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Sini Kinnunen

THE INTERACTION OF TRANSCRIPTION FACTORS GATA4 AND NKX2-5 AND THE EFFECT OF INTERACTION-TARGETED SMALL MOLECULES ON THE HEART

*UNIVERSITY OF OULU GRADUATE SCHOOL;
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UNIVERSITY OF HELSINKI,
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Academic dissertation to be presented with the assent of the Doctoral Training Committee of Health and Biosciences of the University of Oulu for public defence in Auditorium 1041, Biokeskus 2 (Viikinkaari 5, Helsinki), on 15 May 2020, at 12 noon

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Abstract

Heart failure, a progressive disease, is a consequence of various cardiac diseases. It is one of the leading causes of mortality in the world and a challenge for modern medicine. Although the primary prevention of cardiac diseases in developed countries has improved, the absolute number of heart failure patients is increasing. The current treatments relieve the symptoms and improve quality of life, but they only delay the progression of the disease.

Cardiac hypertrophy initially develops as an adaptive response to reduce ventricular wall stress and maintain cardiac function. However, if hypertrophy is prolonged, it activates pathological remodelling processes, e.g. increased fibrosis and cell death, inadequate angiogenesis and upregulation of foetal gene expression. Sequence-specific DNA-binding proteins, transcription factors (TFs), regulate gene expression and coordinate cell specification. The TF GATA-binding protein 4 (GATA4) is involved in several remodelling processes. It mediates the hypertrophic growth of cardiomyocytes, induces angiogenesis, and regulates apoptosis and cell survival. GATA4 and another cardiac TF, NK2 homeobox 5 (NKX2-5), interact physically and synergistically upregulate gene expression of, e.g. the foetal genes. In addition, their physical co-operation is required for mechanical stretch-induced brain natriuretic peptide (BNP) activation and cardiomyocyte hypertrophy. Thus, GATA4-NKX2-5 interaction is an interesting novel drug target.

The aim of this thesis was to (i) characterise the GATA4-NKX2-5 protein-protein interaction and determine the NKX2-5 binding site on the GATA4 protein, (ii) investigate small molecule binding to GATA4 and NKX2-5 and (iii) to study the effect of the small molecule compounds in experimental in vivo models of heart failure. Mutational studies revealed a specific region involving amino acid N272 on the surface of the GATA4 protein that mediates the interaction with NKX2-5. The inhibitor of GATA4-NKX2-5 interaction, 3i-1000, was demonstrated to bind GATA4. This compound presented anti-hypertrophic and cardioprotective effects in vivo. This work demonstrates that cardiac TFs can be targeted and their functions modulated with small molecule compounds. In addition, the results suggest that targeting protein-protein interactions of key TFs may present a novel strategy for treatment of heart failure.

Keywords: cardioprotection, GATA4, heart failure, protein-protein interaction, small molecule, transcription factor

Kinnunen, Sini, GATA4 ja NKX2-5 transkriptiotekijöiden välinen vuorovaikutus ja siihen kohdennettujen yhdisteiden vaikutukset sydämessä.
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Tiivistelmä

Sydämen vajaatoiminta on etenevä sairaus, joka on seurausta tai liittyy useisiin eri sydänsairauksiin. Se on yksi johtavista kuolleisuuden syistä maailmassa ja haaste nykyaikaiselle lääketieteelle. Vaikka kehittyneissä maissa sydänsairauksien ennaltaehkäisy on parantunut, niin vajaatoimintaa sairastavien potilaiden absoluuttinen määrä kuitenkin lisääntyy. Nykyinen lääkehoito helpottaa oireita ja parantaa elämänlaatua, mutta vain hidastaa sairauden etenemistä.

Sydämen liikakasvu vähentää aluksi vasemman kammion seinämän stressiä ja ylläpitää sydämen riittävää toimintaa. Jos tila jatkuu pitkään, patologiset uudelleenmuovautumisprosessit aktivoituvat ja esim. sidekudosta muodostuu lisää, solukuolema ja sikiönaikaisten geenien luenta lisääntyvät. Transkriptiotekijät ovat proteiineja, jotka sitoutuvat sekvenssispesifisesti DNA:han ja siten säätelevät ja koordinoivat geenien luentaa sekä solun toimintaa. Etenkin GATA-sekvenssiin sitoutuva proteiini, GATA4, on mukana useissa uudelleen muovautumisprosesseissa. Se mm. välittää sydänlihassolujen kasvua ja säätelee solukuolemaa. GATA4 ja toinen sydämen transkriptiotekijä NKX2-5 sitoutuvat toisiinsa fyysisesti ja aktivoivat synergistisesti geenien, kuten sikiöaikaisten geenien, luentaa. Lisäksi niiden fyysinen vuorovaikutus on tärkeää mekaanisen venytyksen aiheuttamassa B-tyypin natriureettisen peptidin (BNP) aktivaatiossa ja sydänlihassolujen kasvussa. GATA4-NKX2-5 proteiini-proteiini vuorovaikutus onkin uusi mielenkiintoinen lääkevaikutuskohde.

Tämän väitöskirjatyön tavoite oli (i) tutkia GATA4 ja NKX2-5 proteiini-proteiini vuorovaikutusta ja määrittää NKX2-5:n sitoutumispaikka GATA4:ssä, (ii) tutkia pienimolekyylisen yhdisteen sitoutumista GATA4:ään ja NKX2-5:een sekä (iii) tutkia yhdisteiden vaikutuksia in vivo kokeellisissa sydämen vajaatoimintamalleissa. Mutaatiokokeet osoittivat, että GATA4 proteiinin pinnalla, aminohapon N272 kohdalla oleva alue on tärkeä sitoutumiselle NKX2-5:n kanssa. GATA4-NKX2-5 vuorovaikutuksen estävän yhdisteen 3i-1000:n osoitettiin sitoutuvan GATA4:ään. Lisäksi tällä yhdisteellä oli sydämen liikakasvua estäviä ja sydäntä suojaavia vaikutuksia in vivo. Tämä tutkimus osoittaa, että sydämen transkriptiotekijöihin ja niiden toimintaan voidaan vaikuttaa pienimolekyylisillä yhdisteillä. Keskeisten transkriptiotekijöiden proteiini-proteiini vuorovaikutukset voisivatkin olla uudenlainen lääkevaikutuksen kohde sydämen vajaatoiminnan lääkehoitoon.

Asiasanat: GATA4, pienimolekyylinen yhdiste, proteiini-proteiini vuorovaikutus, sydämen vajaatoiminta, sydäntä suojaava vaikutus, transkriptiotekijä

Never say never

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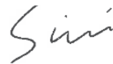
Espoo, January 2020



Abbreviations

ACE	angiotensin converting enzyme
AMI	acute myocardial infarction
Ang	angiotensin
ANP	atrial natriuretic peptide
AP-1	activator protein-1
AT_1R	angiotensin II type 1 receptor
ATPase	adenosine triphosphatase
AV	atrioventricular
AVP	arginine^8-vasopressin
Bax	B-cell lymphoma 2 associated X protein
Bak	B-cell lymphoma 2 homologous antagonist/killer
Bcl	B-cell lymphoma
Bcl-xL	B-cell lymphoma extra large
BH3-only	B-cell lymphoma 2 homology domain 3 only
BNP	B-type natriuretic peptide
BMP	bone morphogenetic protein
CARP	cardiac ankyrin repeated protein
CDK4	cyclin dependent kinase 4
CHD	congenital heart disease
DEPC	diethylpyrocarbonate
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DR	death receptor
DTT	dithiothreitol
E	embryonic day
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene-bis(oxyethylenenitrilo)tetraacetic acid
EMSA	electrophoretic mobility shift assay
EMT	endothelial-mesenchymal transdifferentiation
ERK	extracellular signal-regulated kinase
ET	endothelin
FBS	foetal bovine serum
FGF	fibroblast growth factor
FOG	Friend of GATA family member

GATA	GATA binding protein
GC	guanylate cyclase
GSK3β	glycogen synthase kinase 3β
H3K27	the 27th amino acid in histone H3
HAND	heart and neural crest derivatives expressed
HAT	histone acetyltransferase
Hey	hes related family basic helix-loop-helix transcription factor with YRPW motif
HDAC	histone deacetylase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
HFpEF	heart failure with preserved ejection fraction
HFrEF	heart failure with reduced ejection fraction
HGF	hepatocyte growth factor
HIF1A	hypoxia inducible factor 1 alpha
HL-1	AT-1 mouse atrial cardiomyocyte tumour lineage
HSP70	heat shock protein 70
i.p.	intraperitoneal
IPC	ischaemic preconditioning
I/R	ischaemia/reperfusion
i.v.	intravenous
JMJD1/2	histone demethylase
KLF13	Kruppel like factor 13
LAD	left anterior descending coronary artery
LIM	LIM-type zinc finger-domain
LMCD1	LIM and cysteine rich domains 1 (aka dyxin)
LV	left ventricle
MAPK	mitogen-activated protein kinase
MEF2	myocyte enhancer factor 2 proteins
MEK	MAPK/ERK kinase
MHC	myosin heavy chain
MI	myocardial infarction
MMPs	matrix metalloproteinases
mRNA	messenger ribonucleic acid
NFATc	nuclear factor of activated T cells
NKE	NKX2-5 binding element
NKX2-5	NK2 homeobox 5
NLS	nuclear localisation signal

NO	nitric oxide
NPR	natriuretic peptide receptor
p300	histone acetyltransferase p300
p38	p38 mitogen-activated protein kinase
p53	tumour protein p53
p62	nucleoporin 62 kDa
PCR	polymerase chain reaction
PE	phenylephrine
PKA	protein kinase A
PKB	protein kinase B (aka Akt)
PKC	protein kinase C
PMSF	phenylmethylsulphonyl fluoride
RAAS	renin-angiotensin-aldosterone system
ROS	reactive oxygen species
RT-PCR	reverse transcription-polymerase chain reaction
s.c.	subcutaneous
SEM	standard error of mean
SERCA	sarcoplasmic reticulum Ca^{2+}-ATPase
siRNA	small interfering ribonucleic acid
SMAD	SMAD family member
SNP	sodium nitroprusside
SRF	serum response factor
STAT	signal transducer and activator of transcription
SUMO-1	small ubiquitin-like modifier-1
TAC	transverse aortic constriction
TBX	T-box transcription factor
TF	transcription factor
TGFβ	transforming growth factor β
TNF-α	tumour necrosis factor α
Tris	tris(hydroxymethyl)aminomethane
VEGF	vascular endothelial growth factor
WT	wild type
YY1	YY1 transcription factor

List of original articles

This thesis is based on the following publications, which are referred to throughout the text by their Roman numerals:

I Kinnunen, S.*, Välimäki, M.*, Tölli, M., Wohlfahrt, G., Darwich, R., Komati, H., Nemer, M., Ruskoaho, H. (2015). Nuclear receptor-like structure and interaction of congenital heart disease-associated factors GATA4 and NKX2-5. *PloS One, 10*(12), e144145. https://doi.org/10.1371/journal.pone.0144145

II Jumppanen, M.*, Zore, M.*, Kinnunen, S. M.*, Välimäki, M. J., Talman, V., Boije af Gennäs, G., Ruskoaho, H., Yli-Kauhaluoma, J. (2019). Affinity chromatography reveals a direct binding of the GATA4-NKX2-5 interaction inhibitor (3i-1000) with GATA4 (in revision).

III Kinnunen, S. M.*, Tölli, M.*, Välimäki, M. J., Gao, E., Szabo, Z., Rysä, J., Ferreira, M. P. A., Ohukainen, P., Serpi, R., Correia, A., Mäkilä, E., Salonen, J., Hirvonen, J., Santos, H. A., Ruskoaho, H. (2018). Cardiac actions of a small molecule inhibitor targeting GATA4-NKX2-5 interaction. *Scientific Reports*, *8*(1), 4611. https://doi.org/10.1038/s41598-018-22830-8

IV Karhu, T. S.*, Kinnunen, S. M.*, Tölli, M., Välimäki, M. J., Szabó, Z., Talman, V., Ruskoaho, H. (2020). GATA4-targeted compound exhibits cardioprotective actions against doxorubicin-induced toxicity *in vitro* and *in vivo*: establishment of a chronic cardiotoxicity model using human iPSC-derived cardiomyocytes. *Archives of Toxicology,* in press. https://doi.org/10.1007/s00204-020-02711-8

*co-first author

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1 Introduction

Heart failure is a disease in which the heart is not able to pump enough blood for the needs of the body due to loss of myocytes or impaired function of myocytes. This stage is usually a consequence of another disease: ischaemic heart disease, hypertension, diabetes, valvular heart disease or different cardiomyopathies, e.g. ion-channel disorders, cardiotoxic drugs (cancer therapeutics) and viral infections (Kemp & Conte, 2012; McMurray, 2010; Ziaeian & Fonarow, 2016). Heart failure affects over 37 million people globally (Ziaeian & Fonarow, 2016). Even though the age-adjusted prevalence of heart failure in developed countries has decreased due to the improvement in primary prevention of cardiac diseases, it still affects 1-2% of the adult population and the absolute number of patients is increasing due to shifts in age distribution. Therefore, heart failure is a rapidly growing health problem with increased morbidity and mortality rendering it a substantial burden to the health-care system.

The current treatment of heart failure consists of drugs influencing compensatory mechanisms of hemodynamics like inhibiting the renin-angiotensin-aldosterone system (RAAS), decreasing the workload of the heart and reducing fluid retention (Kemp & Conte, 2012; Ponikowski et al., 2016). In addition to pharmaceutics, other treatment options in the early disease phase are lifestyle modifications (e.g. exercise) and in the late phase, an implantable assist device and surgery (Tham, Bernardo, Ooi, Weeks, & McMullen, 2015). The current treatments relieve the symptoms and improve the quality of life; however, they only delay the progression of the disease. Thus, there is an urgent need for new treatments. Heart failure is usually preceded by left ventricular (LV) hypertrophy and a remodelling process. These cellular processes are the target of new therapeutic approaches, e.g. the signalling pathways, calcium handling, mitochondrial function and gene activation (Tham et al., 2015). The remodelling process involves multiple pathways, cross talk with signalling components and activation/inactivation of various transcription factors eventually leading to alterations in gene expression. Thus, selectively targeting particular transcription factors (TFs) and suppressing their activity is of great therapeutic interest (Kohli, Ahuja, & Rani, 2011).

GATA binding protein 4 (GATA4) and NK2 homeobox 5 (NKX2-5) are crucial TFs for embryonic heart development (Dirkx, da Costa Martins, & De Windt, 2013). In the adult heart, GATA4 is a critical regulator of adaptive cardiac growth in response to pathologic and physiologic stress. Both GATA4 and NKX2-5 regulate the foetal genes which are upregulated during the remodelling process, e.g. atrial

and brain natriuretic peptides (ANP and BNP respectively). Moreover, GATA4 is an important survival factor by regulating apoptosis and inducing cardiac regeneration (Y. Kim et al., 2003; Rysä et al., 2010; Y. J. Suzuki, Nagase, Day, & Das, 2004). GATA4 and NKX2-5 interact physically and act synergistically as key transcriptional controllers of numerous cardiac genes, including ANP (Pikkarainen, Tokola, Kerkelä, & Ruskoaho, 2004). Previously it has been shown that the GATA binding sites in combination with an NKX2-5 binding element are important for the mechanical stretch-induced BNP gene activation and cardiomyocyte hypertrophy (Pikkarainen et al., 2003). Consequently, this indicates that physical co-operation with GATA4 and NKX2-5 is required for the mechanical stretch response, thus making this interaction an interesting drug target.

In the present study, the GATA4 protein structure was explored by mutating amino acids and studying their effect on protein-protein interaction with NKX2-5, binding on deoxyribonucleic acid (DNA) and on transcriptional activity. These results guided the drug design and led to the identification of small molecule compounds which inhibit GATA4-NKX2-5 synergistic activity (Välimäki et al., 2017). The lead compound, 3i-1000, was discovered to inhibit hypertrophic responses, e.g. induced by endothelin-1 (ET-1) and stretching. Here, the compound binding to GATA4 was elucidated by affinity chromatography. Moreover, the cardioprotective effects of the compounds were also studied *in vivo* by using experimental animal models of heart failure, i.e. hypertrophy and pressure overload, myocardial ischaemia and cardiotoxicity models.

2 Review of the literature

2.1 Heart failure

Heart failure can be classified into diastolic and systolic dysfunction (A. M. Katz & Rolett, 2016). The impairment of filling is characteristic for diastolic heart failure usually due to thickening of the left ventricle wall while the left ventricular cavity remains normal-sized or is reduced. Systolic dysfunction is characterised by the dilated left ventricle wall and cavity resulting in reduced ejection of blood. Systolic dysfunction is also known as heart failure with reduced ejection fraction (HFrEF) and diastolic dysfunction as heart failure with preserved ejection fraction (HFpEF). The symptoms and signs are similar in these two forms; however, systolic heart failure occurs more often in younger men with coronary artery disease, whereas diastolic heart failure is more common in older women with hypertension (D. S. Lee et al., 2009; Owan et al., 2006).

The signs and symptoms of heart failure are physiological results of the inadequate pump function of the heart. The ineffective blood flow forward from the left ventricle increases the end-diastolic pressure. This induces the elevation of atrial pressure and, furthermore, increases the pressure in the pulmonary capillaries (Kemp & Conte, 2012). The elevated blood pressure forces fluid out of the capillaries leading to pulmonary congestion and the major clinical symptom of dyspnea, cough and wheezing. Inadequate function of the right ventricle results in lower venous return from the systemic circulation inducing edema in the lower body and ascites. The decreased mean arterial blood pressure reduces oxygen-rich blood flow in the brain and heart causing fatigue. The body tries to stabilise the blood pressure and proper tissue perfusion by several mechanisms including neurohormonal activation, release of natriuretic peptides, and ventricular remodelling (Kemp & Conte, 2012; Nakamura & Sadoshima, 2018; Sergeeva & Christoffels, 2013).

The decreased mean arterial pressure stimulates the sympathetic nervous system and the release of catecholamines (adrenaline and noradrenaline) (Kemp & Conte, 2012). Stimulation of the β-adrenergic receptors in the heart increases the heart rate and contractility resulting in improved cardiac output. In the periphery, stimulation of the sympathetic nervous system leads to vasoconstriction, which increases total peripheral resistance elevating the blood pressure. Sympathetic activation and reduced renal blood flow induce the kidneys to secrete renin. The

activation of the RAAS further increases vasoconstriction, promotes sodium reabsorption and increases the contractility of the heart. In early stages these mechanisms are beneficial and compensatory; however, in the long-term they worsen the heart failure. The current treatment of heart failure consists of drugs which influence these compensatory mechanisms: angiotensin converting enzyme (ACE) inhibitors or angiotensin II type 1 receptor (AT_1R) blockers, β-blocking agents and mineralocorticoid receptor blockers. Diuretics are used to relieve fluid retention, e.g. in the case of pulmonary congestion and peripheral edema (Kemp & Conte, 2012; Ponikowski et al., 2016). For the patients resistant to medical therapy, usually in the end-stage of heart failure, the only treatment options are cardiac transplantation or implantation of a left ventricular assist device (Xie, Burchfield, & Hill, 2013).

2.2 Cardiac hypertrophy

Most of the mammalian cardiac myocytes are terminally differentiated in adults and their ability to proliferate remains an area of active debate (Ponnusamy, Li, & Wang, 2017). However, cardiomyocytes can grow, shrink or die to alter the structure and function of the heart in response to mechanical stress and neurohormonal activation (Nakamura & Sadoshima, 2018; Shimizu & Minamino, 2016). The enlargement of the heart caused by either increase in myocardial mass or expansion of the ventricular chamber is called cardiac hypertrophy (Fig. 1). Left ventricular hypertrophy can be physiological, as a result of exercise or pregnancy, or pathological as in cardiac diseases and myocardial injury. Physiological hypertrophy is a fine-tuned and orchestrated process consisting of reversible growth with normal or enhanced cardiac function while the pathological hypertrophy process is progressive eventually leading to heart failure and death.

Pathological hypertrophy is initially invoked to reduce ventricular wall stress and temporarily preserve cardiac pump function (Nakamura & Sadoshima, 2018). In pressure overload diseases, such as aortic stenosis or hypertension, the heart generally develops concentric hypertrophy (increased wall and septal thickness) where cardiomyocytes typically increase in thickness more than in length due to the parallel arrangement of the new sarcomeres (A. M. Katz & Rolett, 2016; Nakamura & Sadoshima, 2018). In volume overload diseases such as myocardial infarction (MI) or dilated cardiomyopathy, the number of cardiomyocytes is decreased and the surviving cardiomyocytes increase in length characteristic of

sarcomeres arranged in series. This type of remodelling is defined as eccentric hypertrophy or dilatation.

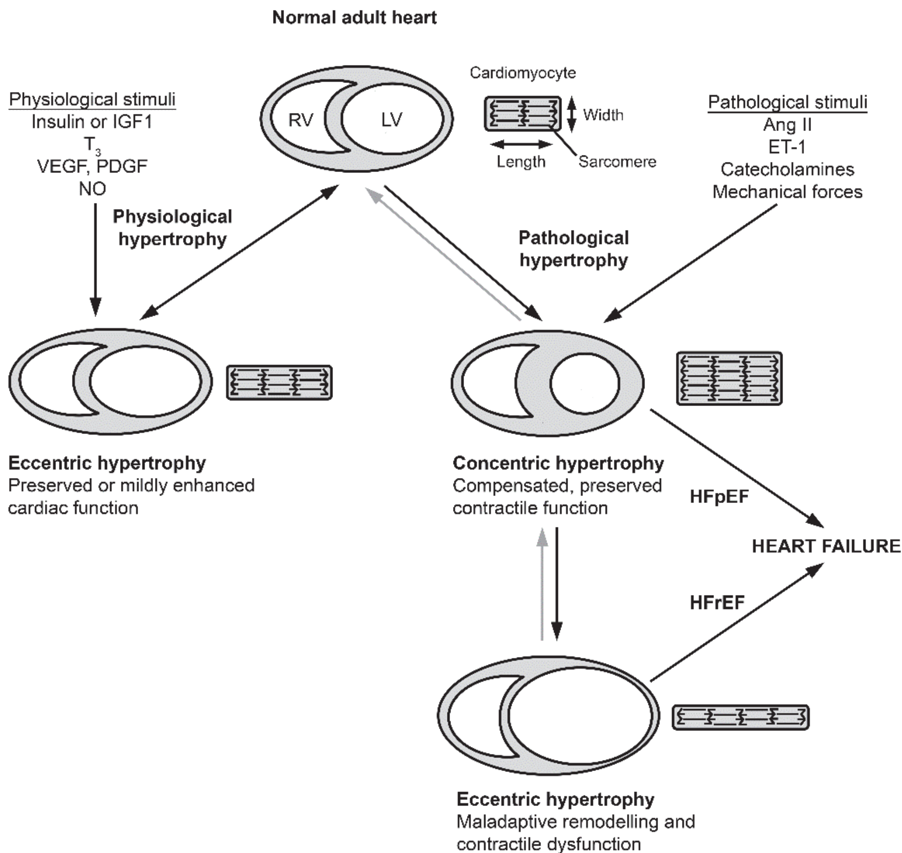

Fig. 1. Different forms of cardiac hypertrophy. Modified from (Nakamura & Sadoshima, 2018). RV, right ventricle; LV, left ventricle; IGF1, insulin-like growth factor 1; T_3, thyroid hormone; VEGF, vascular endothelial growth factor; PDGF, platelet derived growth factor; NO, nitric oxide; Ang II, angiotensin II; ET-1, endothelin-1; HFpEF, heart failure with preserved ejection fraction; HFrEF, heart failure with reduced ejection fraction.

2.3 Myocardial remodelling

Pathological hypertrophy can be distinguished from physiological hypertrophy at the molecular level (Nakamura & Sadoshima, 2018). During physiological hypertrophy, excluding postnatal hypertrophy, the myocytes grow modestly (10-

20%) both in length and width. Hearts with physiological hypertrophy have increased energy production and efficiency as well as sufficient angiogenesis proportional to the ventricular wall growth. For cardiac hypertrophy to be fully adaptive, the activation of responses which antagonise pathological responses is necessary, i.e. cell survival signalling, antioxidant systems, mitochondrial quality control, and cardiomyocyte proliferation and regeneration (Nakamura & Sadoshima, 2018). Pathological hypertrophy, in contrast, is associated with several adverse remodelling processes: fibrosis, cell death, insufficient angiogenesis, induced foetal gene expression, metabolic switch from fatty acids to glucose utilization, altered sarcomere structure, dysregulation of Ca^{2+}-handling proteins, mitochondrial dysfunction, excessive reactive oxygen species (ROS) production, increased autophagy, inflammation as well as impaired protein and mitochondrial quality control (for reviews see: Barry, Davidson, & Townsend, 2008; Nakamura & Sadoshima, 2018; Tham et al., 2015). All three of the most abundant cardiac cell types, endothelial cells, myocytes and fibroblasts, are involved in the myocardial remodelling process (Nakamura & Sadoshima, 2018).

2.3.1 Fibrosis and inflammation

Fibrosis induces stiffness to the myocardium as a compensatory mechanism to chronic pressure or volume overload (Tham et al., 2015). In addition, scar formation is a major remodelling process after myocardial infarction where the loss of cardiomyocytes is replaced with fibrotic tissue (Talman & Ruskoaho, 2016). Extracellular matrix (ECM) surrounding cardiomyocytes is secreted by fibroblasts, which maintain the collagen levels by producing matrix proteins, primarily collagen types I and III, and secreting growth factors, cytokines and collagen degrading matrix metalloproteinases (MMPs) (Frieler & Mortensen, 2015; Nakamura & Sadoshima, 2018; Talman & Ruskoaho, 2016; Tham et al., 2015). Growth factors, including fibroblast growth factors (FGFs) and transforming growth factor β (TGFβ) induce fibroblast proliferation and fibrosis, as well as cardiomyocyte hypertrophy. During pathological remodelling, released inflammatory signalling molecules induce both hypertrophic and fibrotic responses. In patients with heart failure, the circulating levels of inflammatory cytokines such as tumour necrosis factor α (TNF-α), interleukin-6 and interleukin-1β correlate with disease severity (Testa et al., 1996). Furthermore, angiotensin II (Ang II), ET-1 and aldosterone promote vascular and cardiac fibrosis and hypertrophy independently of blood pressure (Frieler & Mortensen, 2015; Talman & Ruskoaho,

2016). In addition to fibroblasts, there are various cell types which can contribute to fibrosis indirectly by secreting fibrogenic mediators, e.g. endothelial cells, macrophages, mast cells, lymphocytes, cardiomyocytes and vascular cells (Talman & Ruskoaho, 2016; Tham et al., 2015). Pro-fibrotic signalling involves numerous factors which affect fibroblast proliferation, transdifferentiation to myofibroblasts, and extracellular matrix deposition (Talman & Ruskoaho, 2016).

2.3.2 Cell death

Based on morphological characteristics, cell death can be classified into three major types: apoptosis, necrosis and autophagy (Moe & Marin-Garcia, 2016). Apoptosis increases normally with ageing and is further accelerated in the presence of pressure overload (Burchfield, Xie, & Hill, 2013). It is a programmed cell death response and reversible up to a point of no return. Myocardial necrosis, however, is the dominant type of cell death in myocardial infarction (Braunwald, 2013). Unlike apoptosis, necrosis involves the release of intracellular contents and triggers an inflammatory response. Some of the released factors can be detected in the blood and used as markers of injury (e.g. creatine kinase, cardiac troponins) (Burchfield et al., 2013). Autophagy, which is characterised by the massive formation of lysosomes, is a process that is considered to be protective but when accelerated may become maladaptive and result in increased cell death (Braunwald, 2013; Moe & Marin-Garcia, 2016). A selective form of autophagy, mitophagy, is also a protective mechanism that allows the elimination of damaged mitochondria and thereby the attenuation of mitochondria-mediated apoptosis and necrosis in the myocardium (Moe & Marin-Garcia, 2016).

Two central pathways mediate apoptotic cell death, an extrinsic pathway involving cell surface death receptors (DR) and an intrinsic pathway initiated by mitochondria and endoplasmic reticulum (Mishra et al., 2019). In the DR pathway, a variety of death ligands (e.g. TNF-α, Fas ligand) bind their cognate receptors to trigger cell death (Konstantinidis, Whelan, & Kitsis, 2012). The intrinsic pathway mediates a diverse array of death stimuli including deprivation of nutrients, oxygen, and survival factors, oxidative stress, DNA damage, proteotoxic stress, and chemical and physical toxins (Konstantinidis et al., 2012). The intrinsic and the extrinsic pathways converge at mitochondria and activate the caspase pathway – a group of cysteine proteases (Moe & Marin-Garcia, 2016). The initiator caspases, caspase-8 and -9, act upstream to initiate and regulate apoptosis, whereas the effector caspases, such as caspase-3, -6, and -7, act downstream of both the

extrinsic and intrinsic pathways to carry out the final biochemical changes in apoptosis (Mishra et al., 2019).

The main regulators of the mitochondrial pathway in both apoptosis and necrosis are the B-cell lymphoma (Bcl) 2 family proteins (Konstantinidis et al., 2012). The Bcl-2 family is composed of both antiapoptotic (e.g. Bcl-2, Bcl-extra large [Bcl-xL], myeloid cell leukemia 1 apoptosis regulator, Bcl-2 family member [Mcl-1]) and proapoptotic members (e.g. Bcl-2 associated X protein [Bax], Bcl-2 homologous antagonist/killer [Bak] and BH3-only proteins). Cardiac-specific overexpression of Bcl-2 decreases infarct size and cardiac dysfunction after ischaemia/reperfusion (I/R) *in vivo* (Brocheriou et al., 2000; Z. Chen, Chua, Ho, Hamdy, & Chua, 2001), whereas deletion of Bax in mice markedly reduces the infarct size and improves cardiac function after I/R or MI (Hochhauser et al., 2003; Hochhauser et al., 2007).

In addition, pressure overload, myocardial infarction and DNA damage with doxorubicin treatment induce tumour protein p53 (p53) accumulation in cardiomyocytes (Morita & Komuro, 2018). p53 induces apoptotic cell death and inhibits autophagic degradation of damaged mitochondria facilitating mitochondrial dysfunction and heart failure (Hoshino et al., 2013).

In addition to the cell death types presented here, other types of cell death have also been recognised in cardiomyocytes, i.e. pyroptosis, necroptosis and ferroptosis (Mishra et al., 2019). Overall, it is likely that more than one form of cell death, and cross-talk among these forms, is involved in the progression of heart failure.

2.3.3 Angiogenesis

Insufficient capillary density and angiogenesis have an observed role in the shift from adaptive cardiac hypertrophy to heart failure (Oka, Akazawa, Naito, & Komuro, 2014; Shimizu & Minamino, 2016). Enhanced vascular growth stimulates myocardial hypertrophy even in the absence of hypertrophic stimuli. The hypertrophic growth may be promoted by increased delivery of nutrients and oxygen to cardiomyocytes or/and increased production of endothelium-derived nitric oxide (NO) – one of the critical factors mediating angiogenesis-induced myocardial hypertrophy. Hypertrophic stimuli activate cardiomyocyte intracellular signalling, e.g. the protein kinase B (PKB/Akt) pathway, and transcription factors such as GATA4 and hypoxia inducible factor 1 alpha (HIF1A). This leads to increased expression of vascular endothelial growth factor (VEGF) – a major angiogenic molecule (Oka et al., 2014; Shimizu & Minamino, 2016). Long-term or

excessive PKB activation is shown to promote pathological hypertrophy (Oka et al., 2014; Shimizu & Minamino, 2016). Moreover, hypoxic stress and doxorubicin induce accumulation of p53, which promotes the ubiquitination and proteosomal degradation of HIF1A. This leads to downregulation of VEGF expression and impaired angiogenesis (Morita & Komuro, 2018; Ravi et al., 2000; Sano et al., 2007). Although much is known, the precise triggers and mechanisms for the disruption of coordinated angiogenesis, which eventually leads to heart failure, remain unclear (Oka et al., 2014).

2.3.4 Foetal gene activation

Pathological cardiac hypertrophy is characterised by the upregulation of foetal genes that are not usually expressed in the adult left ventricle, e.g. ANP and β-myosin heavy chain (β-MHC) (Barry et al., 2008; Dirkx et al., 2013; Tham et al., 2015). *In vitro* the hypertrophic response can be seen within 30 min as elevation of early response genes (e.g. early growth response 1, heat shock protein 70 (HSP70), c-fos, c-jun, c-myc). *In vivo*, this transient response seems to represent a general pattern of growth induction in terminally differentiated cells that have lost the ability to undergo DNA replication (Hefti, Harder, Eppenberger, & Schaub, 1997). Re-expression of foetal genes occurs *in vitro* 6-12 hours after the stimulus. Upregulation of constitutively expressed contractile proteins (ventricular MLC-2 and α-cardiac actin) may follow after 12-24 hours (Hefti et al., 1997). Elevated plasma levels of BNP and N-terminal pro-BNP are used as biomarkers and measured routinely from patients with hypertrophic cardiomyopathy and patients with cardiac dysfunctions (Ruskoaho, 2003). Interestingly, decreased expression of cardiac foetal genes has been observed with heart failure patients after treatment with beta-blockers (Lowes et al., 2002) or implanted ventricular assist devices (Blaxall, Tschannen-Moran, Milano, & Koch, 2003; Margulies et al., 2005). The re-activation of foetal genes involves the combined activation of transcriptional processes, post-transcriptional regulation and chromatin remodelling (DNA methylation, covalent modifications of histones and chromatin conformational changes) (Dirkx et al., 2013; DiSalvo, 2015). Histone lysine acetylation by histone acetyltransferases (HATs) weakens the histone-DNA binding, thereby allowing transcription factors access to DNA. Histone deacetylases (HDACs) have the reciprocal effect; they induce histone-DNA binding and repress transcription. Furthermore, histone methylation by histone methyltransferases and histone demethylases (JMJD1/2) either activate or repress genes. Besides

acetylation/deacetylation/demethylation of histones some HATs, HDACs and JMJDs also modulate the activity of transcription factors either directly or indirectly (Dirkx et al., 2013). The re-activation of foetal genes in the adult failing heart is a complex biological process that involves microRNAs as well as transcriptional, posttranscriptional, and epigenetic regulation of the cardiac genome (Dirkx et al., 2013).

Natriuretic peptides

Natriuretic peptides constitute a family of the three peptide hormones: ANP, BNP and C-type natriuretic peptide (CNP) which act through the transmembrane guanylate cyclase (GC) receptors, i.e. natriuretic peptide receptors (NPRs) (Gardner, 2003; Waschek, 2004). ANP and BNP activate NPR-A/GC-A, whereas CNP activates NPR-B/GC-B (Gardner, 2003). ANP and BNP encoding genes are located very close to each other in the genome, i.e. only about 8 kb from each other (LaPointe, 2005). During mouse cardiac development, ANP and BNP are expressed in both atrial and ventricular chambers and have quite similar expression patterns, although ANP expression is more pronounced than BNP. In mouse embryos ANP and BNP mRNA appear at around day 8-9 when the primitive heart starts regular beating (Cameron & Ellmers, 2003). Both ANP and BNP mRNA expressions have a large peak at the time of progressive septal formation and a smaller peak when the alteration of the heart axis takes place (Cameron & Ellmers, 2003). In human cardiac development, neither BNP mRNA nor BNP peptide was detected in 12–19 week-old foetuses (Sergeeva & Christoffels, 2013), however ANP mRNA was detected in ventricles of 17 and 19 week-old foetuses (Takahashi, Allen, & Izumo, 1992). Around the time of birth ANP becomes restricted to atria in mammals (Small & Krieg, 2004). In both rodents and humans, BNP peptide and mRNA expressions are higher in the atria; however, given the greater mass of the ventricle, it is the primary source for both synthesis and secretion (LaPointe, 2005). In human foetuses, the plasma concentrations of N-terminal proANP and N-terminal proBNP increase markedly after the delivery at the first day of age and thereafter both decrease steadily suggesting that they have a physiological role in the change of perinatal circulation from foetus to neonate (Mir et al., 2003). In adults, ANP and BNP regulation of blood pressure and fluid volume is mediated through several organs: kidney (induces diuresis, natriuresis and inhibits renin secretion), vessels (induce vasodilation), adrenal gland (inhibits aldosterone synthesis) and the central

nervous system (inhibits drinking, salt intake and vasopressin release) (Nishikimi, Maeda, & Matsuoka, 2006).

Besides being a marker of pathological hypertrophic growth, ANP and BNP seem to act as a local brake to control pathological growth of myocytes and fibroblast proliferation (Gardner, 2003; Waschek, 2004). In fibroblasts, ANP reduces growth factor-dependent mitogenesis (Cao & Gardner, 1995). In neonatal ventricular myocytes, ANP inhibits hypertrophic effects, i.e. cell growth and protein synthesis (Calderone, Thaik, Takahashi, Chang, & Colucci, 1998; Horio et al., 2000). Furthermore, BNP is able to reduce fibrosis by decreasing collagen synthesis and increasing matrix metalloproteinase activity in cardiac fibroblasts *in vitro* (Tsuruda et al., 2002). *In vivo* overexpression of BNP in the myocardium by adenovirus-mediated gene delivery improves cardiac function after myocardial infarction or Ang II-induced hypertrophy (Moilanen et al., 2011). In mice, deletion of the BNP gene results in increased interstitial fibrosis in the left ventricle with no signs of systemic hypertension or ventricular hypertrophy (Tamura et al., 2000). Physiological concentrations of ANP increase endothelial cell proliferation and migration thus possibly improving vascularisation; however, large concentrations may be the reason why ANP in some experiments inhibits VEGF signalling (Waschek, 2004). Given the very high levels of natriuretic peptides that circulate in the plasma of heart failure patients, it is interesting that short-term infusion of BNP (6 h) in patients with acute heart failure improves hemodynamic function (Colucci et al., 2000).

Myosin heavy chain

MHCs, the molecular motors of muscle, are expressed in two isoforms in the mammalian heart, α- and β-MHC. The α-MHC (V_1) has a higher adenosine triphosphatase (ATPase) activity than β-MHC (V_3). Thus α-MHC allows for more rapid contractile velocity for the heart than β-MHC, which has greater economy in force generation (Schwartz, Boheler, de la Bastie, Lompre, & Mercadier, 1992). In small mammals, the major isoform is fast α-MHC, whereas in humans, the major form is the slow but more powerful β-MHC (Schwartz et al., 1992). In rodents, β-MHC is the predominant isoform in the developing heart, but during early postnatal development it is replaced by fast α-MHC. Pathological hypertrophic stimuli have been shown to shift α-MHC expression to β-MHC, whereas physiological hypertrophic stimuli increase α-MHC expression. In humans, expression of both isoforms are increased in left ventricles from foetus to adult, β-MHC being the

more pronounced isoform. In the failing heart, the expressions of both isoforms are downregulated (Nakao, Minobe, Roden, Bristow, & Leinwand, 1997) nearly to the levels of foetal expression (Razeghi et al., 2001).

2.4 Transcription factors

TFs are nuclear regulatory proteins, which detect specific sequences in DNA and target the assembly of protein complexes that control gene expression (Lodish et al., 2000). Eukaryotic TFs contain a variety of structural motifs which mediate DNA binding and interactions with other TFs, including homeodomain, leucine zipper, helix-loop-helix and zinc fingers. In addition, TFs contain activation domains which are modified, e.g. phosphorylated by kinases, allowing a TF to interact with its co-activator or enhance its binding to DNA (Latchman, 1993; Lodish et al., 2000).

In the heart, a hypertrophic stimulus enhances TFs GATA4 and myocyte enhancer factor 2 proteins (MEF2) DNA binding, upregulates NKX2-5 expression and downregulates heart and neural crest derivatives expressed (HAND) expression leading to upregulation of foetal gene expression (Akazawa & Komuro, 2003). NKX2-5, myocardin, and GATA4 expressions, quantified from peripheral blood mononuclear cells, were elevated significantly in hypertrophic cardiomyopathy patients (Kontaraki, Parthenakis, Patrianakos, Karalis, & Vardas, 2007).

2.4.1 GATA4

The transcription factor GATA4 belongs to a six member family of GATA-factors, which all bind to a consensus sequence (A/T)GATA(A/G) on DNA (reviewed in Pikkarainen et al., 2004; M. Tremblay, Sanchez-Ferras, & Bouchard, 2018). Classically GATA-factors are divided into haematopoietic (GATA1-3) and cardiac (GATA4-6) GATA-factors according to their first identification in haematopoietic and cardiac development, respectively. However, their expression patterns are more complex. In the case of GATA4, it is expressed in various mesoderm and endoderm-derived tissues: heart, gonad, adrenal gland, intestine and stomach, pancreas, lung, liver and bone (M. Tremblay et al., 2018). GATA4 regulates several genes during cardiac development such as myosin heavy chain, myosin light chain, α cardiac actin, cardiac ankyrin repeated protein (CARP), HAND2/dHAND, connexin40, N-

cadherin, ANP, NKX2-5 and bone morphogenetic protein 4 (BMP4) (reviewed in Peterkin, Gibson, Loose, & Patient, 2005).

GATA4 in cardiac differentiation

GATA4 mRNA is expressed early in the developing mouse embryo E7.0-7.5 and the protein is found at E8.0 during the formation and bending of the heart tube (Heikinheimo, Scandrett, & Wilson, 1994) (Fig. 2). GATA4 deficient mice die between E8.5 and E10.5 due to severe defects in ventral body patterning and a lack of pericardial cavity and heart tube (C. T. Kuo et al., 1997; Molkentin, Lin, Duncan, & Olson, 1997). GATA4 continues to be expressed in cardiac myocytes throughout life (Heikinheimo et al., 1994). In human, over 100 mutations in the GATA4 protein have been reported in association with a variety of forms of congenital heart disease (CHD) (extensively reviewed in Whitcomb, Gharibeh, & Nemer, 2020). In addition, mutations in the 3'-untranslated region of GATA4 mRNA may affect RNA folding and cause CHD (Reamon-Buettner, Cho, & Borlak, 2007). GATA4's role in cardiac differentiation is undeniable as it contributes to diverse cell commitments in various parts of the heart along with cardiac maturation (Fig. 2).

Development of heart valves requires GATA4. During heart development the formation of the atrioventricular canal (later mitral and tricuspid valves) and outflow tract (later aortic and pulmonic valves) is a complex event characterised by a subset of endothelial cells in specified cushion-forming regions differentiating into mesenchymal cells (endothelial-to-mesenchymal transdifferentiation, EMT) (E. J. Armstrong & Bischoff, 2004). Several studies have shown that GATA4 acts as a positive regulator of EMT, since the loss of GATA4 results in hypoplastic endocardial cushions (Rivera-Feliciano et al., 2006; Zeisberg et al., 2005). Specifically in cardiac morphogenesis, the early time point conditional deletion of GATA4 results in the loss of mesenchymal cells within the endocardial cushions, whereas, a late time point deletion has no effect on cushion mesenchyme (Zeisberg et al., 2005). Furthermore, the early deletion of GATA4 showed reduced cardiomyocyte proliferation particularly in the right ventricle, which was also observed in the myocardium with late deletion (Zeisberg et al., 2005). In addition, GATA4 is essential for the genesis of the proepicardium and for correct septum transversum mesenchyme (later epicardium) development (Watt, Battle, Li, & Duncan, 2004).

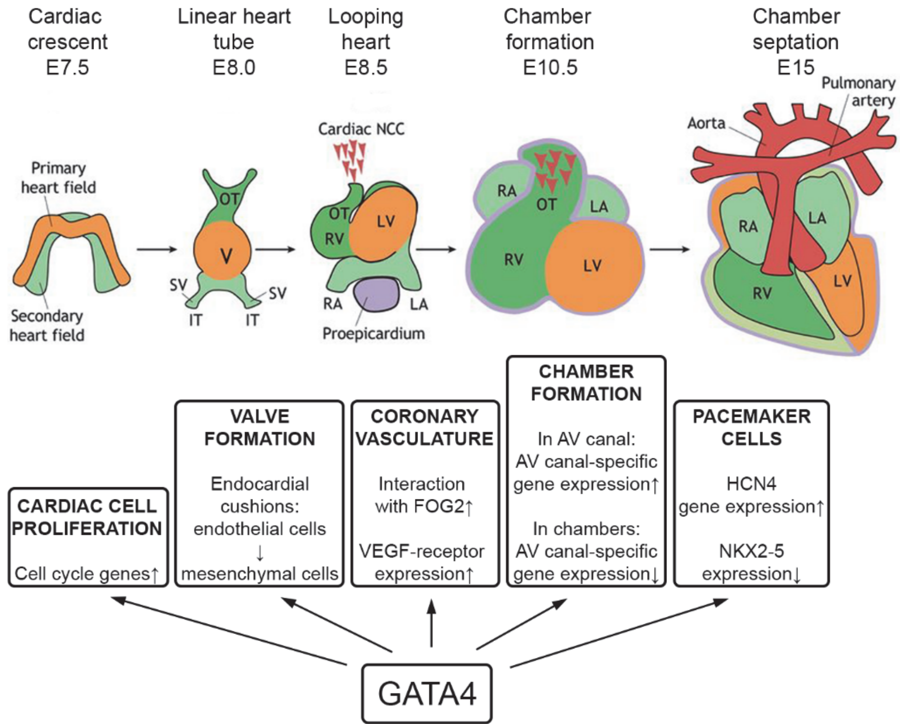

Fig. 2. The role of GATA4 in cardiac differentiation. Embryonic day (E) refers to development of mouse heart. Modified from (M. Tremblay et al., 2018). OT, outflow tract; V, ventricular region; SV, sinus venosus; IT, inflow tract; NCC, neural crest cells; RV, right ventricle; LV, left ventricle; RA, right atrium; LA, left atrium; FOG2, Friend of GATA family member 2; VEGF, vascular endothelial growth factor; AV, atrioventricular; HCN4, hyperpolarization-activated cyclic nucleotide gated potassium channel 4; NKX2-5, NK2 homeobox 5.

The role of GATA4 in chamber development. In the atrioventricular canal, the GATA4/SMAD family member (SMAD) 4 complex recruits HATs like p300 inducing acetylation of the 27th amino acid in histone H3 (H3K27) and atrioventricular (AV) canal-specific gene activity. In contrast, in chambers, GATA4 cooperates with HDAC1, HDAC2 and a chamber-specific hes related family basic helix-loop-helix transcription factor with YRPW motif (Hey) 1 or 2, which leads to H3K27 deacetylation and repression of AV canal-specific genes (Stefanovic et al., 2014). The atrial specific Hey1 and the ventricle specific Hey2 have been shown to repress GATA4 and GATA6 gene expression (Fischer et al., 2005). Furthermore, they can directly bind to GATA4 and GATA6, and block their transcriptional

activity. In addition to all of this, BMP2, required for cardiac development and AV canal-specific gene expression, upregulates GATA4 (Stefanovic et al., 2014; Zheng, Zhu, Lu et al., 2013; Zheng, Zhu, Lv et al., 2013).

GATA4 and the development of coronary vasculature. GATA4 regulates heart muscle vasculature formation during development together with Friend of GATA family member 2 (FOG2/ZFPM2) (Crispino et al., 2001). Crispino et al. (2001) created a mouse expressing mutation V217G in GATA4 ($GATA4^{ki/ki}$) which disrupts the interaction with FOG2 but leaves DNA-binding properties of GATA4 unperturbed. The $GATA4^{ki/ki}$ embryos died between E11.5 and E13.5 due to severe heart malformations and exhibited the absence of coronary vasculature. Closer examination revealed that $GATA4^{ki/ki}$ hearts did not express the receptor for VEGF (Flk-1). Furthermore, the mice with later ablation of the FOG2-GATA4 interaction, $GATA4^{ki\text{-}MC}$, survived normally but showed severely diminished systolic function at 8-14 weeks (Zhou et al., 2009). $GATA4^{ki\text{-}MC}$ mice showed ventricular dilatation and fibrosis, significantly increased cardiomyocyte and endothelial apoptosis and dramatically diminished coronary vasculature, thus indicating that the GATA4-FOG2 interaction is critical for postnatal cardiac function and maintenance of coronary vasculature. In addition, FOG2 seems to act as an attenuator of GATA4 activity during EMT (Crispino et al., 2001; Flagg, Earley, & Svensson, 2007).

GATA4 in cardiac cell differentiation and reprogramming. GATA4-deficient embryonic stem cells have the capacity to differentiate along other lineages, however, disruption of the GATA4 gene results in a specific block in visceral endoderm formation (Soudais et al., 1995). Later it was discovered that GATA4 contributes to cell commitment of all layers of the heart (endocardium, myocardium and epicardium) (Narita, Bielinska, & Wilson, 1997). Interestingly, these experiments suggested that GATA4 is not indispensable in this process and can functionally be compensated by GATA5 or GATA6. Very recently, GATA4 has been shown to mediate BMP4-dependent differentiation of T-box transcription factor (TBX) 18^{+} epicardial progenitor cells into pacemaker-like cells (Wu et al., 2019). BMP4 upregulates GATA4 which promotes hyperpolarization-activated cyclic nucleotide gated potassium channel 4 (HCN4) expression. GATA4 knockdown by siRNA upregulated NKX2-5 indicating that GATA4 participates in the repression of NKX2-5 in pacemaker cells, which distinctively do not express NKX2-5. Furthermore, GATA4 together with other cardiac developmental TFs MEF2C and TBX5 are the key components needed to reprogram fibroblasts to cardiomyocyte-like cells (reviewed in Engel & Ardehali, 2018). In addition, GATA4 is important for cardiomyocyte regeneration by regulating cardiac specific FGF 16 expression

(Yu et al., 2016), a factor that is required for cardiomyocyte replication during development and protects from cardiac injury (Hotta et al., 2008).

GATA4 and cell proliferation

As mentioned above, GATA4 deletion reduces cardiac cell proliferation (Zeisberg et al., 2005). It seems that it is a dosage-sensitive regulator. Mice that are heterozygous for the GATA4 null allele are normal (Xin et al., 2006). Similarly, $GATA4^{flox/flox}$ embryos that express 50% less GATA4 protein survive normally, while $GATA4^{H/H}$ embryos which express 70% less GATA4 protein in the heart die at E13.5-16.5 (Pu, Ishiwata, Juraszek, Ma, & Izumo, 2004). Interestingly, studies with mice expressing a reduced level of functional GATA4 (heterozygous for the GATA4 exon 2 deletion) showed at baseline decreased cardiomyocyte number and mild systolic and diastolic dysfunction (Bisping et al., 2006). The closer examination of $GATA4^{H/H}$ embryos revealed myocardial hypoplasia, which was due at least in part to decreased cardiomyocyte replication at E12.5 (Pu et al., 2004). In addition, the restricted inactivation of GATA4 in the anterior heart field (later outflow tract, right ventricle, and interventricular septum) resulted in hypoplasia of the right ventricle and ventricular septal defects resulting from diminished cardiac proliferation (Rojas et al., 2008). The whole-genome microarray analysis of these GATA4-null hearts showed that expression of cell cycle genes: Cyclin dependent kinase 4 (CDK4), G1/S-specific cyclin D2 (Cyclin D2), and Cyclin A2 were dramatically downregulated, specifically in the right ventricles. Conversely, later inactivation of GATA4 had no effect on Cyclin D2 expression (Zeisberg et al., 2005) or on Cyclin D1–3 expressions in $GATA4^{H/H}$ embryos (Pu et al., 2004). Nevertheless, GATA4 can bind directly to the Cyclin D2 and CDK4 promoters *in vivo* and *in vitro* suggesting that GATA4 could regulate the G1/S transition and cellular proliferation (Rojas et al., 2008; Yamak, Latinkic, Dali, Temsah, & Nemer, 2014).

As it belongs to the DNA binding factors that appear earliest during development, GATA4 is also considered as a pioneer transcription factor since it has the ability to access and open chromatin structure (Zaret & Carroll, 2011). GATA4 was shown to be able to bind *in vitro* reconstituted nucleosomes containing its binding site and to open the local nucleosome-rich domains, even in the absence of ATP-dependent chromatin remodelling enzymes (Cirillo et al., 2002).

GATA4 in the adult heart

In the adult heart, GATA4 regulates genes encoding ANP, BNP, contraction mediating proteins (α-MHC, cardiac troponin-C and –I, Na^{+}/Ca^{2+} exchanger, MHC 1/3), a structural protein (CARP) and receptors (A_1-adenosine receptor and M_2-muscarinic acetylcholine receptor) (reviewed in Molkentin, 2000; Pikkarainen et al., 2004). Furthermore, GATA4 regulates the gene encoding corin, the enzyme that cleaves pro-ANP to active ANP (Pan et al., 2002), and participates in fatty acid metabolism by regulating the carnitinepalmityol transferase-1β protein (Moore, Wang, Belaguli, Schwartz, & McMillin, 2001). The role of GATA4 in the adult heart is summarised in figure 3.

The 70% (Gata4fl/fl$^{\alpha MHC\text{-}Cre}$) or 95% (Gata4fl/fl$^{\beta MHC\text{-}Cre}$) reduction of GATA4 in the adult cardiac myocytes results in the dysregulation of approximately 1.0% of all cardiac-expressed genes, reduces cardiomyocyte survival, and impairs the ability of the myocardium to hypertrophy and compensate in response to pressure overload or to exercise stimulation (Oka et al., 2006). For example, the genes that were down-regulated include ANP, α-MHC, protein kinase C (PKC), and those upregulated include β-MHC, caspase-12 and Bcl-6. This indicates increased apoptosis, which was also observed in the histological stainings as increased DNA-fragmentation (TUNEL). Cardiomyocyte apoptosis increased in mice as they aged and the apoptosis was dramatically increased following pressure overload.

In the normal adult myocardium, GATA4 overexpression induces angiogenesis (Rysä et al., 2010). This was shown by intramyocardial injection of GATA4 overexpressing adenoviral vector in adult rats after two weeks. Similarly, the adult cardiomyocyte-specific overexpression of GATA4 (induction started with mice at the age of 1 month) enhances angiogenesis in the myocardium and mild, not pathological, hypertrophy (Heineke et al., 2007). Conversely, the adult cardiomyocyte-specific deletion of GATA4 (Gata4fl/fl$^{\beta MHC\text{-}Cre}$) leads to a decrease in capillary density (Heineke et al., 2007). In adult mice, GATA4 enhances angiogenesis by inducing the expression of VEGF-A.

Recently, GATA4 has been shown to be involved in cellular senescence (Kang et al., 2015). In normal conditions, GATA4 binds to the nucleoporin 62 kDa (p62) autophagy adaptor and is degraded by selective autophagy in mesenchymal stem cells. Senescence induction suppresses this autophagy through decreasing the interaction between GATA4 and p62, thereby stabilizing GATA4 that accumulates in cells. It is suggested that the function of GATA4 in the adult heart is to regulate both adaptive and maladaptive growth.

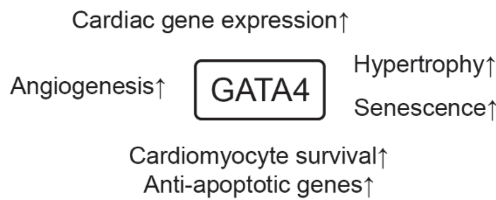

Fig. 3. GATA4 in the adult heart remodelling processes.

GATA4 and hypertrophy

Transgenic overexpression of GATA4 induces hypertrophic myocardial cell growth with hypertrophic gene expression (Liang et al., 2001). Similarly, overexpression of GATA4 in cell culture by adenoviral gene transfer induces cardiomyocyte hypertrophy (Liang et al., 2001) and sarcomere reorganization as efficiently as ET-1 or phenylephrine (PE) (Charron et al., 2001). Hypertrophic stimuli, including pressure overload, Ang II, PE, isoprenaline, and ET-1 lead to activation of GATA4 DNA-binding and transcriptional activity in cardiac myocytes (Hasegawa, Lee, Jobe, Markham, & Kitsis, 1997; Kerkelä, Pikkarainen, Majalahti-Palviainen, Tokola, & Ruskoaho, 2002; Majalahti et al., 2007; Morimoto et al., 2000; Morisco et al., 2001).

PE and ET-1 induce GATA4 DNA binding activity in neonatal cardiac myocytes (Kerkelä et al., 2002; Liang et al., 2001; Pikkarainen et al., 2002). The blocking of GATA4 expression by dominant-negative GATA4 (engrailed fusion) or anti-sense GATA4 mRNA prevents cardiomyocyte hypertrophy induced by PE and ET-1 in culture (Charron et al., 2001; Liang et al., 2001). ET-1 and PE induce phosphorylation of GATA4 Ser-105 through activation of p38 MAPK and extracellular signal-regulated kinases (ERK) 1/2 (Charron et al., 2001; Kerkelä et al., 2002; Liang, Wiese et al., 2001; Morimoto et al., 2000). Furthermore, ET-1 and PKC activator phorbol myristate acetate (PMA) activate MAPK/ERK kinase (MEK) inducing the GATA4 phosphorylation by ERK (MEK/ERK pathway) in adult AT-1 mouse atrial cardiomyocytes (HL-1 cell line) (Clement, Tan, Guo, Kitta, & Suzuki, 2002; Kitta, Clement, Remeika, Blumberg, & Suzuki, 2001). Interestingly, in HL-1 cells, PE or H_2O_2 are not able to induce GATA4 phosphorylation.

Mechanical stretch increases transiently GATA4 DNA binding activity and transcript levels followed by increases in the expression of BNP, ANP and skeletal

alpha-actin genes (Pikkarainen et al., 2003). Inhibition of GATA4 protein production by adenovirus-mediated transfer of GATA4 antisense cDNA blocks stretch-induced increases in BNP mRNA and sarcomere reorganization (Pikkarainen et al., 2003).

Isoprenaline increases GATA4 upregulation and binding to the ANP promoter (Morisco et al., 2001). As a consequence of β-adrenergic stimulation, the Akt-glycogen synthase kinase 3β (GSK3β) pathway is inactivated and GSK3β predominantly localises in the cytoplasm which allows GATA4 to accumulate in the nucleus. In the absence of a β-adrenergic stimulus, active GSK3β exists in the nucleus, phosphorylates GATA4, and stimulates nuclear export of GATA4 with nuclear exportin chromosomal maintenance 1 (Crm1/Exportin 1) (Morisco et al., 2001).

In vivo, aortic coarctation with rats induces pressure overload and upregulation of an intra-myocardially injected β-MHC-promoter luciferase reporter (Hasegawa et al., 1997). This induction was mediated through GATA-binding sites on the promoter presumably by GATA4. In another similar *in vivo* experiment, pressure overload increased the AT_1R gene expression likewise through GATA-binding sites (Herzig et al., 1997). Furthermore, this response appears to be mediated, in part, by a functional interaction between activator protein-1 (AP-1) and GATA4 transcription factors on the AT_1R promoter. The pressure overload induced by intravenous administration of arginine^{8}-vasopressin (AVP) increases left ventricular BNP mRNA expression and GATA4 binding activity, but not GATA5 and GATA6 binding, via ET-1 (Hautala et al., 2001). Interestingly, AVP did not increase GATA4 mRNA levels. Ang II infusion also increases GATA4 binding activity on the BNP promoter, as well as BNP mRNA and protein expression in the left ventricle (Majalahti et al., 2007). Furthermore, ex vivo rat hearts subjected to stretch, by inflating a balloon in the ventricle, show induction of p38 MAPK and MEK1/2 kinases, increase in GATA4 DNA-binding and upregulation of BNP mRNA (Hautala, Tenhunen, Szokodi, & Ruskoaho, 2002; Tenhunen et al., 2004). In mice expressing a reduced level of functional GATA4 (heterozygous for the GATA4 exon 2 deletion), transverse aortic constriction (TAC) pressure overload increases apoptosis and fibrosis plus cardiomyocytes hypertrophied by increasing in length more than width (Bisping et al., 2006). In hypertrophy, phosphorylation of GATA4 at Ser-105 is critically important since S105A mutant mice failed to develop productive hypertrophy in response to pressure overload and PE (van Berlo, Elrod, Aronow, Pu, & Molkentin, 2011).

Interestingly, overexpression of GATA6 also induces cardiac hypertrophy in cultured cardiac myocytes, however, GATA6 mRNA and protein are not regulated by hypertrophic signals (Liang et al., 2001; Liang, Wiese et al., 2001). Correspondingly, pressure overload and stretch induce specifically GATA4- but not GATA5- and GATA6-binding activity (Hautala et al., 2001; Hautala et al., 2002) suggesting that GATA4 may be more highly specialized for controlling these processes compared to GATA5 and GATA6. This further emphasises the role of GATA4 as a mediator of hypertrophic signalling.

GATA4 and apoptosis

Ischaemia and toxins act as apoptotic stimuli in the myocardium and cause a decline in GATA4 protein levels. Ischaemia/reperfusion (Y. J. Suzuki et al., 2004), anthracyclines/doxorubicin (Aries, Paradis, Lefebvre, Schwartz, & Nemer, 2004; Bien et al., 2007; Esaki et al., 2008; Kobayashi et al., 2006; Kobayashi et al., 2010; Koka et al., 2010; Riad et al., 2008) and mercury (Y. J. Suzuki, 2003) all induce apoptosis and downregulation of GATA4. The increased apoptosis is also observed in mice with reduced GATA4 levels and GATA4 knock-out in adult cardiomyocytes (Bisping et al., 2006; Oka et al., 2006) as well as in neonatal cardiomyocytes where GATA4 has been depleted by adenoviral anti-sense transcripts (Aries et al., 2004). *In vivo* overexpression of GATA4 by intramyocardial delivery of GATA4 adenoviral vector prevents both myocardial infarction induced apoptosis and adverse remodelling as well as induces stem cell recruitment (Rysä et al., 2010). Similarly, anthracycline-induced apoptosis in cardiomyocytes is prevented by overexpression of GATA4 by adenovirus-mediated gene transfer *in vitro* in neonatal cardiomyocytes and HL-1 cells (Aries et al., 2004; Y. Kim et al., 2003; Kobayashi et al., 2006) and *in vivo* in GATA4 overexpressing TG mice (Kobayashi et al., 2006). In addition, the α_1-adrenergic agonist PE (100 nM) is able to preserve doxorubicin depleted GATA4 levels (Aries et al., 2004).

GATA4 is a transcriptional regulator of the anti-apoptotic genes Bcl-xL (Aries et al., 2004; Kitta et al., 2003; Park, Nagase, Vinod Kumar, & Suzuki, 2007) and Bcl-2 (Kobayashi et al., 2006). Kitta et al. (2003) have shown that adenoviral overexpression of GATA4 in HL-1 cells is enough to induce upregulation of Bcl-xL and that GATA4 Ser-105 phosphorylation is required for this. Similarly, transgenic overexpression of GATA4 in mice induces upregulation of Bcl-2 mRNA and protein levels (Kobayashi et al., 2006). Kobayashi et al. (2006) demonstrated that GATA4 directly binds the Bcl-2 promoter. Furthermore, hepatocyte growth

factor (HGF), released in response to myocardial infarction, was found to prevent the anthracycline-induced apoptosis in HL-1 cells (Kitta et al., 2003). HGF induces GATA4 Ser-105 phosphorylation via the MEK/ERK signalling pathway and DNA-binding, thereby increasing the expression of Bcl-xL (Kitta et al., 2003). Furthermore, *in vivo* GATA4 is involved in erythropoietin (EPO) -induced cardioprotection against ischaemia/reperfusion injury in the mouse heart (Shan et al., 2009). EPO rescued the decrease in GATA4 and phospho-GATA4 in the myocardium following I/R as well as increased the levels of cardioprotective phospho-PKB and Bcl-2. The anti-apoptotic effect of EPO was abolished both *in vivo* and *in vitro* by blocking of GATA4.

A well-known mechanism of anthracyclines is their ability to produce ROS, and it has been discovered that the DNA-binding activity of GATA4 is oxidant-sensitive, since nuclear protein extracts treated with ROS producing substances, H_2O_2 or a sulphhydryl oxidant, diamide, potently inhibit GATA4 DNA-binding (Y. Kim et al., 2003). However, antioxidants (overexpression of the antioxidant enzyme catalase in HL-1 cells or pretreatment with a sulphhydryl reductant N-acetylcysteine) are not able to prevent the daunorubicin-mediated decrease of GATA4 activity. Furthermore, the treatment of HL-1 cells with a high concentration of H_2O_2 (producing more ROS than what is expected to be generated by low micromolar anthracycline) did not suppress GATA4 DNA-binding activity. Kim et al. (2003) propose that the cellular actions of anthracyclines on GATA4 may not be involved in generation of ROS or alteration of the mRNA stability but may occur at the transcriptional level (Y. Kim et al., 2003). A mechanism for anthracycline induced down-regulation of GATA4 gene transcription involves the p53-dependent inhibition of the CCAAT-binding protein/ nuclear factor for Y box protein (CBF/NF-Y) binding within the GATA4 promoter (Park et al., 2011). Another mechanism discovered involves doxorubicin induced activation of autophagy which is suppressed by the preservation of GATA4 (Kobayashi et al., 2010). A recent study proposed that the doxorubicin-induced GATA4 depletion is caspase-1 dependent rather than via the ubiquitin-proteasome pathway (Aries et al., 2014). Caspase-1 inhibition of GATA4 transcriptional activity is rescued by HSP70, which binds directly to GATA4 and masks the caspase recognition motif (Aries et al., 2014). Thus, it is yet unclear how GATA4 protects from anthracycline induced cardiotoxicity.

One interesting strategy against apoptosis is ischaemic preconditioning (IPC), which refers to the ability of short periods of ischaemia to make the myocardium more resistant to a subsequent ischaemic insult (Salvi, 2001). IPC created in ex

vivo rat hearts with four cyclic episodes of perfusion (5 min ischaemia and 10 min reperfusion) increases the DNA binding activity of GATA4 (Y. J. Suzuki et al., 2004). However, this IPC did protect from I/R induced downregulation of GATA4 mRNA expression. Interestingly, Suzuki et al. (2004) found that IPC induced acetylation of lysine residues within the GATA4 molecule but no Ser-105 phosphorylation was observed. In HL-1 cells, an apoptotic concentration of the NO donor sodium nitroprusside (SNP) downregulated GATA4 mRNA expression via protein kinase G (PKG). SNP/NO induced GATA4 acetylation was independent of PKG (Y. J. Suzuki et al., 2004). Acetylated GATA4 may be more resistant to I/R-driven degradation possibly by preventing the ubiquitination of these sites. The mice subjected to acute intermittent hypoxia (IH) (2 min of 10% O_2 and 2 min of 21% O_2 for 5 cycles) showed increased GATA4 gene expression and DNA-binding on the Bcl-xL promoter in the left ventricles (Park et al., 2007). Similarly, no change in GATA4 phosphorylation was observed (studied by electrophoretic mobility shift assay (EMSA)), which led to the proposition that the increase in DNA-binding was due to an increase in GATA4 expression. Park et al. (2007) discovered that acute IH activates factors which bind to the proximal 100-bp region of GATA4.

The α_1-adrenoreceptors play an important role in mediating IPC by inhibiting apoptosis by increasing the levels of the antiapoptotic protein Bcl-2 (Salvi, 2001). The *in vitro* experiment with adult cardiomyocytes from rats, which have a cardiac myocyte specific deletion of the α1-adrenergic receptor subtypes A and B (α1ABKO mice), showed that overexpression of GATA4 is sufficient to protect from noradrenaline (1 μM) induced cell death (Huang et al., 2008). However, Huang et al. (2008) found that the α1A-subtype did not induce phosphorylation or increase the activity of GATA4 in adult mouse cardiomyocytes in culture or *in vivo*. GATA4 phosphorylation did not increase *in vitro* after treatment of wild type adult cardiomyocytes for 24 h with 20 μM α1-selective activator PE or after a TAC operation in α1ABKO mice. Thus, Huang et al. (2008) hypothesise that GATA4 is not required for α1-mediated survival signalling in adult cardiac myocytes. In contrast, in neonatal cardiomyocytes, PE induced GATA4 Ser-105 phosphorylation (Charron et al., 2001; Liang, Wiese et al., 2001; Morimoto et al., 2000) suggesting a different regulation in neonatal vs. adult cardiomyocytes. Interestingly, 10 μM PE is shown to induce apoptosis in neonatal cardiomyocytes (Pu, Ma, & Izumo, 2003). PE increases the GATA4-p300 interaction, their DNA-binding and transcriptional activity in neonatal cardiac myocytes, and that interaction is inhibited by FOG2

(Hirai et al., 2004). It seems that also in the heart acute or chronic stress has harmful effects whereas short-term or mild stress may have protective and beneficial effects.

GATA4 interactions with co-factors and transcriptional activation

All GATA-family members contain two zinc fingers on their structure, one of which, the DNA-binding carboxyl-terminal finger, is conserved among the members (Omichinski et al., 1993). The N-terminal zinc finger alone is not capable of binding DNA, but influences the stability and specificity of DNA binding (reviewed in Pikkarainen et al., 2004). GATA4, 5 and 6 contain two evolutionarily conserved N-terminal activation domains, i.e. amino acids 1-74 and 130-177 in GATA4 (Morrisey, Ip, Tang, & Parmacek, 1997) (Fig. 4). Mutational analyses revealed more specifically that the residues Gln-3, Phe-26, His-28, Tyr-38, Tyr-53 and Gln-55 in activation domain I as well as Trp-172 in activation domain II were required for high level transcriptional activity (Morrisey et al., 1997). However, more or less the whole GATA4 protein is needed for transcriptional transactivation and the basic region (aa 302-324) adjacent to the C-terminal zinc finger is required for GATA4 binding to DNA (Morrisey et al., 1997). In addition, amino acid residues 270-324 contain a nonclassical nuclear localisation signal (NLS) (Morrisey et al., 1997; Philips, Kwok, & Chong, 2007). More specifically, four arginines within the NLS mediate active transport predominantly through the nonclassical pathway via interaction with importin β. Simultaneous mutation of residues Arg-282, Arg-283, Arg-317 and Arg-319 (mGATA4) is required for complete inhibition of nuclear import (Philips et al., 2007). Furthermore, GATA4 may contain additional NLSs (Hunter et al., 2014; Morrisey et al., 1997).

N-terminal interaction partners. The N-terminal region of GATA4 (aa 1-181) is important for the physical interaction with the Jumonji (JARID2) TF that inhibits GATA4 transcriptional activity (T. G. Kim, Chen, Sadoshima, & Lee, 2004). The N-terminal activation domain, especially Ser-160, is important for interaction with Cyclin D2 (Yamak et al., 2014). Amino acids 129-152 of GATA4 are required for interaction with the mammalian chromatin remodelling complex Brahma-related gene 1 associated factor 60C (BAF60c) and are important for cardiogenesis (Gallagher, Komati, Roy, Nemer, & Latinkic, 2012). The amino-terminal zinc finger of the GATA4 protein interacts with other zinc finger transcription factors: FOG2, which generally represses GATA4 transcriptional activity (Lu et al., 1999; Svensson, Tufts, Polk, & Leiden, 1999), and Kruppel like factor 13 (KLF13) (Lavallee et al., 2006). More specifically, Glu-215 (mGATA4) is shown to be

important for the GATA4-FOG2 interaction (Svensson, Huggins, Dardik, Polk, & Leiden, 2000). Later it was shown that Val-217 corresponds to a conserved amino acid among GATA-factors that mediates interaction with FOG1 or FOG2 (Crispino et al., 2001).

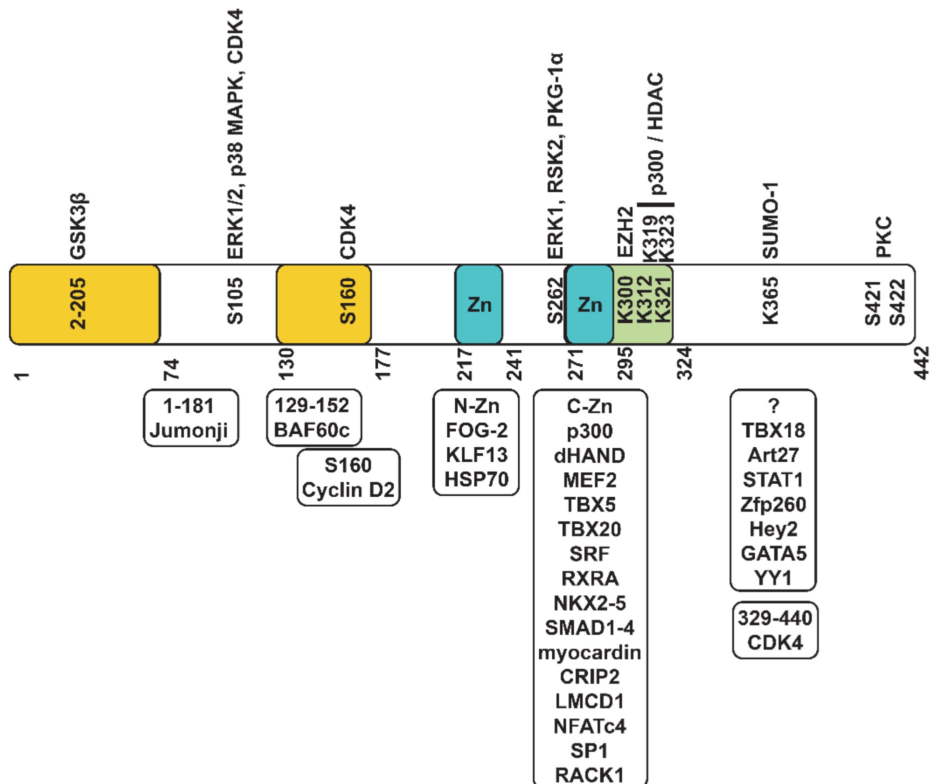

Fig. 4. GATA4 protein structure, posttranslational modifications and interaction partners. The amino acid numbering corresponds to human GATA4. Yellow boxes indicates transaction domains, blue boxes zinc fingers and green box nuclear localisation signal. Zn, zinc finger; N-Zn, amino terminal zinc finger; C-Zn, carboxy terminal zinc finger.

C-terminal interaction partners. GATA4 physically interacts with several other TFs and cofactors mostly through its C-terminal zinc finger: histone acetyltransferase p300 (Dai & Markham, 2001), HAND2/dHAND [especially amino acids WRR 281-283] (Dai, Cserjesi, Markham, & Molkentin, 2002), MEF2A, -C and -D (Morin, Charron, Robitaille, & Nemer, 2000), TBX5 (Garg et

al., 2003), TBX20 (Stennard et al., 2003), serum response factor (SRF) (Belaguli et al., 2000; Morin, Paradis, Aries, & Nemer, 2001), retinoid X receptor alpha (Clabby, Robison, Quigley, Wilson, & Kelly, 2003), NKX2-5 (Durocher, Charron, Warren, Schwartz, & Nemer, 1997; Y. Lee et al., 1998; Shiojima et al., 1999), SMAD family members 1,2,3 and 4 (Brown et al., 2004), myocardin [especially amino acids WRR 281-283] (Oh et al., 2004), GATA-6 (Charron, Paradis, Bronchain, Nemer, & Nemer, 1999), LIM-type zinc finger-domain (LIM) containing cysteine rich protein 2 (CRIP2) (Chang et al., 2003), LIM and cysteine rich domains 1 (LMCD1/dyxin) (Rath, Wang, Lu, & Morrisey, 2005), nuclear factor of activated T cells 4 (NFATc4/NFAT3) (Molkentin et al., 1998), Sp1 transcription factor (SP1) (Hu et al., 2011) and receptor for activated C kinase 1 (RACK1) (H. Suzuki et al., 2016) (Fig. 4).

Other interaction partners. In addition, there are direct interaction partners of GATA4; however, their binding sites have not yet been determined in detail. TBX18 (Farin et al., 2007), transcriptional repressor Art27 (Carter, Buckle, Tanaka, Perdomo, & Chong, 2014), signal transducer and activator of transcription (STAT) 1 (Wang et al., 2005), zinc finger protein 260 (Zfp260/PEX1) (Debrus et al., 2005), Hey2 (Xiang et al., 2006), GATA5 (Kassab et al., 2015) and the YY1 transcription factor do not physically interact with GATA4 but synergistically activate transcription together with the cyclic adenosine monophosphate response element-binding protein (CREB) (Bhalla, Robitaille, & Nemer, 2001). Furthermore, CDK4 physically interacts with the C-terminal region of GATA4 (aa 329-440) even though it phosphorylates the serine residues at the N-terminal domain (Gallagher et al., 2014). In addition to these interaction partners, GATA4 physically interacts with several TFs in cell types other than cardiac cells that are not discussed here.

Posttranslational modification of Ser-105. Transcriptional activity of GATA4 is regulated by several posttranslational modifications (Fig. 4) of which acetylation, phosphorylation and sumoylation generally activates and methylation inhibits GATA4 transcriptional activity (reviewed in Katanasaka, Suzuki, Sunagawa, Hasegawa, & Morimoto, 2016). The fundamental regulators of GATA4 activity in hypertrophy are p38 MAPK and ERK1/2, which phosphorylate Ser-105 (Charron et al., 2001; Liang, Wiese et al., 2001). CDK4 phosphorylates Ser-105 and Ser-160 (Nakajima et al., 2011; Yamak et al., 2014). Interestingly, phosphorylation of Ser-105 is necessary for GATA4-dependent cardiac myocyte survival and hypertrophy but not for GATA4-induced cardiogenesis (Gallagher et al., 2012). Moreover, Ser-105 is differentially required for transcriptional synergy between GATA4 and SRF but not TBX5 and NKX2-5 (Gallagher et al., 2012).

Posttranslational modifications of other residues. Ser-261 of GATA4 is phosphorylated by ERK1, ribosomal protein S6 kinase 2 (RSK2) (Li, Liu, Hu, Ma, & Zhou, 2012) and PKG-1α (Ma, Wang, Yu, & Schwartz, 2016). EPO-induced ERK signalling activation increased GATA4 phosphorylation and acetylation, partly via an increase in the association between GATA4 and p300, and these processes required the phosphorylation of GATA4 at the Ser-261 residue (Jun, Shim, Ryoo, & Kwak, 2013). Moreover, protein kinase A (PKA) phosphorylates Ser-261 in gonadal cells (J. J. Tremblay & Viger, 2003); however, the role of PKA in regulating GATA4 in cardiac cells is not known. In embryonic cardiomyocytes, PKC phosphorylates Ser-419/Ser-420 and is, together with STAT, involved in Ang II-induced GATA4 dependent gene transcription (Wang et al., 2005). GSK3β phosphorylates the N-terminal domain of GATA4 (2–205 aa) and negatively regulates it by increasing nuclear export of the GATA4 protein (Morisco et al., 2001). Acetylation of Lys-312/319/321/323 by p300 mediates hypertrophic responses (Takaya et al., 2008). Lys-366 is sumoylated by the small ubiquitin-like modifier-1 (SUMO-1) which results in enhanced GATA4 activity (Komatsu et al., 2004; Wang, Feng, & Schwartz, 2004). The sumoylation regulates nuclear localization of GATA4 in cardiac myocytes (Wang et al., 2004) but not necessarily in other cell types (Belaguli, Zhang, Garcia, & Berger, 2012). Enhancer of zeste homolog 2 (EZH2), the catalytic subunit of the polycomb-repressive complex 2 (PRC2), methylates GATA4 at Lys-299 and attenuates its transcriptional activity by reducing its interaction with and acetylation by p300 (He et al., 2012). Phosphorylated HDAC2 interacts with GATA4 and deacetylates lysines 311, 318, 320, and 322 resulting in suppression of transcriptional activity (Trivedi et al., 2010). Furthermore, RACK1, which has no catalytic activity, binds GATA4 and inhibits p300/GATA4 interactions and p300/GATA4-dependent hypertrophic responses (H. Suzuki et al., 2016).

GATA4 activity is also regulated on protein levels. ROS production induced by H_2O_2 in P19 embryoid bodies increases GATA4 mRNA expression in low and high concentrations. However, the high concentration of H_2O_2 induces GATA4 protein degradation in a proteasome-dependent manner (Li, Zhang, Jiang, Liu, & Liu, 2018). HSP70, which prevents caspase-1 cleavage of GATA4, interacts directly with the N-terminal of GATA4 (Aries et al., 2014). The GATA4 amino acids Asp-168 and Asp-230 have been recognised as caspase-1 cleavage sites and the double mutation of amino acids 168 and 230 blocks GATA4 cleavage by caspase-1.

2.4.2 NKX2-5

NKX2-5 starts to be expressed very early in development, at day E7.5 like GATA4, and the expression continues in the adult heart (Komuro & Izumo, 1993). It is needed for rightward looping and later for chamber specification, septation and development of the conduction system (Akazawa & Komuro, 2005). Human mutations in the NKX2-5 gene cause CHD with various malformations, e.g. atrial or ventricular septal defects, atrioventricular bloc, Tetralogy of Fallot or combinations of these (Reamon-Buettner & Borlak, 2010). In NKX2-5 knockout mice, the heart forms but its development stops at the looping stage (Lyons et al., 1995). NKX2-5 is considered to be cardiac specific, however, there are some contradictory reports as to whether it is expressed only in the adult heart (Komuro & Izumo, 1993; Shiojima et al., 1996) or also in spleen and tongue in adults (Lints, Parsons, Hartley, Lyons, & Harvey, 1993). The NKX2-5 protein has been detected from foetal mouse spleen, stomach, liver, tongue, anterior larynx and cranial skeletal muscles; however, NKX2-5 expression in these organs is markedly downregulated after birth (Kasahara, Bartunkova, Schinke, Tanaka, & Izumo, 1998). Thus far the role of NKX2-5 in other adult organs, except the heart, is unknown.

Protein structure and regulation of the transcriptional activity

NKX2-5 belongs to the NK-2 class of NK homeobox genes (Harvey, 1996). NK-2 members share an evolutionary conserved protein structure with a TN-domain at the N-terminal end, a homeodomain in the middle and an NK-2 domain located C-terminal next to the homeodomain (Fig. 5). The homeodomain has a helix-loop-helix motif consisting of three alpha helix structures which determines the specificity for DNA binding (Harvey, 1996). Homeodomains of the NK-2 class proteins have a conserved tyrosine at position 54 (Tyr-190 in the mNKX2-5 sequence) which is suggested to mediate the contact of the third helix with the major groove of DNA (Harvey, 1996). NKX2-5 binds on DNA with high affinity to the NK-element (NKE) 5'-(C/T)AAGTG-3' and with lower affinity to a 5'-TAAT-3' core sequence (C. Y. Chen & Schwartz, 1995). The NK-2 domain is included in a proline-alanine rich region which has been shown to act as a transcriptional inhibitor (C. Y. Chen & Schwartz, 1995). Removal of the C-terminal end increased the NKX2-5 transcriptional activity over 50-fold (C. Y. Chen & Schwartz, 1995). The nuclear localisation signal sequence locates N-terminally

next to the homeodomain (Kasahara & Izumo, 1999). The function of the TN domain is not yet clear (Akazawa & Komuro, 2005). It is suggested that the N-terminal domain of NKX2-5, without the homeodomain, could interact with other transcription factor(s) and mediate the transactivation activity without direct binding to DNA (Shiojima et al., 1996).

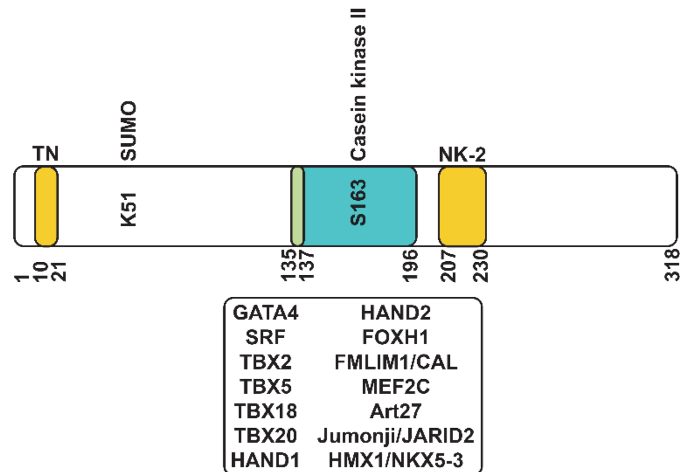

Fig. 5. NKX2-5 protein structure, posttranslational modifications and interaction partners. The amino acid numbering corresponds to mouse NKX2-5. Yellow boxes indicates TN-domain and NK-2 domain, blue box homeodomain and green box nuclear localisation signal.

Transcriptional activity of NKX2-5 is regulated by posttranslational modifications. First of all, it can form homodimers through the homeodomain that stabilises the protein binding on DNA (Kasahara et al., 2001). Lys-193 and Arg-194 (mNKX2-5 sequence) at the third α-helix are critical for homodimerisation. In addition, NKX2-5 is also a target of p38γ and casein kinase II phosphorylation (Kasahara & Izumo, 1999; Ramachandra, Mehta, Wong, & Shim, 2016). Ser-163 in the homeodomain is the target of casein kinase II phosphorylation which increases the affinity to the DNA. Lys-51 is sumoylated (Wang, Zhang, Iyer, Feng, & Schwartz, 2008). Several transcription factors modulate the transcriptional activity of NKX2-5 by interacting physically or by affecting its ability to bind the consensus sequence (Fig. 5). These include: GATA4, SRF, TBX2, 5, 18 and 20, HAND1 and HAND2, forkhead box H1 (FOXH1), filamin binding LIM protein 1 (FBLIM1/CAL), MEF2C, Art27, Jumoni/JARID2, nuclear receptor subfamily 2 group F member 1 (NR2F1/COUP-TF1) and H6 family homeobox 1

(HMX1/NKX5-3) (Akazawa & Komuro, 2005; Carter et al., 2014; Farin et al., 2007; T. G. Kim et al., 2004; Pradhan et al., 2016; Vincentz, Barnes, Firulli, Conway, & Firulli, 2008).

The role of NKX2-5 in cardiac hypertrophy

NKX2-5 regulates the expression of several important cardiac proteins, e.g. ANP, MEF2C, A_1-adenosine receptor, HOP homeobox, myocardin, actin alpha cardiac muscle 1 (aka cardiac α-actin), calreticulin, gap junction protein alpha 5 (connexin 40), endothelin converting enzyme 1, Mov10 like RISC complex RNA helicase 1 (aka Csm), CARP and the sodium/calcium exchanger (NCX1/SLC8A1) (Akazawa & Komuro, 2005). NKX2-5 overexpression with transgenic mice induces hypertrophic gene expression, i.e. the expression of its target genes, ANP, BNP, CARP, MLC2v and endogenous NKX2-5; however, the hearts are normal-sized (Takimoto et al., 2000). These results suggest that NKX2-5 is not sufficient for the generation of cardiac hypertrophy.

NKX2-5 is upregulated during hypertrophy as shown in several *in vivo* studies with different hypertrophic models, e.g. PE and isoprenaline (Saadane, Alpert, & Chalifour, 1999). Its target genes are upregulated also in right ventricular hypertrophy induced by banding of the feline pulmonary artery (Thompson, Rackley, & O'Brien, 1998). Doxorubicin downregulates NKX2-5 transcription in cardiomyocytes (Poizat, Sartorelli, Chung, Kloner, & Kedes, 2000). In adults, a lack of NKX2-5 impaired contractile function and showed histological abnormalities (Toko et al., 2002). This was studied in the model of transgenic mice which overexpress a dominant negative mutant of human NKX2-5 under the control of the α-MHC promoter (DN-TG mice). Furthermore, in DN-TG mice, doxorubicin induced more severe cardiac dysfunction in association with a significant increase in cardiomyocyte apoptosis, while doxorubicin-induced myocardial damage was mild in transgenic mice overexpressing the wild-type NKX2-5. This suggests a cardioprotective role of NKX2-5 in postnatal hearts. Furthermore, overexpression of a NKX2-5 mutant with truncation of the C-terminus induced apoptosis in cultured rat neonatal cardiomyocytes, although this mutant functioned as a transcriptional activator of the ANP promoter (Zhu et al., 2000).

2.4.3 GATA4-NKX2-5 physical interaction and co-operation

GATA4-NKX2-5 protein-protein interaction takes place between the homeodomain of NKX2-5 and the C-terminal zinc zinger of GATA4 (Durocher et al., 1997; Y. Lee et al., 1998; Sepulveda et al., 1998; Shiojima et al., 1999). More specifically, the third α-helix in the NKX2-5 homeodomain is required for physical interaction with GATA4 (Y. Lee et al., 1998; Sepulveda et al., 1998) and especially Lys-193 seems to be essential (Kasahara et al., 2001). However, there are conflicting reports concerning whether or not amino acids 300-327 on the C-terminal extension are needed (Durocher et al., 1997; Y. Lee et al., 1998). Interestingly, a GATA4 mutation mG295S, which corresponds to hG296S causing CHD in humans, is able to interact with NKX2-5 but not with TBX5 (Garg et al., 2003).

GATA4 and NKX2-5 activate synergistically ANP (Durocher & Nemer, 1998; Y. Lee et al., 1998), cardiac α-actin (Sepulveda et al., 1998), CARP (H. Kuo et al., 1999), NADPH oxidase 2 (Jin et al., 2017) and A_1-adenosine receptor (Rivkees, Chen, Kulkarni, Browne, & Zhao, 1999) promoters *in vitro*. For synergistic activation, the whole GATA4 protein is needed (Durocher et al., 1997; Y. Lee et al., 1998), although perhaps not including the first 127 N-terminal amino acids (Sepulveda et al., 1998). For NKX2-5, the homeodomain with the C-terminal extension is needed (Durocher et al., 1997). GATA4 binding to the NKX2-5 C-terminal autorepressive domain seems to induce a conformational change that unmasks NKX2-5 activation domains and improves NKX2-5 DNA binding activity (Durocher et al., 1997; Sepulveda et al., 1998). Moreover, for synergistic activation NKX2-5 binding to NKEs is required but no GATA sites are needed (Durocher & Nemer, 1998; Shiojima et al., 1999). NKX2-5 reduces the DNA binding affinity of GATA4 to GATA sites (Shiojima et al., 1999) and reduces GATA4 transcriptional activity on promoters containing only GATA sites (Durocher & Nemer, 1998). Furthermore, GATA5, but not GATA6, is able to synergise with NKX2-5 on the ANP promoter (Durocher et al., 1997; Durocher & Nemer, 1998).

The interaction of GATA and NK2 family members is evolutionarily conserved between invertebrates and vertebrates (Patient & McGhee, 2002). GATA4 and NKX2-5 regulate the chamber specific expression of ANP (Small & Krieg, 2003). Studies with transgenic frogs showed that the NKE site and proximal GATA site are important for repressor complex assembly which is needed for atrial-specific expression of the *ANP* gene. Mutation of either the GATA or NKE site resulted in failure of ANP transcription to be restricted to the atria or, in other words, inhibit

the expression in the ventricle (Small & Krieg, 2003). The GATA4 and NKX2-5 interaction seems to be important also for development of the mechanical stretch response in cardiac myocytes (Pikkarainen et al., 2003). Mechanical stretch activates GATA4 binding to -90 tandem GATA sites on the BNP promoter and induces the stretch response, as reflected by increased BNP expression. Mutation of these sites decreases the stretch response 40%. When mutation of the upstream NKX2-5 binding site (-387 NK-element) was introduced with GATA site mutations, the stretch response was abolished almost completely. However, mutation of the NKX2-5 binding site alone had no effect on the stretch response. This indicates that for the mechanical stretch response *in vitro*, physical co-operation with GATA4 and NKX2-5 is required.

Both GATA4 and NKX2-5 can induce P19 pluripotent embryonic carcinoma cells to differentiate into cardiomyocytes in the presence of dimethyl sulphoxide (DMSO) (Grepin, Nemer, & Nemer, 1997; Monzen et al., 2002). Overexpression of both cardiac transcription factors NKX2-5 and GATA4, but not either one alone, induces differentiation of P19CL6noggin cells (constitutively overexpressing the BMP antagonist noggin) into cardiomyocytes (Monzen et al., 1999). It has been also shown that sarcomeric MHC as well as three cardiac-specific transcription factors (NKX2-5, GATA4, and MEF2C) further enhance the DMSO induced differentiation (Wen et al., 2007).

2.4.4 Small molecule compounds acting on transcription factors

The remodelling process is regulated by multiple pathways involving cross talk with signalling components and various transcription factors eventually leading to alterations in gene expression (Tham et al., 2015). Thus transcription factors are potential and attractive therapeutic targets (Kohli et al., 2011). Transcription factors have ligand-binding, dimerisation, DNA-binding, nuclear-localisation, and regulatory domains, which may be targeted directly by small molecule drugs. For a long time, transcription factors (other than nuclear receptors) and their protein-protein interactions (PPI) were considered as undruggable targets (Arkin, Tang, & Wells, 2014; Bushweller, 2019; Lambert, Jambon, Depauw, & David-Cordonnier, 2018). The increased knowledge about the mechanism of action and the use of all the modern tools of target-based discovery (structure, computation, screening, and biomarkers) have made this possible. For cancer treatment, there are already a few drugs in clinical use that inhibit the transcription factor PPI (i.e. inhibitors for p53 and Murine Double Minute 2 interaction) or in clinical trials (i.e. STAT3 dimer

inhibitor) (Bushweller, 2019; Lambert et al., 2018). In addition, several new drugs in clinical trials target epigenetic mechanisms (HDAC inhibitors, histone methyltransferase inhibitors, bromodomain-protein 4 inhibitors) or transcription factor DNA binding.

In the cardiac field, targeting transcription factors with small molecules is at an early stage. Moreover, only a few studies where small molecules have been used to improve cardiac function *in vivo* have been reported. A cardiogenic small molecule Shz increases NKX2-5 expression and induces stem cell differentiation into more cardiomyocyte-like cells *in vitro* (Sadek et al., 2008). In *in vivo* experiments, human mobilised peripheral blood mononuclear cells (M-PBMCs) were pre-treated with Shz prior to injection into the cryo-injured rat heart resulting in improvement in left ventricular function. Intramyocardial injections of GATA4, MEF2C and TBX5 (GMT)-encoding retrovirus and 2 weeks i.p. injections of SB431542 (transforming growth factor-β inhibitor) and XAV939 (glycogen synthase kinase 3β inhibitor) after MI induced improvement of the ejection fraction and reduced the scar size by remuscularisation (Mohamed et al., 2017). And finally, Isx1, a small molecule capable of enhancing NKX2-5 expression *in vitro*, was able to improve cardiac function in an experimental acute myocardial infarction (AMI) model in mice after one week i.p. dosing (Russell, Goetsch, Aguilar, Frantz, & Schneider, 2012). However, it was discovered later that Isx1 acts as an activator of the G protein-coupled receptor rather than through transcription factors (Russell et al., 2012).

2.5 Experimental *in vivo* models of heart failure

To study cardiac function and the cardiovascular effect of drugs, whole animal experimental models are needed. In principle, the closer the heart or body weight of the animals is to human heart or body weight, the more similar are the hearts (Milani-Nejad & Janssen, 2014). In this sense, small animals (rats and mice) are not ideal, however, they are easier to handle and the maintenance costs are lower than for larger animals. Furthermore, 99% of human genes have murine orthologs and various *in vivo* cardiac parameters can be measured in small rodents (Camacho, Fan, Liu, & He, 2016; Milani-Nejad & Janssen, 2014). In the following tables the small animal models of heart failure are divided to models of HFrEF (Table 1) which is typically associated with loss of cardiomyocytes and increased wall stress, as reflected by higher levels of natriuretic peptides compared to HFpEF (Table 2) (Riehle & Bauersachs, 2019). However, the pathogenesis of heart failure is

multifactorial and it is often impossible to distinguish the underlying mechanisms. Furthermore, it is important to note that some of the HFpEF models may also have systolic contractile dysfunction or they may precede the later onset of systolic dysfunction and thus they can be considered as HFrEF models as well.

Table 1. Small animal models of HFrEF.

Model	HF stimulus	Clinical congruence
Ischaemia/MI		
LAD ligation	Tissue damage, interruption of blood flow	AMI, DCM, LVEF↓
Ischaemia/reperfusion	Temporary ischaemia	Reperfusion of the occluded vessel during coronary angiography after an AMI, DCM, LVEF↓
Cryoinjury	Tissue damage, interruption of blood flow	DCM
Pressure Overload		
TAC	Acute increase in LV afterload	Hypertension, aortic stenosis, aortic valve stenosis/malformations, LVEF↓, concentric hypertrophy
Ascending aortic constriction	Arterial hypertension	Gradual progression of arterial hypertension in patients (elderly)
Abdominal aorta coarctation (suprarenal)	Renal hypoperfusion↑, hypertension, LV hypertrophy	Slower progression of the HF
Volume Overload		
Aortocaval fistula (shunt)	Decrease in arterial pressure, increase in venous pressure	Patients with mitral valve regurgitation, LV preload↑, eccentric hypertrophy
Cardiotoxicity		
Doxorubicin	Oxidant stress, mitochondrial dysfunction, myocyte apoptosis	Anthracycline induced cardiomyopathy, DCM
Isoprenaline	Stimulation of ß-adrenergic receptor signalling	TTS/PPG? (Angelini & Gamero, 2019), DCM
Ethanol	Decreased myocardial contractility, cardiomyocyte loss	

MI, myocardial infarction; LAD, left anterior descending coronary artery; DCM, dilated cardiomyopathy; TAC, transverse aortic coarctation; TTS, Takotsubo syndrome; PPG, pheochromocytoma and paraganglioma; Modfied from (Houser et al., 2012; Riehle & Bauersachs, 2019)

Table 2. Small animal models of HFpEF.

Model	Physiological signs	Clinical congruence
Hypertension		
Ang II infusion (Also pressure overload model)	Chronic stimulation of AT_1R signalling	The slow progression of hypertension and HF as in patients with HHD
Dahl salt-sensitive rat	Hypertension following a high salt diet (inbred strain)	Same as above
Spontaneously hypertensive rat	Inbred strain with hypertension	Same as above
Kidney nephrectomy	Salt and water retention, hypertension	Early chronic kidney disease
Restrictive cardiomyopathy		
Radiation fibrosis	Myocardial injury	LV stiffness
Tight skin mouse	Excessive fibrosis induced by fibrillin-1 overexpression	Scleroderma
Metabolic syndrome		
ob/ob	Hyperphagia, hyperglycemia, and hyperinsulinemia, diastolic dysfunction	Obesity, T2D
db/db	Hyperphagia based on leptin resistance, diastolic dysfunction	Obesity, T2D
Zucker fatty rats	Insulin resistance, express non-functional leptin receptors	T2D
Zucker diabetic fatty rats	Inbred strain with high serum glucose levels	T2D
High-caloric diet (± low-dose STZ)	High caloric intake (± pancreatic β-cell toxin)	Additional low-dose STZ treatment mimics β-cell failure and late stage T2D
Ageing		
Senescence-accelerated prone mice	Age dependent diastolic dysfunction, adverse remodelling, endothelial cell dysfunction	Elderly patients with HFpEF

AT_1R, angiotensin II (Ang II) type 1 receptor; HF, heart failure; HHD, hypertensive heart disease; ob/ob, leptin-deficient mouse spontaneously develops obesity and type 2 diabetes; db/db, point mutation in the diabetes (db) gene encoding the leptin receptor; T2D, type 2 diabetes; STZ, streptozotocin. Modfied from (Houser et al., 2012; Riehle & Bauersachs, 2019).

In addition to models presented in the tables, there are other animal models of HF that were excluded including models of RV hypertrophy, additional toxic models, type 1 diabetes models as well as numerous transgenic animal models (Houser et al., 2012; Riehle & Bauersachs, 2019).

There are some limitations with the use of rat and mouse models regarding differences in cardiac function compared to the human heart that have to be kept in mind when designing the experiments and interpreting the results. Rodent hearts have very high heart rates (five times that of humans) thus the systolic contraction and diastolic filling occur rapidly (Hasenfuss, 1998; M. G. Katz et al., 2019; Milani-Nejad & Janssen, 2014). Ventricular action potential duration is much shorter in rats (50 ms) than in humans (250 ms) and it lacks a prominent plateau phase. Ventricular myocytes of small rodents express fast α-MHC (>94-100%) and human ventricles slow β-MHC (>90-95%). Calcium removal from the cytosol in rats is dominated by the activity of the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) (90-92%) whereas in humans, SERCA contributes to 76% with the Na^{+}/Ca^{2+} -exchanger accounting for the majority of the rest. In addition, in most animal studies cardiac incident (e.g. sudden occlusion of coronary artery) is generated in healthy animals with a defined genetic background, whereas in humans, cardiovascular disease development is complex and progressive with underlying vascular disorder, genetic and environmental components (Houser et al., 2012). Nevertheless, the small animal models of heart failure do exhibit the critical features of disease phenotype observed in clinics, at least to some extent (Houser et al., 2012).

2.5.1 Angiotensin II induced hypertension

Ang II is an octapeptide (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe), Ang (1–8), that increases blood pressure by inducing vasoconstriction. In addition, it induces cardiac remodelling by promoting the growth of cardiac myocytes, fibroblast and vascular smooth muscle cells and producing ROS via NAD(P)H oxidase activation through AT_1R (Forrester et al., 2018). Ang II plasma levels in healthy humans are ~8.7 fmol/ml, in rats ~50 fmol/ml and in mice ~130 fmol/ml. The half-life of Ang II is quite short (1-2 min in plasma) and it metabolises to Ang (1-7), Ang III and Ang IV (Forrester et al., 2018; Griffin et al., 1991). It is noteworthy to point out, that the metabolites bind to other angiotensin receptors, which induce vasodilation and reverse remodelling. Continuous infusion via osmotic minipump increases the circulating levels of Ang II in *in vivo* models. In rats, Ang II infusion (200

ng/min/kg) increased the plasma levels to ~130 fmol/ml, and in mice, 12 days of infusion (400 ng/min/kg) increased the levels to 370 fmol/ml (Forrester et al., 2018). Systolic blood pressure starts to rise immediately after the pumps have been implanted (Griffin et al., 1991). The increased circulating levels of Ang II in animals have important effects on other organs, especially kidney (Riehle & Bauersachs, 2019). In addition, it is questionable if high levels of Ang II and chronic stimulation of AT_1R reconstitute the increased RAAS activity seen in human pathology (Forrester et al., 2018). Nevertheless, the angiotensin model is technically easy to perform and it induces the slow progression of hypertension and HF as in patients with hypertensive heart disease (HHD). Whether Ang II infusion in animals induces HFpEF or HFrEF seems to be under debate (Forrester et al., 2018; Valero-Munoz, Backman, & Sam, 2017). This issue might also be strain specific, as C57BL/6J mice develop compensatory concentric hypertrophy and fibrosis, whereas Balb/c mice show severe LV chamber dilatation, which is rarely seen in HFpEF and is more characteristic of dilated cardiomyopathy (Valero-Munoz et al., 2017).

2.5.2 Coronary artery ligation

The permanent ligation of the LAD artery is considered a standard model for MI which progressively develops the dilated cardiomyopathy (DCM) (Hasenfuss, 1998; Houser et al., 2012). It is important to recognise that, in this model, the degree of long-term LV remodelling, chamber dilatation and reduction in systolic function is directly proportional to the initial infarct size. The challenge to this model is the anatomy of the rat heart. First, the LAD artery transverses to the apex completely intramurally, therefore, any external inspection is difficult (M. G. Katz et al., 2019). Secondly, in 25–30% of rats, there are anatomical variations of the coronary arteries. The most common types of LAD arteries are those with 2–3 diagonal branches. In some rats, there are two LAD arteries or the LAD artery bifurcates early and does not reach the heart's apex (M. G. Katz et al., 2019). The equivalence of the infarct size between groups should be demonstrated when comparing subsequent remodelling responses in different groups of animals (Houser et al., 2012; M. G. Katz et al., 2019). There is some discrepancy in how fast HF develops after MI creation (M. G. Katz et al., 2019). On average, clinical signs of HF develop after 3–6 weeks. However, in cases of larger infarct, the ventricle becomes distended and dilated already after 7 days. The animal survival and functional abnormalities are directly related to the size of myocardial infarction (M. G. Katz et al., 2019).

Depending on the ligation location the forming scar can be roughly 30–35% or 40–55% of the LV creating moderate to severe HF, respectively (M. G. Katz et al., 2019). In the same respect, in small MI (< 20%), the survival rate at 60 days is ~ 85%, and in large MI (> 45%) ~ 60%. In addition, the genetic background of the animal strain influences post-MI adaptations (Riehle & Bauersachs, 2019). In 129S6 mice, infarct rupture is most frequently observed, while in Swiss mice, cardiac dilatation is most prominent. Similarly, it has been shown that LAD artery ligation in Lewis inbred rats produces a uniformly large infarct with lower mortality compared to the Sprague-Dawley rat model (Liu et al., 1997). The branching and position of the LAD artery is also different in Sprague-Dawley outbred rats compared to Lewis inbred rats.

2.5.3 Doxorubicin induced cardiotoxicity

Doxorubicin is a cytotoxic drug belonging to the group of anthracyclines which have a prominent role in treating many forms of cancer (e.g. solid tumours and leukaemia) (Corremans, Adao, De Keulenaer, Leite-Moreira, & Bras-Silva, 2018). However, cardiotoxic side effects represent a serious limitation to their use. The main mechanisms of anthracycline-induced toxicity in cardiomyocytes are thought to be the same as the antitumour actions in cancer cells: inhibition of topoisomerase 2 (TOP2), DNA intercalation and production of free radicals due to its metabolism. All these cause DNA damage and induce cell death pathways. In addition to these, numerous other mechanisms for doxorubicin-induced cardiomyopathy have been discovered (Renu, V, P, & Arunachalam, 2018).

In *in vivo* models, doxorubicin has been given to animals with various doses (1-20 mg/kg) and treatment schedules (single, daily or weekly doses), most commonly intraperitoneally (i.p), but also intravenously (i.v.) (Herman & Ferrans, 1997; Ueno et al., 2006). *In vivo* studies where GATA4 has been shown to be protective in doxorubicin-induced cardiomyopathy, the cardiotoxicity was induced with a single 15 mg/kg or 20 mg/kg dose of doxorubicin i.p. (Aries et al., 2004; Kobayashi et al., 2006). Mice dosed with doxorubicin (20 mg/kg i.p.) and imaged by ultrasound five days later showed significantly reduced left ventricular performance (FS, cardiac output, and stroke volume) relative to control animals (Weinstein, Mihm, & Bauer, 2000).

3 Aims of the research

The main aim of this study was to characterise the protein-protein interaction of cardiac transcription factors GATA4-NKX2-5 and the small molecule compounds targeted to inhibit this interaction. More specifically, the objectives were:

1. To characterise the protein-protein binding site for the transcription factors GATA4 and NKX2-5.
2. To investigate the small molecule inhibitors binding to GATA4 and NKX2-5.
3. To study the effect of the small molecule inhibitors in experimental *in vivo* models of heart failure.

4 Materials and methods

A summary of experimental methods used in the original publications is presented in Table 3.

Table 3. Summary of the experimental protocols.

Experimental method	Cells/animal	Study
In vitro		
Site directed mutagenesis		I
Immunoprecipitation	COS-1 cells	I
EMSA	COS-1 cells	I
Luciferase reporter assay	COS-1 cells	I
Affinity chromatography	COS-1 cells	II
In vivo		
Ang II induced hypertrophy	Rat	III
Coronary artery ligation	Rat	III
Coronary artery ligation	Mouse	III
Doxorubicin induced cardiotoxicity	Rat	IV

EMSA, electrophoretic mobility shift assay; Ang II, angiotensin II

4.1 Plasmids

The plasmid expressing the mouse GATA4 (pMT2-GATA4) and the empty pMT2 plasmid were gifts from D.B. Wilson (Department of Pediatrics, St. Louis Children's Hospital) (Arceci, King, Simon, Orkin, & Wilson, 1993). The mutations were created in pMT2-GATA4 using a Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer’s protocol with the primers listed in Table 4. The mutations were designated by their position in the mouse GATA4 protein sequence (GenBank Accession Number NP_032118). The plasmid expressing the mouse NKX2-5 (pEF-FLAG-NKX2-5) was provided by R. P. Harvey (The Victor Chang Cardiac Research Institute, Darlinghurst, Australia) (Ranganayakulu, Elliott, Harvey, & Olson, 1998). The mouse NKX2-5 cDNA sequence was cloned from the pEF-plasmid to the EcoRI-site in the pMT2-plasmid by using the following oligos forward: ATATATGAATTCTCCAGATCTTTCGAAATCACCATGGACTAC, reverse: ATATATGAATTCCTTGCGTTACGCACTCACTTTAATGGGAAG. Three high affinity binding sites (NKE) for NKX2-5 containing luciferase vector p3xHA were created by modifying previous studies (C. Y. Chen & Schwartz, 1995; Ranganayakulu et al., 1998). Two oligos containing three NKE (underlined

sequence) with MluI and BglII restriction sites and phosphorylated 5' ends (sense; CGCGTCTCAAGTGGGTCTCAAGTGGAGCCTCAAGTGGA, antisense; AGAGTTCACCCAGAGTTCACCTCGGAGTTCACCTCTAG) were obtained from Oligomer (Helsinki, Finland). The oligos were annealed and ligated to the pGL3 basic reporter vector containing a rat albumin minimal promoter with a TATA-box (-40 - +28), a kind gift from J. Hakkola (Department of Pharmacology and Toxicology, University of Oulu, Finland) (Hakkola, Hu, & Ingelman-Sundberg, 2003). A rat BNP minimal promoter containing a luciferase reporter vector and minimal promoter together with a -90 tandem GATA-site have been described previously (Grepin et al., 1994). The internal control vector pRL-TKd238 was a gift from J.F. Strauss III (University of Pennsylvania School of Medicine, Philadelphia) (Ho & Strauss, 2004). All constructs were verified by restriction and sequencing the whole gene.

Table 4. Sense primer sequences used to create mutations to GATA4.

Mutation	Sequence 5' to 3' direction
V217Y	CAGAAGGCAGAGAGTGT**TAC**AATTGTGGGGCCATGTC
H234S	GAGATGGGACGGGA**AGC**TACCTGTGCAATGCC
R264A	CTGTCCGCTTCC**GCC**CGGGTAGGCCTC
S269C	GGGTAGGCCTC**TGC**TGTGCCAACTGCCAG
A271V	GCCTCTCCTGT**GTC**AACTGCCAGACTACC
N272S	CCTCTCCTGTGCC**AGT**TGCCAGACTACCACC
N272D	GTAGGCCTCTCCTGTGCC**GAT**TGCCAGACTACCAC
Q274H	CCAACTGC**CAT**ACTACCACCACCACGCTG
R283A	GCTGTGGCGT**GCT**AATGCCGAGGGTGAGC
R283Q	CGCTGTGGCGT**CAA**AATGCCGAGGGTGAGC
E288G	GCCGAGGGT**GGG**CCTGTATGTAATGCCTG
E288K	GCGTCGTAATGCCGAGGGT**AAG**CCTGTATG
M298Y	CGGCCTCTAC**TAC**AAGCTCCATGGGGTTCCCAGG
K299A	CGGCCTCTACATG**GCG**CTCCATGGGGTTC
R319C	CCAGAAAA**TGT**AAGCCCAAGAACCTGAATAAATCTAAGACGCC
R319S	CGGAAGGAGGGGATTCAAACCAGAAAA**AGC**AAGCCCAAGAAC
P321C	GGAGGGGATTCAAACCAGAAAACGGAAG**TGC**AAGAACC
S327A	GCCCAAGAACCTGAATAAA**GCT**AAGACGC

Highlighted nucleotides denote mutated codons.

4.2 COS-1 cell culture

Continuous COS-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 100 U/ml of penicillin, and 100 µg/ml of streptomycin. The cells were originally obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were a kind gift from J. Hakkola (Department of Pharmacology and Toxicology, University of Oulu, Finland) (Hukkanen et al., 2003). All the cell cultures were maintained at 37 °C in a humidified atmosphere with 5% CO_2. For immunoprecipitation and EMSA experiments the cells were seeded into 175 cm^2 culture flasks and transfected 24 h later with 20 µg of the indicated expression vectors using the FuGENE 6 (Promega) reagent at a 1:3 DNA:reagent ratio. The transfection reagent was removed after 24 h and cells were collected 48 h post-transfection. For affinity experiments, the cells were seeded on a 6-well plate and transfected for 24 h with 0.6 µg of pMT2-GATA4 or pMT2-NKX2-5 using the FuGENE 6 reagent at a 1:3 DNA:reagent ratio. For the cell lysate and the control samples the cells were not transfected.

4.3 Protein extractions *in vitro* and Western Blot

The nuclear and cytosolic proteins were extracted by adapting the protocol described by Schreiber et al. (Schreiber, Matthias, Muller, & Schaffner, 1989). For nuclear protein extractions, the cells were resuspended in low salt buffer consisting of 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10 mM KCl, 0.1 mM ethylenediaminetetraacetic acid (EDTA) and 0.1 mM ethylene-bis(oxyethylenenitrilo)tetraacetic acid (EGTA) supplemented with protease (P8340, Sigma) and phosphatase (P0044, Sigma) inhibitor cocktails (1:100 volume). The suspensions were then allowed to swell on ice. After that, membrane proteins were solubilised and isolated by adding 10% Igepal CA-630 detergent and vortexing vigorously followed by centrifugation. The supernatants were collected as the cytosolic fractions. The pellets were resuspended in high salt buffer containing 20 mM HEPES, 0.4 M NaCl, 1 mM EDTA and 1 mM EGTA, with protease and phosphatase inhibitors and then rocked for 15 min. After centrifugation the supernatant was collected as the nuclear fraction. The entire procedure was carried out at 4 °C or on ice.

For total protein samples, the cells were lysed into non-denaturing lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton-X100,

2.5 mM sodium pyrophosphate, pH 7.5) with phosphatase inhibitors (1 mM betaglycerophosphate, 1 mM Na3VO4, 50 mM NaF) and protease inhibitors (Protease Inhibitor Mini Tablets, #88666, Pierce). Protein concentrations were determined with the Bradford protein assay (Bradford, 1976).

Protein samples were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Optitran BA-S 85 Reinforced, Schleicher & Schuell or Amersham Protran Premium 10600004, GE Healthcare Life Sciences). Non-fat milk or bovine serum albumin both as 5% in TBS-Tween was used to block nonspecific background. The primary antibodies anti-GATA4 (sc-9052 Santa Cruz Biotechnology) and anti-NKX2-5 (sc-8697 Santa Cruz Biotechnology) were all used in 1:1000 dilution. The membranes were incubated with the primary antibodies overnight at 4 °C, followed by one hour incubation in the secondary antibody at room temperature. The secondary antibodies anti-rabbit IgG (#7074 Cell Signaling Technology) and anti-goat IgG (sc-2020 Santa Cruz Biotechnology) were used at 1:2000. ECL Plus reagents (Amersham Biosciences) or Pierce SuperSignal kit (#34078, Thermo Scientific) were used to induce chemiluminescence. The signal was digitalised with a Luminescent Imager Analyzer LAS-3000 (Fujifilm) and analysed with Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA). For a second Western, the membrane was stripped for 30 minutes at +60 °C in stripping buffer (62.5 mM Tris pH 6.8, 2% SDS and 100 mM β -mercaptoethanol).

4.4 Co-immunoprecipitation

The relative expression levels of native and mutant GATA4 proteins were evaluated by Western blot and used to adjust the required volume of cell extract from transfected cells in order to standardise the amount of GATA4 protein in each immunoprecipitation. For each immunoprecipitation, 50 μg of FLAG-NKX2-5 protein and 30-84 μg of GATA4 protein were used. The total protein concentration was kept equal between the samples by the addition of 0–54 μg of protein extract from untransfected cells as required. Agarose bound anti-FLAG M2 antibody (Sigma) was used at 30 μl per immunoprecipitation reaction followed by overnight incubation in lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100 and 2.5 mM sodium pyrophosphate) with protease and phosphatase inhibitors (20 μg/ml leupeptin, 2 μg/ml pepstatin, 20 μg/ml aprotinin, 1 mM phenylmethylsulphonyl fluoride (PMSF), 50 mM NaF, 6 μg/ml N-tosyl-L-phenylalaninyl-chloromethylketone (TPCK) and 6 μg/ml N-alpha-tosyl-L-lysinyl-

chloromethylketone (TLCK)) at 4 °C with gentle agitation. The beads were collected by centrifugation with a table top microcentrifuge and washed with the lysis buffer three times. The immunoprecipitated proteins were eluted from the agarose beads by boiling the samples in 20 µl of SDS-loading buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 1.25% 2-mercaptoethanol, 0.1% bromophenol blue) for 4 min and vortexed vigorously to elute the bound proteins. A 21 µl sample of supernatant was analysed with Western blotting.

4.5 Affinity chromatography

The affinity chromatography protocol was based on the immunoprecipitation protocol. After solid phase synthesis, compounds immobilised on Sepharose® were suspended into 20% ethanol. To approximate equal amounts of Sepharose® in each sample, the quantity of the dry and packed Sepharose® originally used in synthesis reactions was calculated to correspond to 15 µl of packed Sepharose® in affinity reactions. The reactions were done in 1.5 ml microcentrifuge tubes and kept on ice. To remove ethanol, the Sepharose® was washed twice with the lysis buffer similar to that used in the co-immunoprecipitation experiment, but without inhibitors. The tubes were gently inverted several times and the Sepharose® collected by centrifugation at 10 000 g for 10 s at 4 °C. The binding reactions were performed overnight under gentle agitation at 4 °C with 30 µg of total protein lysate in 0.95 ml of lysis buffer with phosphatase inhibitors (0.2 mM Na3VO4, 50 mM NaF) and protease inhibitors (Protease Inhibitor Mini Tablets, #88666, Pierce). The samples were then washed gently three times with 0.7 ml of lysis buffer (with inhibitors). The buffer was then removed and the samples boiled in 20 µl of SDS sample buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 1.25% 2-mercaptoethanol, 0.1% bromophenol blue) for 4 min and vortexed vigorously to elute the bound proteins from the Sepharose®. A 21 µl sample of supernatant was analysed with Western blotting.

For method validation and to verify sufficient washing of the unbound proteins, 28 µl samples taken before washes and from the final third wash were subjected to Western blotting. The quantity of total proteins was visually examined by using Ponceau S (P7170, Sigma) staining and immunoblotting with GATA4 and NKX2-5 antibodies. Total proteins and GATA4 or NKX2-5 protein were detected before washes but not in the samples from the final wash, confirming that unbound proteins were successfully removed during the washing steps. Furthermore, affinity chromatography reactions with untransfected cell lysates showed no

immunoreactivity for GATA4 or NKX2-5, validating the specificity of the antibodies used.

4.6 Electrophoretic mobility shift assay

For the analysis of GATA4 DNA binding activity, double-stranded oligonucleotides containing the -90 GATA binding region of the BNP gene were labelled with [α-32P]-dCTP using the Klenow enzyme. The probes used are shown in Table 5.

Table 5. Oligonucleotide probes used in EMSA.

Probe	Sequence
rBNP -90 tandem GATA	
sense	5'-*TGTG*TCT**GATA**AATCAGA**GATA**ACCCCACC-3'
antisense	5'- GGTGGGGTTATCTCTGATTTATCAGA-3'
rBNP GATAmut -91	
sense	5'- *TGTG*TCT**<u>TGC</u>A**AATCAGA**GATA**ACCCCACC-3'
antisense	5'-GGTGGGGTT ATCTCTGATTT<u>GCA</u>AGA-3'
rBNP GATAmut -80	
sense	5'-*TGTG*TCT**GATA**AATCAGA**<u>TGC</u>A**ACCCCACC-3'
antisense	5'-GGTGGGGTT<u>GCA</u>TCTGATTTATCAGA-3'
rBNP -91GATA	
sense	5'-*TGTG*TCT**GATA**AATCAGAG-3'
antisense	5'-CTCTGATTTATCAGA-3'

GATA binding sites are indicated in **bold**, the mutated nucleotides are <u>underlined</u> and 5’ overhangs that were filled in the labelling reaction are in *italics*. rBNP, rat BNP; GATAmut, mutated GATA site.

The binding reactions contained 3 μg of total protein extract and 2 μg of poly(deoxyinosinic-deoxycytidylic) acid sodium salt in a buffer containing 10 mM HEPES (pH 7.9), 1 mM MgCl2, 50 mmol/l KCl, 0.1 mM EDTA, 10% glycerol, 0.025% NP-40 or Igepal CA-630, 1 mM dithiothreitol (DTT), 0.25 mM PMSF and 1 μM of each aprotinin, leupeptin and pepstatin. After a 10 min preincubation of extract, the probe was added and binding was allowed to proceed at room temperature for 20 min. For supershift analysis, after the preincubation the protein was incubated for another 10 minutes with 2 μl of the anti-GATA4 polyclonal antibody (sc-1237X, Santa Cruz Biotechnology) before adding the probe. The reaction mixes were then analysed by electrophoresis on a 5% polyacrylamide gel in 0.5x Tris-borate-EDTA buffer at 225 V for 2 h at 4 °C. After electrophoresis the gels were dried and exposed to PhosphorImager screens (Molecular Dynamics,

Sunnyvale, CA, USA), which were scanned using a Biorad Molecular Imager FX Pro Plus (Bio-Rad Laboratories, Hercules, CA, USA). All the results were quantified using the Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).

4.7 Luciferase assay

COS-1 cells were co-transfected with 150 ng of Firefly luciferase reporter vector, 35 ng Renilla luciferase vector and 75 ng of expression plasmid pMT2-GATA4 per 1 cm^2 well using FuGENE 6 (Promega) at a 1:3 DNA:reagent ratio. In GATA4-NKX2-5 interaction studies with p3xHA, the same amount of luciferase reporter vectors were used along with 75 ng of pMT2-NKX2-5 and 75 ng of pMT2-GATA4 plasmids. Particular GATA4 mutant proteins required adjustment in the amount of transfected plasmid to achieve equal protein levels. Twenty-four hours after transfections the cells were lysed with 1x Passive Lysis Buffer (E194A, Promega). The samples were processed with a Dual-Luciferase Assay system according to the manufacturer's protocol (E1960, Promega) and measured with a luminometer (Luminoskan RS, Labsystems, Helsinki, Finland). For the analysis of the results, the Firefly values were divided by the Renilla values and then normalised to the control values. Each value from biological and technical replicates was treated separately and combined for statistical calculations.

4.8 *In vivo* experiments

Animal experiments were carried out in accordance with the 3 R principles of the EU directive 2010/63/EU governing the care and use of experimental animals, and following local laws and regulations [Finnish Act on the Protection of Animals Used for Scientific or Educational Purposes (497/2013, Government Decree on the Protection of Animals Used for Scientific or Educational Purposes (564/2013)]. The protocols were approved by the national Animal Experiment Board of Finland. In all animal experiments the compounds were diluted with DMSO and administered to the animals in a 1:1 dilution of corn oil. The control animals received an equivalent volume of vehicle (DMSO with corn oil).

4.8.1 Angiotensin II induced hypertension

Ang II (33.3 μg/kg per hour) was administered to male Sprague-Dawley rats weighing 250-300 g via subcutaneously implanted osmotic minipumps (Alzet model 2002; Scanbur BK AB, Sollentuna, Sweden) for 2 weeks. The minipumps were installed through a neck incision under isoflurane anesthesia. Compounds 3i-1000 or 3i-0595 were administered (30 mg/kg/day) i.p. for 2 weeks. The dose of compound 3i-1000 was divided into three portions and the dose of compound 3i-0595 was divided into two portions. Echocardiography was performed before decapitation.

4.8.2 Myocardial infarction in rats

Male Sprague-Dawley rats (250-300 g) were anaesthetised with medetomidine hydrochloride (Domitor, 250 μg/kg i.p.) and ketamine hydrochloride (Ketamine, 50 mg/kg, i.p.). The rats were connected to the respirator through a tracheotomy. A left thoracotomy and pericardial incision were performed, and the LAD was ligated. After the operation, anaesthesia was partially antagonised with atipamezole hydrochloride (Antisedan, 1.5 mg/kg ip) and the rats were hydrated with 5 ml physiological saline solution given subcutaneously. For postoperative analgesia, buprenorphine hydrochloride (Temgesic, 0.1 mg/kg s.c.) was administered twice daily and carprofen (Rimadyl, 5 mg/kg s.c.) once a day for three days. The sham-operated (SHAM) rats underwent the same surgical procedure without the ligation of LAD as a placebo surgery. Compound was administered (30 mg/kg/day) i.p. divided into two portions, the first dose given after the operation and the next the following morning. At one week post-infarction the echocardiography was performed, the rats were decapitated and LV tissues collected for further analysis.

4.8.3 Myocardial infarction in mice

Myocardial infarction in male 8-10-week-old C57BL/6 mice was produced by coronary artery ligation by a method which does not require ventilation (Gao et al., 2010). The SHAM mice underwent the same surgical procedure without ligation of the descending coronary artery. The mice were medicated pre-operatively with carprofen 5.0 mg/kg s.c. All animals were monitored after the surgery and received buprenorphine 0.1 mg/kg within 6 hours after surgery. Carprofen and buprenorphine were administered s.c. the following morning. Afterwards

buprenorphine was administered in drinking water for 3 days. Compound 3i-1000 was administered two times a day by i.p. injections 30 mg/kg/day. The first dose was right after the operation and the next the following morning. Echocardiography was performed at 3 days and at 1 week after the operation.

4.8.4 Doxorubicin induced cardiotoxicity

Doxorubicin was administered to 7-week-old male Sprague Dawley rats with an average weight of 216 g (189-245 g). The doxorubicin was administered 1 mg/kg i.p. for 10 days (Hayward & Hydock, 2007) diluted to 3 mM in saline. For control animals, the equivalent volume of saline was administered. From week 7 to 9 of the experiment, compound 3i-1000 or 3i-0595 was administered at 30 mg/kg/day i.p. divided into two portions for two weeks. Transthoracic echocardiography analysis was performed at 2, 7 and 9 week time points. The rats were sedated with isoflurane or with terminal anaesthesia with ketamine (50 mg/kg i.p.) and xylazine (10 mg/kg i.p.). The apex of the LV was immersed in liquid nitrogen and stored at −70 °C for further analysis.

4.8.5 Echocardiography

Transthoracic echocardiography was performed using the Vevo2100 high-frequency high-resolution linear array ultrasound system (FujiFilm VisualSonics, Toronto, Canada) and MS-250 transducer (13–24 MHz, axial resolution 75 μm, lateral resolution 165 μm) for rats or MS-550S transducer (Visual Sonics Vevo 2100, 40 MHz, axial resolution 40 μm, lateral resolution 90 μm) for mice. The echocardiography was performed on animals sedated with isoflurane or in terminal anaesthesia. Using two-dimensional imaging, a short axis view of the LV at the level of the papillary muscles was obtained and a two-dimensionally guided M-mode recording through the anterior and posterior walls of the LV were acquired. The images were carefully analysed with Vevo Workstation software 1.7 by a blinded observer. End-systolic and end-diastolic LV dimensions (ESD and EDD) as well as the thickness of the interventricular septum and posterior wall were measured from the M-mode tracings. LV fractional shortening (FS) and ejection fraction (EF) were calculated from the M-mode LV dimensions using equations 1 and 2. An average of three measurements of each variable was used. After echocardiographic measurements, the terminally anaesthetised animals were

decapitated, the hearts were excised, and the apex of the LV was immersed in liquid nitrogen and stored at -70 °C for further analysis.

$$(1)\ FS(\%) = \{(LVEDD - LVESD) / LVEDD\} \times 100$$
$$(2)\ EF(\%) = \{(LVEDD)^3 - (LVESD)^3) / LVEDD^3\} \times 100$$

4.9 RNA analysis from tissue samples

The LV tissue was ground in liquid nitrogen to a powder of which 1/3 was used for total RNA isolation using the guanidine thiocyanate–CsCl method (modified from Cathala et al., 1983). Briefly, tissue powder was homogenised in 3 ml lysis buffer containing 4 M guanidinium thiocyanate, 0.1 M Tris-HCl (pH 7.5), 7% β-mercaptoethanol and 1.0–2.0% Na-lauroylsarcosine with an Ultra-Turrax® (IKA®) and the cell debris was pelleted for 10 min at 3000 rpm (1791 x g) at 4 °C. The supernatant containing RNA was stored in -80 °C for further treatment. RNA was isolated by ultracentrifugation overnight through a 5.7 M CsCl cushion at 4 °C. The resulting pellet was resuspended in lysis buffer and RNA was precipitated with 3 M sodium acetate (pH 5.2) (1/10 vol) and ice-cold absolute ethanol (3 x vol) at least for 1 h at -20 °C. The precipitated RNA was pelleted by centrifugation for 15–20 min at 12,000 rpm (13,520 x g) at 4 °C and washed with 70% ethanol in diethylpyrocarbonate (DEPC)-treated water followed by another centrifugation for 5–10 min as described above. The washing was repeated and the RNA pellet was air dried before dissolving in DEPC-H2O. For quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analyses, cDNA was synthesised from total RNA with a First-Strand cDNA Synthesis Kit (GE Healthcare Life Sciences) following the manufacturer's protocol. RNA was analysed by RT-PCR on an ABI 7300 sequence detection system (Applied Biosystems) using TaqMan chemistry. The results were quantified using the ΔΔCT method and normalised to 18S RNA quantified from the same samples. The following sequences of the primers and the fluorogenic probe were used in the assay: atrial natriuretic peptide (ANP, forward: GAAAAGCAAACTGAGGGCTCTG, reverse: CCTACCCCCGAAGCAGCT, probe: TCGCTGGCCCTCGGAGCCT) and B-type natriuretic peptide (BNP, forward: TGGGCAGAAGATAGACCGGA, reverse: ACAACCTCAGCCCGT CACAG, probe: CGGCGCAGTCAGTCGCTTGG).

4.10 Protein analysis from tissue samples

Two thirds of the ground LV tissue was homogenised in 4 ml of lysis buffer (20 mM Tris, 10 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, pH 8.0) containing protease and phosphatase inhibitors (1 mM β-glycerophosphate, 1 mM Na_3VO_4, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml aprotinin, 2 mM benzamidine, 1 mM PMSF, 50 mM sodium fluoride, 1 mM dithiothreitol). Of the homogenate, 0.8 ml was used for total protein extraction and the rest for nuclear protein extraction. Then, 0.2 ml of lysis buffer (100 mM Tris-HCl, 750 mM NaCl, 5 mM EDTA, 5 mM EGTA, 5% Triton-X100, 12.5 mM sodium pyrophosphate, 5 mM β-glycerophosphate, 5 mM Na_3VO_4, pH 7.5) was added into the total protein homogenate and vortexed for 30 s. After a 20 min centrifugation at 12,500 rpm (14,670 x g) at 4 °C the supernatant containing total proteins was collected. For nuclear protein extraction, the homogenate was divided into two sets, which were later combined. The homogenate was kept on ice for 15 min after which NP-40 was added to a final concentration of 0.6%. The sample was vortexed vigorously for 15 s and centrifuged for 30 s at 12,500 rpm (14,670 x g) at 4 °C. The pellet was suspended into buffer (20 mM Hepes, 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, pH 8.0) including the inhibitors mentioned above and the parallel samples were combined. The samples were then vortexed vigorously for 45 min at 4 °C. After the final centrifugation at 12,500 rpm for 5 min at 4 °C, the supernatant containing nuclear proteins was collected. The protein concentrations were determined with the Bio-Rad Protein Assay and analysed by the Western blot method as described with *in vitro* proteins.

4.11 Statistical analysis

The results are expressed as means with error bars representing standard error of the mean (SEM) or standard deviation (SD). Statistical analyses were performed using IBM SPSS Statistics 21 or 24 software. (Study I) To determine the statistical difference between two groups, Student's t-test was used. In the luciferase assays one group was considered as a control and all other groups were compared against it with Dunnett's t-test. (Study III) Statistical significance was evaluated by one-way analysis of variance (ANOVA) followed by a least significant difference (LSD) post hoc test for multiple comparisons. To determine the statistical difference between two groups, the independent samples t-test was used. (Study IV) Welch's t-test or independent samples t-test were used for comparison of two groups at one

time point and Levene's test to analyse the equality of variances. Differences at the level of $P<0.05$ were considered statistically significant.

5 Results

5.1 Mutational analysis of amino acids important for physical interaction of GATA4 and NKX2-5

5.1.1 Immunoprecipitation (I)

To define the important amino acids involved in the physical interaction of the GATA4-NKX2-5 complex, a total of 19 amino acids were mutated on the protein surface of GATA4. The selection of the amino acids was based on molecular modelling work (I). Thirteen mutations were located on the C-terminal zinc finger (R264A, S269C, A271V, N272D, N272S, Q274H, S269C+Q274H, R283A, R283Q, E288G, E288K, M298Y, K299A), four on the C-terminal extension (R319C, R319S, P321C, S327A) and two on the N-terminal zinc finger (V217Y, H234S). Mutations were created in the gene encoding the mouse GATA4 protein by using site-directed mutagenesis. Mutated proteins were produced in mammalian COS-1 cells and their expression levels were quantified by Western blot. Equalised GATA4 levels were used in co-immunoprecipitation studies (see input GATA4, Fig. 6) with N-terminal FLAG-tagged wild type NKX2-5. Immunoprecipitated protein samples were analysed by Western blot using GATA4 and NKX2-5 antibodies (Fig. 6) and quantified (Fig. 7).

At first, amino acids E288 and R283 were mutated since it has been shown that those have an effect on the GATA4-NKX2-5 interaction when multiple adjacent amino acids have been mutated (Y. Lee et al., 1998). Single amino acid mutations E288K or E288G had no effect on the interaction, however, R283A strongly inhibited GATA4-NKX2-5 interaction (84%). This effect was verified by substituting the positively charged arginine with the similarly sized but uncharged amino acid glutamine. R283Q inhibited the interaction similarly. Since R283 turned out be effective, mutations R264A and K299A, locating on the same surface, were created. Interestingly, those decreased the GATA4-NKX2-5 interaction as well.

Amino acid M298 is orientated opposite to K299 and in the haematopoietic GATA1-3 subclass this amino acid is tyrosine. Mutation M298Y decreased the interaction with NKX2-5 by 62%. In the folded GATA4 protein, N272 locates on the same surface as M298 and the corresponding mutation in humans (N273S) causes congenital heart defects (Reamon-Buettner & Borlak, 2005).

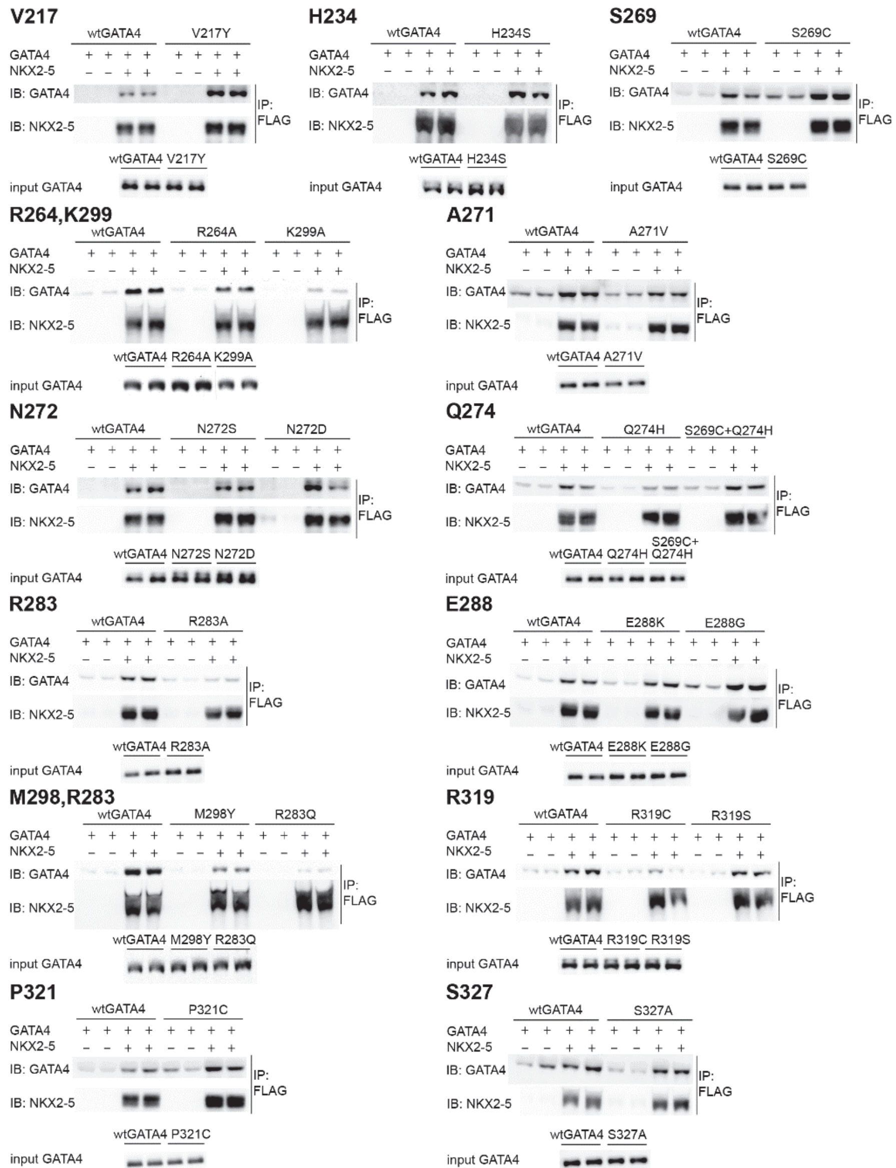

Fig. 6. Representative Western blots from co-immunoprecipitation of mutant GATA4 and FLAG-NKX2-5 proteins. Each mutation was studied in two independent experiments and analysed on Western blot with two technical replicates. Input GATA4 shows the levels of the protein before immunoprecipitation. IP, immunoprecipitation; IB, immunoblotting. Reprinted with permission from Study I (S. Kinnunen et al., 2015).

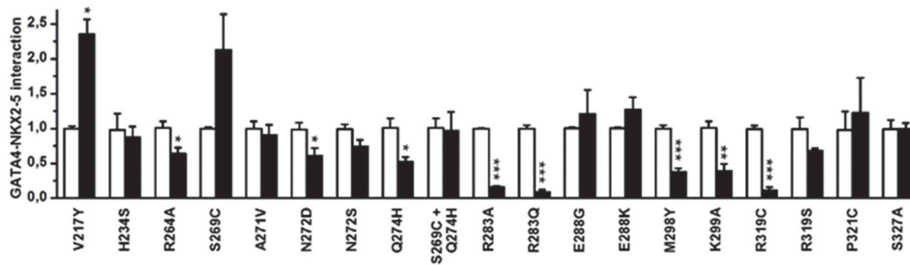

Fig. 7. Effect of mutations on GATA4-NKX2-5 protein-protein interaction quantified after immunoprecipitation. Mutated GATA4 protein binding to wild type FLAG-NKX2-5 (black bar) was compared to wild type GATA4 binding (white bar). The results show the mean of two independent experiments expressed as fold changes +SEM. * P<0.05, ** P<0.01 and *** P<0.001 vs. control group (independent samples Student's *t*-test). Reprinted with permission from Study I (S. Kinnunen et al., 2015).

In the present experiments, the effect of an N272S mutation on NKX2-5 interaction was not significant; however, mutation to a negatively charged aspartic acid, N272D, decreased the interaction. Moreover, amino acid A271, the amino acid next to N272 in the loop area, had no effect on the GATA4-NKX2-5 interaction.

GATA4 and GATA5 share a sequence identity of 45% for the whole protein and 79% for the zinc fingers, but are expressed differentially (Molkentin, 2000). GATA5 and NKX2-5 have been shown *in vitro* to activate the ANP promoter co-operatively almost as well as GATA4 and NKX2-5 (70% activity in comparison to GATA4) (Durocher et al., 1997). At the C-terminal zinc finger, GATA5 contains an extra cysteine which is absent in other members of the GATA family. This cysteine is located on the surface of GATA5 where the presumed protein-protein interactions could take place. S269C, Q274H and their combination were chosen to mimic the structure of GATA5. Interestingly, the mutation S269C tended to increase the GATA4-NKX2-5 interaction, but this change was not statistically significant. The Q274H mutation decreased the interaction and the combination S269C + Q274H abolished this effect.

It has been shown that the C-terminal extension of GATA4 (aa 303-390) is important for NKX2-5 interaction (Durocher et al., 1997). Therefore, a hypothetical phosphorylation site S327 locating outside of the zinc fingers was mutated. However, this mutant had no effect in immunoprecipitation studies. C-terminal extension mutations R319C, R319S or P321C were inserted to create an amino acid combination similar to that observed in the protein structure of NKX2-5. The aim was to design a similar internal competing motif in GATA4 that could block NKX2-

5 binding. Consequently, R319C strongly (89%) decreased the GATA4-NKX2-5 interaction.

Two mutations were also made to the N-terminal zinc finger. Amino acid V217 has been shown to be important for the GATA4-FOG2 interaction (Crispino et al., 2001) and H234 is situated next to V217. Interestingly, V217Y strongly increased (235%, $p<0.05$) the GATA4-NKX2-5 interaction.

5.1.2 DNA-binding (I)

The aim was to mutate only amino acids on the protein surface of native folded GATA4. To confirm that the secondary structure had been preserved, the effect of the mutation on GATA4 DNA-binding properties was studied by EMSA. The same equalised protein amounts were used here as in immunoprecipitation studies. GATA4 binding to the -90 tandem GATA element on the rBNP gene promoter generates two bands on a gel (Fig. 8A-E). In the presence of GATA4 antibody, instead of two bands only one band can be observed confirming that both bands contained GATA4 (Fig. 8B). The total intensities of these two bands were quantified and proportioned to wtGATA4 binding on the same gel (Fig. 8F). The strongest decrease (72%) in DNA-binding was noted with mutation R283A. Mutations locating on the same surface, R264A and K299A, showed consistently distinct binding patterns (Fig. 8D). R264A enhanced the binding in the upper band and K299A in the lower band, however, there was no effect on total DNA binding (Fig. 8F).

Mutations of N272 and the GATA5 mimicking mutations, S269C and S269C+Q274H, slightly decreased DNA binding. Mutations on the N-terminal zinc-finger or on the C-terminal extension had no significant influence on DNA-binding, except with amino acid R319 mutations.

To examine the GATA4 binding on tandem GATA-sites and the formation of two bands, further studies with the probes containing only one binding site were performed (Fig. 8A, E). When the probe contained the N-terminal GATA-site, rBNP 91 GATA or rBNP GATAmut -80, only the lower band was formed. This indicates one GATA4 molecule binding on these probes. Correspondingly, GATA4 binding on the C-terminal GATA-site (rBNP GATAmut -91) promotes one GATA4 binding; however, the presence of a faint upper band suggests a second GATA4 can bind in some cases.

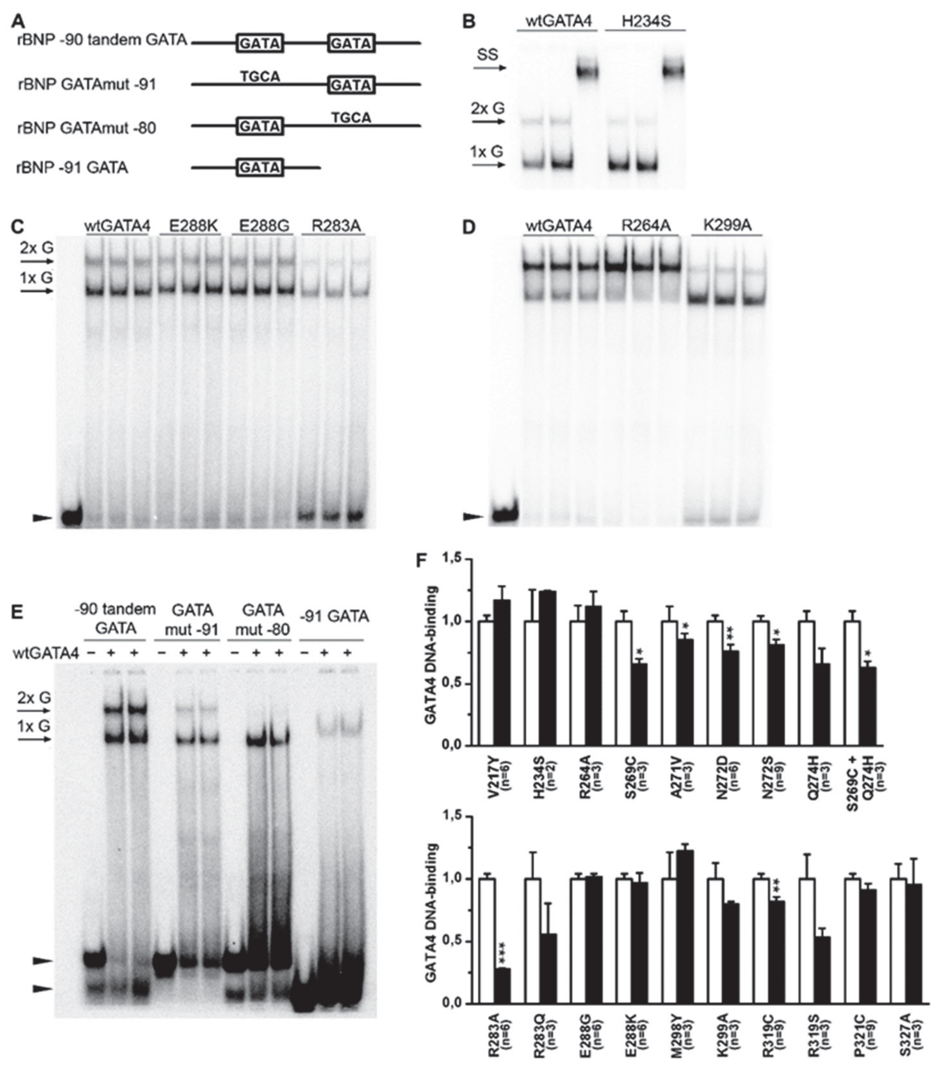

Fig. 8. DNA-binding properties of mutated GATA4 proteins. A) The probes used contained either a native GATA binding site or a mutated TGCA site. B) The two bands shift up with GATA4 antibody (SS, supershift). C) Representative images of EMSA studies with mutant GATA4 proteins. D) Mutations R264A and K299A caused different binding patterns. E) Mutation or deletion of the other GATA binding site promotes only one GATA4 molecule binding. F) Effect of mutations on GATA4 DNA-binding, quantified total intensity of the two bands (black bar) relative to wtGATA4 binding (white bar). The results are mean (+SEM) from two to nine independent reproducible EMSA samples using the -90 tandem GATA probe (n=1-3). 2xG = binding of two GATA4 molecules, 1xG

= binding of one GATA4 molecule. *p<0.05, **p<0.01 and ***p<0.001 vs. control group (independent samples Student's t-test). Reprinted with permission from Study I (S. Kinnunen et al., 2015).

5.1.3 Transcriptional activation (I)

The effect of the mutations on GATA4 transcriptional activity was studied in COS-1 cells by luciferase reporter assay. The cells were transfected with luciferase reporter plasmids containing the rat BNP minimal promoter (Fig. 9A), BNP -90 tandem GATA-sites in front of the minimal promoter (Fig. 9B) or a sequence containing three artificial NKX2-5 binding sites in front of the rat albumin minimal promoter (Fig. 9D). The amount of the transfected GATA4 mutant plasmids was adjusted to achieve equal expression levels of the mutant proteins and wtGATA4 (Fig. 9C).

All GATA4 mutations locating on a C-terminal zinc finger or C-terminal extension inhibited the activity of the minimal promoter (Fig. 9A). In the case of the promoter containing tandem GATA-sites, mutations R283A, R283Q, K299A, N272D, N272S and R319C abolished the transcriptional activity of GATA4 (Fig. 9B).

GATA4 and NKX2-5 are able to activate transcription synergistically on certain promoters. This synergism is dependent on NKX2-5 binding to the NKE site on the promoter, but not on the GATA4-DNA interaction (Shiojima et al., 1999). To study the effect of the mutant GATA4 proteins on the synergistic interaction with NKX2-5, COS-1 cells were transfected with three NKE sites containing reporter together with GATA4 mutants and wtNKX2-5 expressing plasmids (Fig. 9D). Wild type GATA4 alone had only a slight effect on this promoter (13% of synergy), while NKX2-5 activated 57% of the synergy and together the synergistic effect was 100% (Fig. 9D). The same GATA4 amino acid mutations inhibited synergistic activation as did the tandem GATA-sites promoter (R283A, R283Q, K299A, N272D, N272S and R319C). In addition to these the C-terminal extension mutations, R319S, P321C and S327A, inhibited synergistic activation as well. Interestingly, the N-terminal mutation V217Y increased the synergism.

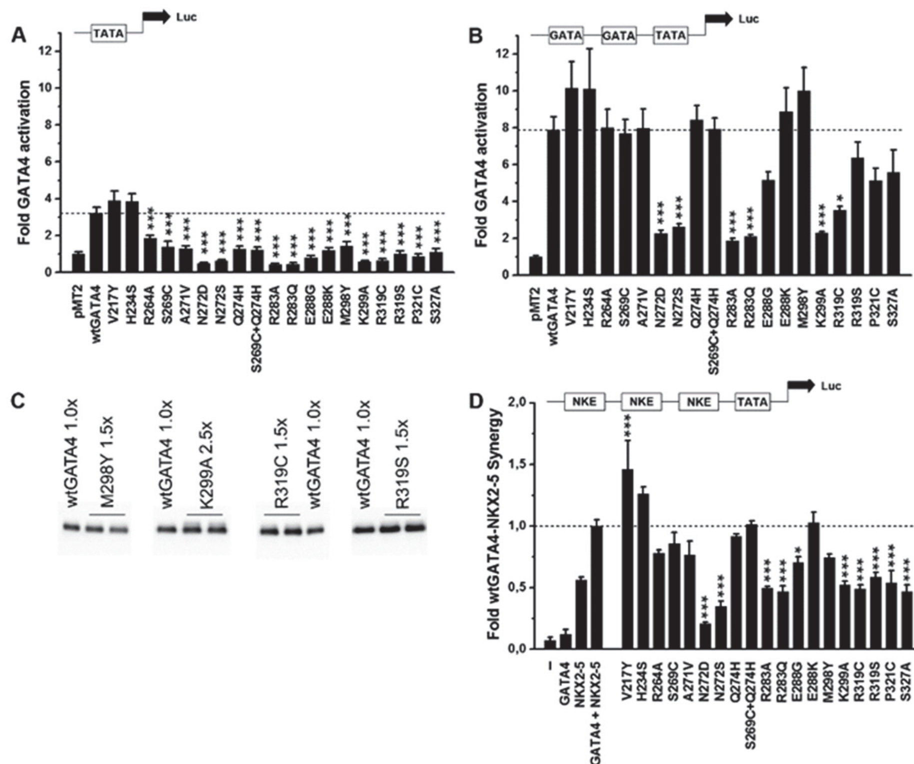

Fig. 9. Functional effects of the GATA4 mutants. Mutant GATA4 protein transcriptional activity was studied with luciferase reporter assays on A) rat BNP minimal promoter and B) tandem GATA site containing promoter. Results are mean (+SEM) expressed as fold-changes compared to empty vector pMT2 from three independent experiments with parallel samples (n=3). C) Protein expression levels were equalised by transfecting an adjusted amount of mutant GATA4 plasmids. D) The effect of mutations on synergistic activation with NKX2-5 was studied with a luciferase reporter containing three NKX-2.5 binding sites. The results are mean (+SEM) expressed as fold-change compared to the wtGATA4-NKX2-5 control group from two independent experiments with parallel samples (n=2). *p<0.05, **p<0.01 and ***p<0.001 vs. wtGATA4 in A and B and vs. wtGATA4-NKX2-5 in D (Dunnett's t-test). Reprinted with permission from Study I (S. Kinnunen et al., 2015).

5.2 Affinity chromatography of the lead compound (II)

The information gained about the protein-protein interface was used to virtually screen small molecule libraries (Välimäki et al., 2017). As a primary screening

method, the luciferase assay of GATA4-NKX2-5 synergy was used to screen around 800 molecules. The most potent compounds were further studied *in vitro* in neonatal cardiac myocytes induced by different hypertrophic stimuli, i.e. ET-1, stretch and PE (Välimäki et al., 2017) & (II). The active compounds in the luciferase screening assay significantly decreased ANP and BNP gene expression induced by hypertrophic stimuli. The effect of the most potent compound, 3i-1000, binding to GATA4 and NKX2-5 was further studied by an affinity chromatography method. Here, 3i-1000 was attached to Sepharose® beads through a PEG_3 linker from two different points: from central (14) and north (15) positions. In addition, an inactive derivative of 3i-1000 (13) and PEG_3 linker alone (12) were used as controls. GATA4 and NKX2-5 proteins were overexpressed in COS-1 cells separately and the total cell lysate was extracted in non-denaturing conditions to preserve native protein conformation. Immobilised compounds were incubated with protein lysates overnight, washed several times to remove unbound proteins and finally boiled in SDS sample buffer to elute bound proteins. The samples were resolved by SDS-PAGE and immunoblotted with GATA4 and NKX2-5 antibodies (Fig. 10). As shown in figure 10A, the active compound (14 and 15) bound to GATA4, but the inactive compound 13 did not. On the other hand, NKX2-5 bound to the inactive compound 13 and active compound 15 similarly (Fig. 10B). Furthermore, NKX2-5 binding to the linker (12) was even more pronounced, indicating this binding to be unspecific.

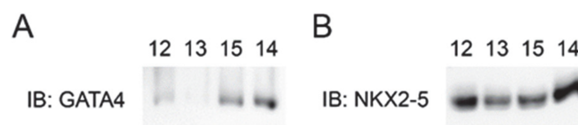

Fig. 10. Binding of GATA4 and NKX2-5 proteins to immobilised compounds. Compounds were attached to Sepharose® through a PEG_3 linker and the binding to GATA4 or NKX2-5 was studied by an affinity chromatography method. A) GATA4 and B) NKX2-5 antibodies were used to visualise the binding. PEG_3 linker (12), inactive derivative of 3i-1000 (13) and active 3i-1000 (14 and 15). The experiment was repeated two to three times with similar outcomes. IB, immunoblotted.

5.3 The effects of compounds in *in vivo* experimental models of heart failure

5.3.1 Angiotensin II induced hypertrophy and pressure overload (III)

Two active compounds from the *in vitro* tests with different profiles, 3i-1000 and 3i-0595, were studied further *in vivo* using Ang II induced hypertrophy and the pressure overload model. Continuous infusion of Ang II *in vivo* induces classical hypertrophic responses: protein synthesis, upregulation of foetal gene expression (e.g. ANP, BNP), fibroblast proliferation and fibrosis (S. Kim & Iwao, 2000; Suo et al., 2002). Ang II infusion for 2 weeks increases blood pressure and ventricular weight–to–body weight ratio and induced changes in diastolic function (Serpi et al., 2011; Suo et al., 2002). The rats were subcutaneously implanted with osmotic minipumps which release Ang II at the rate of 33,3 μg/kg/h for two weeks. The compounds or vehicle DMSO were administered i.p. three times a day (3i-1000) or two times a day (3i-0595) at a total dose of 30 mg/kg/day for two weeks. Interestingly, compound 3i-1000 significantly improved cardiac function by increasing left ventricular EF (Fig. 11A) and FS (Fig. 11B) and had a tendency to downregulate natriuretic peptide gene expression (Fig. 11C and D). In addition, it significantly increased heart rate and reduced left ventricular inner diameter during diastole and systole. In contrast, compound 3i-0595 significantly decreased EF and FS, thus impairing the systolic function.

5.3.2 Myocardial infarction (III)

AMI was induced by ligation of the LAD coronary artery both in mice and rats. Control animals went through a SHAM operation without ligation. Administration of compound 3i-1000 at the dose of 30 mg/kg/day or vehicle DMSO was started right after the operation. The mice were treated with 3i-1000 for four days and followed up to one week. The compound 3i-1000 significantly improved the left ventricular EF (Fig. 12A) and FS (Fig. 12B). Improvement of function was reflected by downregulation of elevated natriuretic peptide gene expression (Fig. 12C and D).

The experimental protocol in rats was similar to that in mice, except that rats were treated with 3i-1000 (30 mg/kg/day i.p.) for one week after AMI and finally examined by echocardiography. Even though in this experiment the decrease in left ventricular EF and FS due to the AMI was modest (Fig. 12E and F), there was

a tendency for improvement of cardiac function and attenuation of natriuretic peptide gene expression by 3i-1000 (Fig. 12G and H).

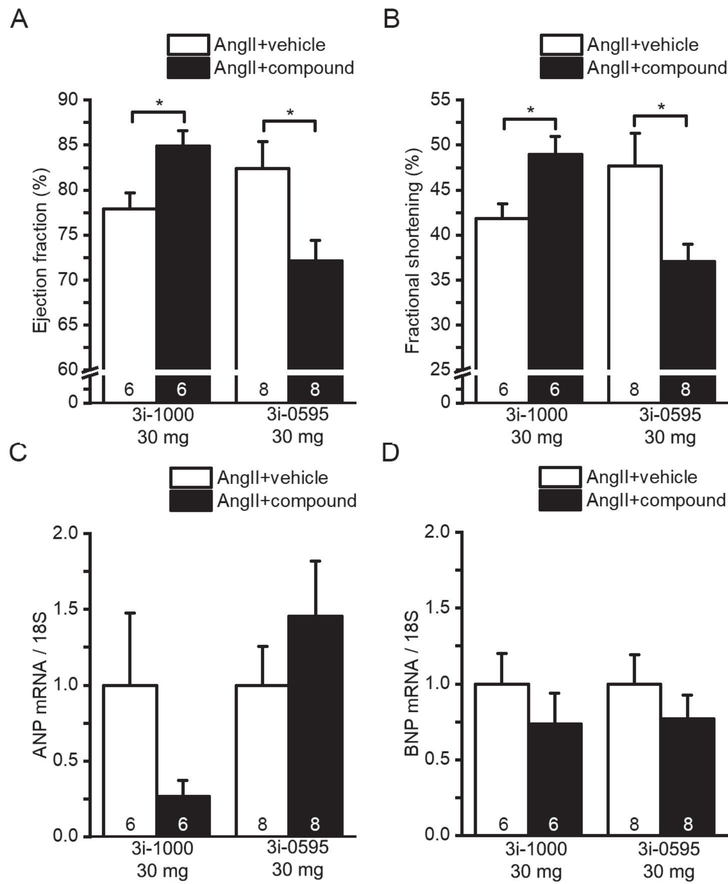

Fig. 11. The effect of compounds 3i-1000 and 3i-0595 on Ang II induced hypertrophy and pressure overload in rats. Animals received Ang II at the rate of 33,3 μg/kg/h and either 3i-1000 or 3i-0595 at the dose of 30 mg/kg/day i.p. for two weeks. Results are average +SEM. The number of animals in each group is denoted on the column. ****p*<0.05 (independent samples Student's *t*-test).**

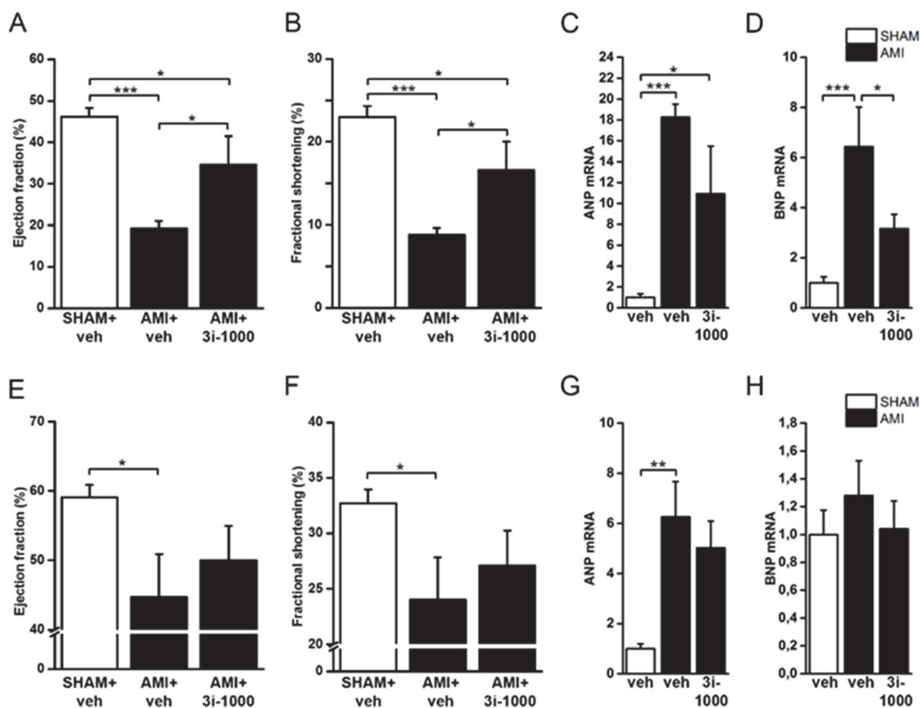

Fig. 12. Acute myocardial infarction induced by LAD-ligation to mice and rats. A-D) The mice underwent either an AMI or SHAM operation, were treated with vehicle (DMSO) or 3i-1000 at the dose of 30 mg/kg/day i.p. for four days and followed up to one week. The number of animals was 15 in SHAM+veh, 4 in AMI+veh and 3 in AMI+3i-1000 groups. E-H) The rats were treated with vehicle or 3i-1000 (30 mg/kg/day i.p.) for one week before echocardiographic measurements. The number of animals was 6 in SHAM+veh, 7 in AMI+veh and 8 in AMI+3i-1000 groups. The results are mean +SEM. ****p*<0.05, *****p*<0.01 and ******p*<0.001 (one-way ANOVA followed by least significant difference post hoc test). Reprinted with permission from Study III (S. M. Kinnunen et al., 2018).

5.3.3 Doxorubicin induced cardiotoxicity (IV)

To study whether the compounds have protective effects on doxorubicin induced cardiotoxicity, an animal model was established first. Several experimental conditions were tested for rats as presented in figure 13, and for mice. A high bolus dose of 15 mg/kg or 7.5 mg/kg/week three times i.p. (Fig. 13A and B) did not show any changes in ejection fraction; however, doxorubicin had a strong impact on the welfare of the animals. Acute diarrhea, ascites and serious weight loss (18-20%)

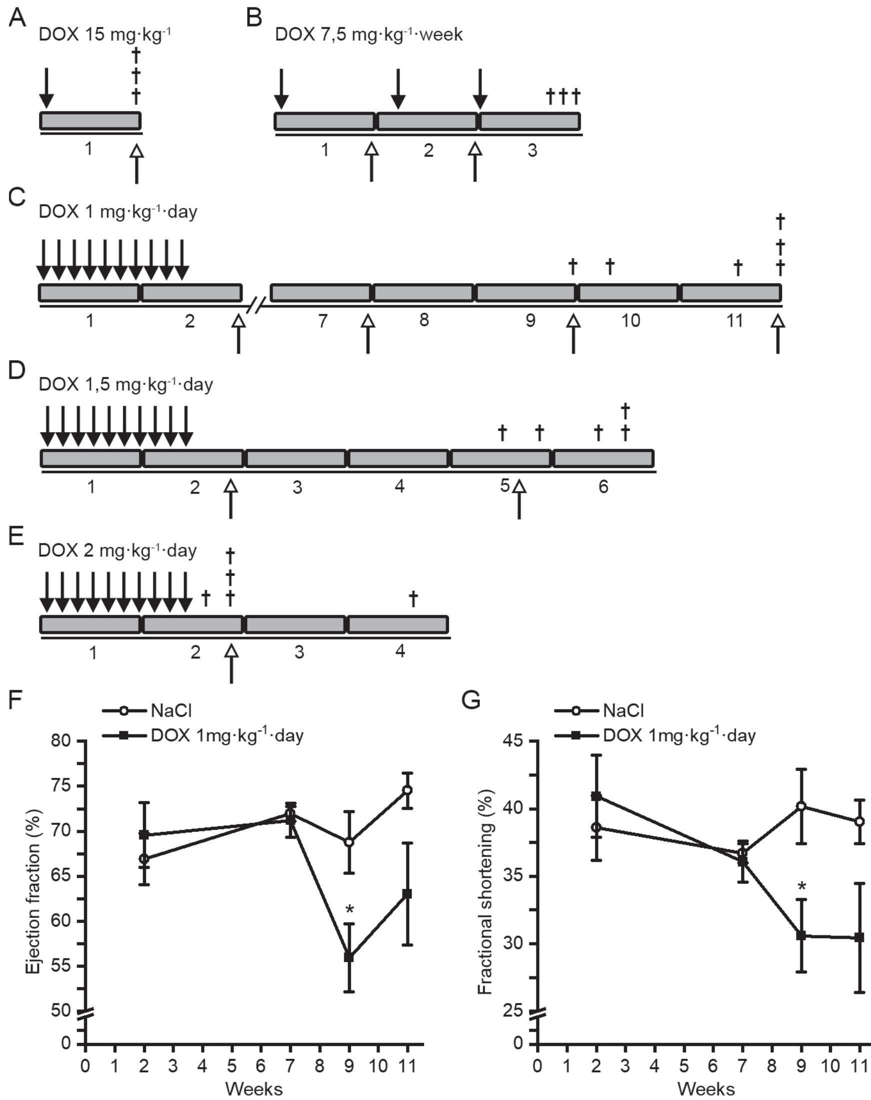

Fig. 13. Development of the model for doxorubicin cardiotoxicity with rats. A-E) The models with different doxorubicin dose and administration schedules. The black arrows denote doxorubicin injection times and white arrows echocardiographic imaging. The grey bars represent duration of the experiment in weeks and the crosses the time of decease of the animal. F and G) Echocardiographic results from the model where doxorubicin was given at 1 mg/kg/day for 10 consecutive days. The number of animals

in the NaCl group in each experiment was n=3 and in DOX-groups n=3-6. The results are averages ±SEM. *p<0.05 (independent samples Student's t-test).

were observed after 7 days in 2/3 of the rats. Neither did the similar experimental designs in mice, single dose of 15 mg/kg nor 7.5 mg/kg or 7.5 mg/kg/week for two weeks, show any changes in cardiac function. The dose of 15 mg/kg resulted in serious weight loss (>20%) already after 5 days in 2/3 of the mice, which was the criterion to stop the experiment. Finally, the model for rats with 1 mg/kg/day for 10 days developed by Hayward & Hydock (Hayward & Hydock, 2007) resulted in cardiomyopathy over time (Fig. 13C). This was observed as the decline in both ejection fraction and fractional shortening at nine weeks (Fig. 13F and G). The efforts to speed up the process by administering 1.5 or 2.0 mg/kg/day for 10 days (Fig. 13D and E) did not result in a decrease in EF or FS, but instead worsened animal health.

Based on the experiments, the compounds were studied in the model where animals were given doxorubicin at 1 mg/kg/day (i.p.) for 10 days (Fig. 14A). After seven weeks, the animals were treated with compound 3i-1000 or 3i-0595 two times a day (i.p.) at the dose of 30 mg/kg/day for two weeks and finally imaged by echocardiography. The treatment of rats with compound 3i-1000 after doxorubicin treatment significantly improved left ventricular EF and FS compared to animals which had received vehicle DMSO (Fig. 14B and C). ANP and BNP gene expression, measured from left ventricle tissue, increased due to doxorubicin treatment, whereas 3i-1000 had no significant effect on ANP and BNP mRNA levels (Fig. 14D and E). Compound 3i-0595 had no effect on left ventricular function or on natriuretic peptide gene expression in this model. Nuclear proteins extracted from LV tissue were analysed by Western blot and immunoblotted with GATA4 antibody. Neither doxorubicin nor either of the compounds had an apparent effect on GATA4 protein levels (IV). Interestingly, Western blot analysis of the total proteins showed that the doxorubicin induced significant down regulation of phospho-p38 MAPK was restored by treatment with 3i-1000 (IV).

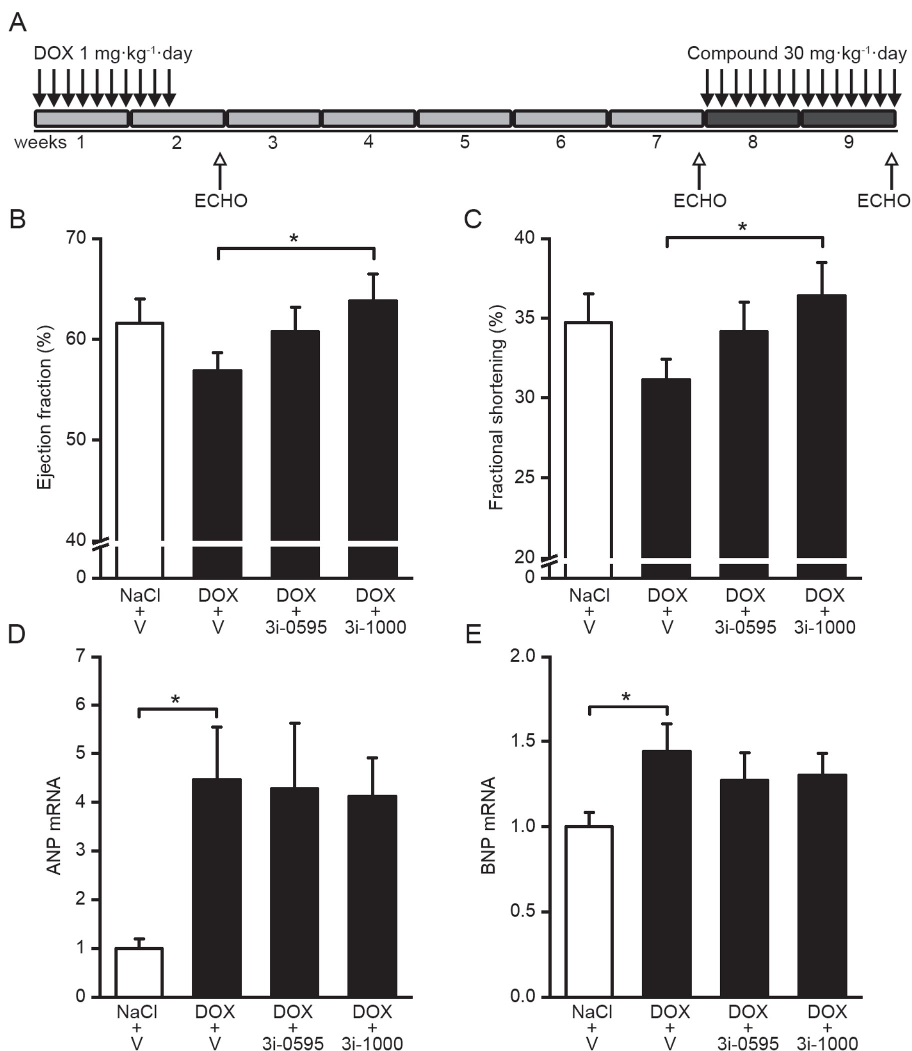

Fig. 14. The effect of compounds on doxorubicin induced cardiotoxicity in rats. A) The experimental protocol. The animals received either vehicle DMSO or compounds 3i-1000 or 3i-0595 for two weeks. The black arrows at the beginning denote doxorubicin or NaCl injection times and white arrows echocardiographic imaging. The black arrows at the end denote compound or vehicle treatment. The grey bars represent duration of the experiment in weeks. B-E) The echocardiographic parameters and the mRNA levels measured by RT-PCR in the left ventricular tissue after nine weeks. The number of animals was 10 in NaCl+V, 9 in DOX+V, 10 in DOX+3i-0595 and 8 in DOX+3i-1000. The results are averages +SEM. *p<0.05 (independent samples Student's *t*-test).

6 Discussion

6.1 Identification of GATA4-NKX2-5 interaction site

GATA4 mutations constructed during this study locate and cluster on specific regions on the protein surface. The group of amino acids R264, R283 and K299 form one such region-specific cluster and the mutations of these resulted in inhibition of the GATA4-NKX2-5 interaction, especially R283, which also strongly inhibited GATA4 DNA-binding in EMSA. Interestingly, mutations R264A and K299A consistently created distinct binding patterns on EMSA gels while having no effect on total GATA4 DNA binding (Fig. 8). This phenomenon implies that these amino acids could coordinate GATA4 binding to tandem GATA-site on DNA: mutation K299A promoting one GATA4 binding and R264A two GATA4 binding. Based on the modelling work, amino acids R264, R283 and K299 locate on the protein surface which partly contacts DNA and coordinate the packing of C- and N terminal zinc fingers (I). Mutation R264A allows the zinc fingers to pack more closely together and thus two GATA4 molecules would be able to bind on a tandem GATA-site sequence simultaneously. In contrast, mutation K299A favours a more open conformation allowing binding of only one GATA4 at a time on a tandem GATA-site. The transactivation studies support this hypothesis since R264A was able to activate a tandem GATA-site promoter similarly to wtGATA4 whereas K299A resulted in reduced activation (Fig. 9B). Apparently, the internal coordination of the zinc fingers is important for the assembly of the GATA4-NKX2-5 protein complex since all these mutations abolished the interaction in immunoprecipitation studies. In addition, R283A and K299A reduced also synergistic transactivation (Fig. 9C).

The other clustered region involving amino acids M298 and N272 locates opposite to the DNA-interfering surface of GATA4. Mutations of these residues resulted in inhibition of protein-protein interaction with NKX2-5. M298Y had no effect on GATA4 DNA binding or transcriptional activity confirming the preserved protein structural fold of the GATA4 protein and showing the importance of this single amino acid to protein-protein interaction. However, mutation N272S, which causes an atrioventricular septal defect in humans (Reamon-Buettner & Borlak, 2005), also resulted in reduced DNA binding, BNP-promoter activity and synergistic transactivation. Further studies showed that N272S inhibited synergistic activation specifically with NKX2-5 on the ANP promoter but not with KLF13 (I).

According to modelling work, the C-terminal zinc finger of GATA4 and the homeodomain of NKX2-5 resemble the DNA-binding domain of the nuclear receptors (I). For instance, the estrogen receptor contains the conserved residues R63, K66, and C67 in a helical structure that mediates interaction between the two zinc fingers (Schwabe, Chapman, Finch, & Rhodes, 1993) & (I). Previously it has been shown for NKX2-5 that K193 in the third helix of the homeodomain is specifically required to form the association with the C-terminal zinc finger of GATA4 and this specific sequence around K193 contains the pattern of amino acids R, K and C in the same distance (RxxKC) as in the estrogen receptor (Kasahara et al., 2001) & (I). Thus, M298 and N272 form an active site on the protein surface of GATA4 that could interact with the RxxKC containing sequence of the third helix of the NKX2-5 homeodomain (I).

The idea with the cysteine mutations on the GATA4 C-terminal extension was to create an internal competing motif that mimics the cysteine on the NKX2-5 RxxKC sequence. Indeed, R319C prevented protein-protein interaction and synergistic transactivation with NKX2-5. However, it also inhibited GATA4 DNA binding and BNP promoter transactivation. Previously it has been shown that the basic region (aa 302-327) adjacent to the C-terminal zinc finger is needed for GATA4 binding to DNA (Morrisey et al., 1997). Interestingly, mutations P321C and S327A locating on this basic region did not have an effect on DNA binding or BNP promoter activity. However, all mutations on the C-terminal extension inhibited synergistic activation with NKX2-5.

Even though previous studies have shown that the C-terminal zinc finger of GATA4 is sufficient for the physical interaction with NKX2-5 (Durocher et al., 1997; Y. Lee et al., 1998; Shiojima et al., 1999), mutation V217 indicates that protein-protein association may arise also from N-terminal interactions. It has been shown that the N-terminal zinc finger of the GATA4 protein interacts with other zinc finger transcription factors like FOG-2, which reduces the GATA4 transcriptional activity (Crispino et al., 2001; Lu et al., 1999). The mutation V217Y increased considerably the physical binding and synergy of GATA4 with NKX2-5 (Fig. 6, 7 and 9D) without having an effect on transactivation of the BNP promoter (Fig. 9A and B). Increased GATA4-NKX2-5 activity with mutation V217Y may arise due to reduced GATA4-FOG-2 interaction and subsequent lack of gene repression. On the other hand, further studies showed that V217Y did not increase the synergy with NKX2-5 on the ANP promoter (I). Instead, V217Y increased the synergy with KLF13 on the ANP promoter (I). In agreement with this, KLF13 has been shown to interact with the N-terminal zinc finger of GATA4 and the mutation

V217G increased the GATA4-KLF13 synergy also on the BNP promoter (Lavallee et al., 2006). The mechanism of this increased activity needs further exploration.

The BNP promoter contains a specialised TATA box with a GATA motif that is conserved between species (e.g. rat, dog, human) (Grepin et al., 1994). A luciferase study showed that GATA4 enhances BNP transcription through this site (Fig. 9A). It seems that this site is very sensitive to any GATA4 mutation on the C-terminal zinc finger or C-terminal extension since all of the mutations locating on these sites decrease the transcriptional activation (Fig. 9A). The distal -90 GATA binding site is also conserved between these species on the BNP promoter; however, in humans there is only an N-terminal GATA motif whereas in rats and dogs this site contains tandem GATA-sites (Grepin et al., 1994). In the case of a promoter containing tandem GATA-sites, only R283A, R283Q, K299A, N272D, N272S and R319C abolished the transcriptional activity of GATA4 (Fig. 9B).

Furthermore, the GATA4 mutation G296S causing CHD has been shown to specifically disrupt the interaction with TBX5 and SMAD4 but not with NKX2-5 (Garg et al., 2003; Moskowitz et al., 2011). Similarly, the G303E mutation in GATA4 diminishes the interaction with SMAD4 (Moskowitz et al., 2011).

6.2 Identification of small molecule inhibitors

A luciferase reporter assay of GATA4-NKX2-5 synergy was used to screen small molecules targeting the protein-protein interaction. Cell based luciferase assays are commonly used for high throughput drug screening (Auld & Inglese 2018). However, the compounds may exhibit beneficial effects by interfering with luciferase or by modulating transcription machinery resulting in false positives. In addition, compounds may aggregate, quench light, have redox behaviour or enzymatically inhibit the reporter. Therefore, the positive hits were further studied *in vitro* in neonatal cardiomyocytes under various hypertrophic stimuli (Välimäki et al., 2017) & (I).

The compounds studied here, *N*-[4-(diethylamino)phenyl]-5-methyl-3-phenylisoxazole-4-carboxamide (3i-1000) and 4-[(4-propoxybenzylidene)amino]-5-(pyridin-4-yl)-4H-1,2,4-triazole-3-thiol (3i-0595), are structurally related but belong to different compound families (Fig. 15). They both inhibited the GATA4-NKX2-5 interaction in a luciferase synergy assay, 3i-0595 with IC_{50} 10 μM, and 3i-1000 with IC_{50} 3 μM (compounds 4 and 3, respectively, in Välimäki et al., 2017). Interestingly, they had opposite effects on ANP and BNP basal gene expression, 3i-0595 increasing and 3i-1000 decreasing expression. In addition, 3i-0595 appeared

to have the opposite action on ET-1 stimulated gene expression by slightly increasing ANP and decreasing BNP, whereas 3i-1000 inhibited both gene expressions. In addition, 3i-1000 significantly reduced mechanical stretch-induced hypertrophic growth of neonatal cardiomyocytes (Välimäki et al., 2017) and down regulated stretch- and PE-induced ANP and BNP mRNA expression (III). Interestingly, PE induced GATA4 Ser-105 phosphorylation was inhibited by 3i-1000 (III). The compounds 3i-1000 and 3i-0595 were not cytotoxic for neonatal cardiomyocytes at 50 µM concentration (Välimäki et al., 2017). The toxicity of the compound 3i-1000 has been further studied in several other cell types, e.g. fibroblasts, human induced pluripotent stem cells (hiPSCs) and hiPSC-derived cardiomyocytes (Karhu et al., 2018). At the concentration of 30 µM, 3i-1000 was not toxic except for stem cells which were very sensitive already to 3 µM concentration.

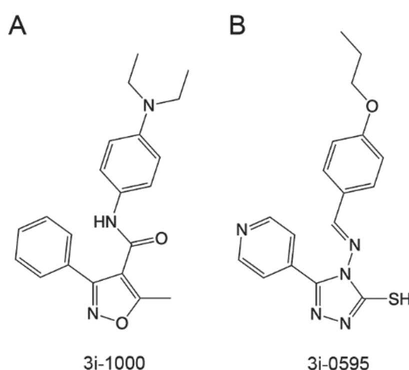

Fig. 15. The structures of the compounds. A) *N*-[4-(diethylamino)phenyl]-5-methyl-3-phenylisoxazole-4-carboxamide (3i-1000), B) 4-[(4-propoxybenzylidene)amino]-5-(pyridin-4-yl)-4H-1,2,4-triazole-3-thiol (3i-0595).

6.3 Target validation

Demonstration of binding of the compounds to a target turned out to be challenging since for the majority of ligand binding assays a large amount of pure protein is needed. During the purification processes GATA4 tends to degrade, which was the reason NMR-structure analysis failed (unpublished observation). For microscale thermophoresis a smaller amount of pure protein is required, however, the protein needed to be labelled with a fluorescent dye. The labelling process resulted in poor

quality GATA4 protein (unpublished results). The advantage of affinity chromatography is that the target protein can be pulled down directly from the whole cell lysate with immobilised ligand. Here, the active compound 3i-1000 and the related compound, inactive in luciferase assays, were attached to Sepharose® through a PEG_3 linker. Since the compound was targeted to the GATA4-NKX2-5 interaction, binding to both proteins was studied. NKX2-5 binding to 3i-1000 was considered to be unspecific since it bound to the inactive and active compound to the same degree and to the PEG_3 linker with even higher affinity. GATA4, however, showed specific binding to the active compound. GATA4 did not bind to the inactive compound and only slightly to the PEG_3 linker. It is noteworthy that in these studies, the compound binding specifically to GATA4 and NKX2-5 was studied, and therefore does not exclude the possibility that the compound could also bind to other proteins as well. In addition, it is possible that the compound binding to GATA4 is likely to modulate the protein conformation such that it reduces binding with NKX2-5 or inhibits or induces binding of other GATA co-factors. Thus, the precise mechanism(s) of action of 3i-1000 needs to be further investigated.

6.4 Cardioprotective effect of 3i-1000

The actions of 3i-1000 and 3i-0595 were studied *in vivo* in an Ang II induced hypertrophy and pressure overload model in rats. In the present experiments, 3i-1000 at the dose of 30 mg/kg/day significantly improved the cardiac function (ventricular EF and FS), whereas 3i-0595 significantly impaired the cardiac function. In addition, 3i-1000 had a tendency to decrease ANP and BNP gene expression, while 3i-0595 increased expression of ANP and decreased BNP. Remarkably, the compounds had similarly opposite effects on ANP and BNP gene expression *in vivo* as previously observed *in vitro* (Välimäki et al., 2017).

Acute myocardial infarction produced by LAD ligation induces left ventricular dilation and wall thinning with subsequent reduction in systolic function (Rysä et al., 2010). Previously, it has been shown that GATA4 adenoviral overexpression protects infarcted heart by restoring systolic function and ventricular wall morphology (Rysä et al., 2010). Interestingly, the GATA4 acting compound 3i-1000 exhibited protective effects in this model by improving systolic function (EF and FS) and reducing natriuretic peptide gene expression. The protective effect was more pronounced in the mouse experiment where infarction was more severe than in rat experiments (more significant reduction in LV systolic function with AMI+veh mice). Furthermore, 3i-1000 was studied in an ischaemia re-perfusion

model which resembles the clinical scenario of acute myocardial infarction in humans (III). Here the compound was administered one week before 30 min of ischaemia and 24 hours re-perfusion time. Although 3i-1000 showed only a tendency to improve LV systolic function, it significantly reduced I/R-elevated ANP gene expression. As experiments in rats showed rapid metabolism of 3i-1000 after i.p. administration (III), the compound was packed into biodegradable and thermally oxidized porous silicon (TOPSi) microparticles and injected locally into myocardium at the site of infarction (III). While cardiac function, analysed after one week, remained unchanged with 3i-1000 loaded TOPSi particles, MI elevated collagen 1A1 gene expression was significantly downregulated. In addition, 3i-1000 showed a tendency to down-regulate ANP, tumour necrosis factor-alpha (TNF-α) and osteopontin (OPN, also known as SPP1) mRNA levels. Overall, these studies in experimental models of myocardial infarction indicate significant potential for 3i-1000 to promote myocardial repair.

6.5 Doxorubicin induced cardiotoxicity and GATA4 targeted compound

Doxorubicin among other anthracyclines is a widely used and very efficient drug in cancer chemotherapy (Corremans et al., 2018). The most serious side effect for this drug is cardiotoxicity that can develop into cardiomyopathy and heart failure even after decades (G. T. Armstrong et al., 2012). Many years of extensive research on the anthracycline cytotoxicity and cardiotoxicity has revealed several mechanisms and it seems that the same mechanisms are involved in both processes (Corremans et al., 2018; Renu et al., 2018). At present there is only one FDA-approved cardioprotective agent for anthracycline-induced cardiotoxicity, dexrazoxane. However, the most effective strategy against cardiotoxicity is prevention by using a low dose, continuous infusions and liposomal formulations of doxorubicin.

For studies of doxorubicin induced cardiotoxicity different animal models, doses and schedules have been used (Aston et al., 2017). The aim here was to use the same model (a single 15 mg/kg dose) as previously described in GATA4 *in vivo* cardioprotection studies and in various other studies, but this failed. Neither rats nor mice treated with a single dose of 15 mg/kg showed any decrease in cardiac function examined by echocardiography after 5 days in mice and after one week in rats. Instead the animals had severe health problems and weight loss, which were the criteria to stop the experiments. Most likely this is due to the genetic variation

between the animal strains. The *in vivo* model, in which Weinstein et al. (2000) used a single doxorubicin dose of 20 mg/kg and measured reduction in cardiac function 5 days later, used CF-1 male mice. In the present experiment, C57BL/6 male mouse and Sprague-Dawley male rat strains, inbred in the Oulu laboratory animal centre, were used. These strains did not tolerate such a high dose of doxorubicin. The dose of 7.5 mg/kg/week was better tolerated; however, the experiment with mice had to be discontinued after 2 weeks and with rats after 3 weeks due to health concerns. Moreover, no effect on cardiac function was observed by echocardiography. Furthermore, the clinical value of the studies with a single high bolus dose of doxorubicin has been questioned, since they simulate acute cardiotoxicity (Corremans et al., 2018; Gianni et al., 2008). The more relevant protocol would be the doxorubicin-induced chronic toxicity with low dose and repeated administrations as is used in clinics (Vejpongsa & Yeh, 2014). In clinical use, doxorubicin is administered at the doses of 40-90 mg/m^2 through intravenous infusion at least 15 min-long every third week (Vejpongsa & Yeh, 2014). For pediatric patients and together with chemotherapy, lower doses are used. Moreover, intraperitoneal administration reduces doxorubicin bioavailability (43.8%) compared to intravenous administration (Nagai, Nogami, Egusa, & Konishi, 2014). Thus, to evaluate the doxorubicin dose used in the present experiments to the doses used in humans, plasma concentrations should be compared due to different pharmacokinetics and dosing forms (i.p. in rats vs i.v. in humans). In one experiment, when mice were administered a single dose of 12 mg/kg i.p. doxorubicin, the plasma concentration was 60 ng/ml after 2 hours and 20 ng/ml after 24 hours (Johansen, 1981). Correspondingly, in humans a 60 mg/m^2 dose i.v. resulted in doxorubicin plasma concentrations of 480 ng/ml after 1 hour and 40 ng/ml after 24 hours (Barpe, Rosa, & Froehlich, 2010). Thus, in the present model in rats, the total cumulative dose of 10 mg/kg over 10 ten days resembles roughly the subchronic cardiotoxicity model. On the other hand, the cumulative dose of 10 mg/kg in rats has been estimated to correspond to 400 mg/m^2 in humans (80 kg, 183 cm) (Hayward & Hydock, 2007).

GATA4 is shown to be involved in doxorubicin induced cardiotoxicity. Doxorubicin treatment decreases GATA4 protein levels and downregulates GATA4 gene expression *in vitro* in cardiac myocytes (Aries et al., 2004; Aries et al., 2014; B. Chen et al., 2012; Kobayashi et al., 2006; Kobayashi et al., 2010) as well as *in vivo* (Aries et al., 2004; Kobayashi et al., 2006). In neonatal cardiomyocytes, doxorubicin downregulates also GATA4 target genes ANP and/or BNP as well as survival factors Bcl-xL and Bcl-2 (Aihara et al., 2000; Aries et al., 2004; S. Chen,

Garami, & Gardner, 1999; Kobayashi et al., 2006). However, in H9c2 murine cardiac myoblast cells ANP gene expression is upregulated by doxorubicin (Karagiannis et al., 2010; Zordoky & El-Kadi, 2008). Similarly, doxorubicin induced cardiotoxicity in humans elevates ANP and BNP levels (Hayakawa et al., 2001). Several experiments have also shown the protective role of GATA4. In cardiac myocytes overexpression of GATA4 with adenovirus decreases doxorubicin induced apoptosis and restores the ANP, Bcl-xL and Bcl-2 expression levels (Aries et al., 2004; Y. Kim et al., 2003; Kobayashi et al., 2006). Similarly, transgenic mice overexpressing GATA4 in the heart showed resistance to doxorubicin effects (downregulation of GATA4 and Bcl-2) compared to wild type mice (Kobayashi et al., 2006**.** More specifically, this recovery was shown to be mediated through the GATA4 Ser-105 phosphorylation site (Gallagher et al., 2012). Furthermore, PE infusion prevents GATA4 depletion in doxorubicin-treated GATA4+/- mice (Aries et al., 2004). Therefore, there is strong support that manipulation of GATA4 activity could prevent doxorubicin induced cardiotoxicity. Interestingly, compound 3i-1000 showed improvement in cardiac function in doxorubicin-treated rats. However, in this animal model there was no detectable decrease in GATA4 protein or mRNA levels by doxorubicin treatment suggesting that the beneficial effects observed in this study by 3i-1000 are not related to the regulation of the GATA4 protein levels. The studies in which doxorubicin has induced GATA4 protein depletion have been short-term experiments using a high dose. As observed during the cardiotoxicity animal model development, these acute models do not show changes in cardiac function, which is characteristic for cardiomyopathy in humans.

The use of short-term animal experiments also in preclinical studies of cancer therapeutics has been criticised (Corremans et al., 2018; Gianni et al., 2008). This is a dilemma concerning also development of new drugs. The preclinical studies do not predict the possible cardiovascular toxicity, which remains one of the leading causes of early and late attrition in drug development, as well as a major reason for the withdrawal of marketed drugs (Fermini, Coyne, & Coyne, 2018). Approximately eight out of every nine of the compounds fail in clinical trials. Fermini et. al. (2018) estimated that 10% of “future failure” drugs could be eliminated with proper cardiotoxicity testing alone.

7 Summary and conclusions

The aim of this present study was to characterise the protein-protein binding mode of two cardiac transcription factors GATA4 and NKX2-5 as well as the lead compound targeted to inhibit this interaction. The most important findings are summarised below and in Fig. 16.

1. To explore the GATA4 protein structure and the protein-protein interaction with NKX2-5, a total of 19 amino acids were mutated on the surface of GATA4. Altogether the immunoprecipitation, DNA-binding and functional studies revealed that GATA4 interaction with NKX2-5 is mediated through the GATA4 protein surface that is opposite to the DNA-binding site and contains amino acids N272 and M298. Moreover, the internal arrangement of the zinc finger domains of GATA4 has an impact on GATA4-NKX2-5 interaction as well, and the zinc finger arrangement was particularly important for GATA4 DNA-binding and transcriptional activation.
2. The affinity chromatography method was utilised to examine the binding of the small molecule inhibitor 3i-1000 of the GATA4-NKX2-5 interaction to its target. The active compound 3i-1000, immobilised to Sepharose®, showed more specific binding towards GATA4 than NKX2-5. The finding that the inactive derivative of 3i-1000, which did not bind GATA4 but bound NKX2-5 to the same degree as the active compound, implies that the binding with NKX2-5 is unspecific.
3. The effect of compound 3i-1000 was studied *in vivo* in experimental models of heart failure. In all models, an Ang II induced hypertrophy and pressure overload model, myocardial ischaemia created by LAD and chronic cardiotoxicity induced by doxorubicin, 3i-1000 significantly improved cardiac function by increasing left ventricular EF and FS, indicating cardioprotective properties for the compound.
4. In the future, a more detailed assessment and validation of the molecular mechanism(s) and the optimisation are needed to fully evaluate the potential of GATA4–NKX2-5 interaction modulators for heart repair and regeneration.

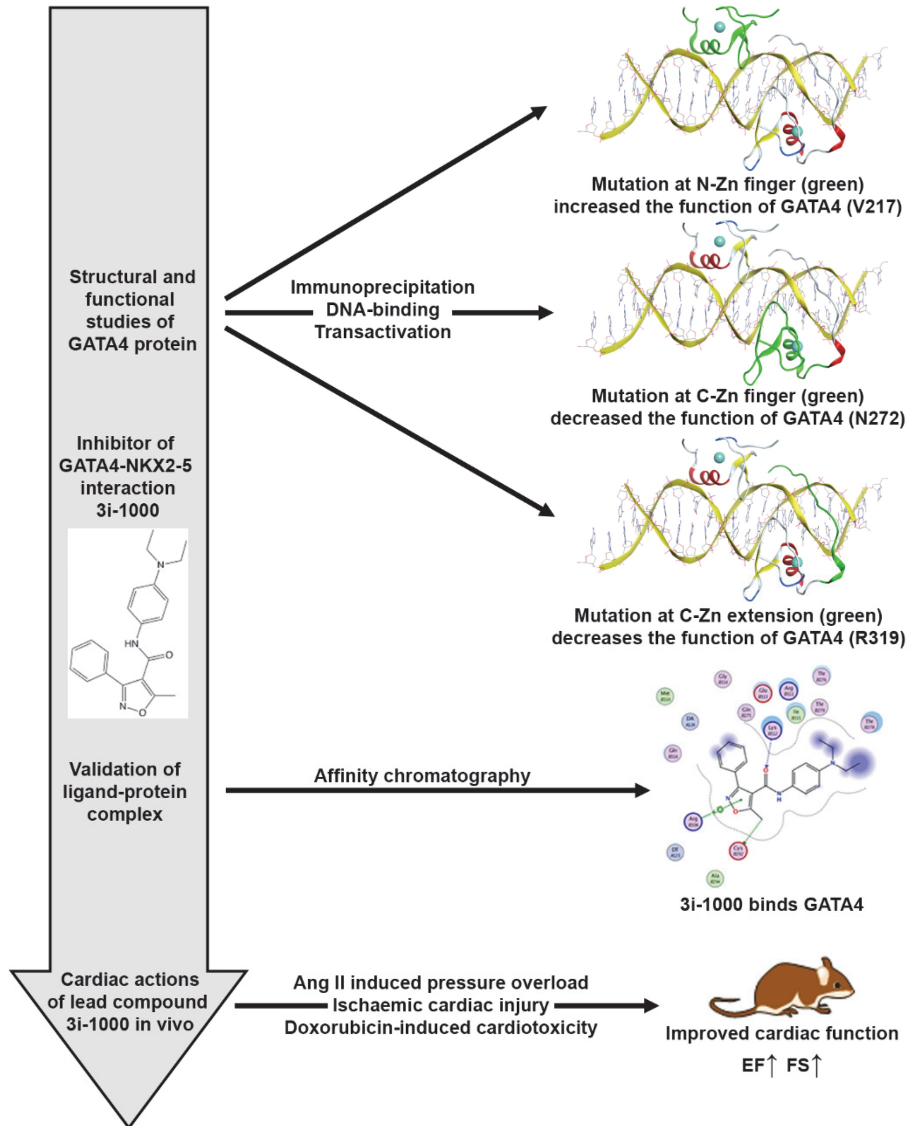

Fig. 16. Summary of the most important results of the thesis. N-Zn, N-terminal zinc finger; C-Zn, C-terminal zinc finger; EF, ejection fraction; FS, fractional shortening.

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