun-dependent molecular and cellular mechanisms.

Chromatin immuno-precipitation (ChIP) can be performed to confirm direct suppressive and activatory transcriptional regulation of identified fibrotic and sarcomeric genes. These analyses could be followed by promoter analyses and promoter activity assays. Alternatively, direct occupation of promoters by c-Jun could be assessed on a genome-wide level using the ChIP-on-chip technology.

Immunostainings as well as Western blotting of sarcomeric proteins should be performed to further confirm cytoskeletal defects in cardiomyocytes in the absence of c-jun.

More generally, it will be important to complement these analyses with in vivo experiments concerning other members of the AP-1 family and to study dimerization of AP-1 factors specifically in the cardiomyocytes to understand the complex regulation of heart physiology by this group of transcription factors.
XI. REFERENCES


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