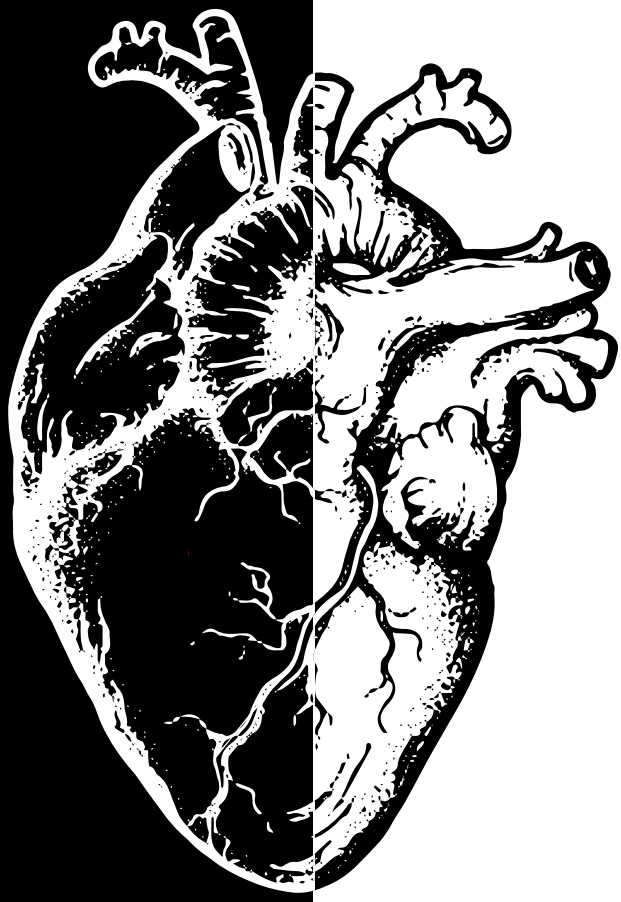
LncRNA Bigheart in cardiac disease and development



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Chapter 1

General introduction

Heart failure (HF) is a major public health issue, affecting at least 26 million people worldwide, with an incidence of 20 cases per 1000 people in individuals between 65 to 69 years, and a significantly higher rate in individuals 85 years or older.[1]The formal definition of HF is the inability of the heart to supply the peripheral tissue with the required amount of blood and oxygen to satisfy their metabolic demands. HF is responsible for 300,000 annual deaths, with a 5-year mortality approaching 50%. This percentage is expected to massively increase by 2030 in the USA. [2,3] Europe currently shows a slightly lower 5-year mortality rate of about 41%. Unfortunately, the European rate is forecast to match American values in the upcoming years [4], with a consequent increase in the cost of global healthcare.[5] It is estimated that direct and indirect HF related costs are more than $39 billion annually in the United States with an average cost associated to HF hospitalization as high as $10,000 per patient[6]. A similar trend

can be observed in Europe where the average cost for HF hospitalization is €11.000 [6,7]. One of the reasons for these incredible numbers derives from the initial difficulty of diagnosing HF. Patients can experience a wide range of symptoms due to the elevation in cardiac filling pressures or reduced cardiac output. The main symptoms are Palpitations, dyspnoea, Fatigue, and rapid or irregular heart rate[8]. However, early stages of HF can be asymptomatic, making early diagnosis challenging. In order to standardize the diagnostic process, clinicians divide heart failure into subtypes standardised according with the guidelines laid down by the New York Heart Association (NYHA) and the American College of Cardiology (ACC) to evaluate and manage this condition. Consistently with NYHA and ACC, the left ventricular ejection fraction (LVEF), measured by echocardiographic imaging, is one of the major criteria to evaluate the HF severity. Using this parameter, it is possible to distinguish two main classes of HF, patients with heart failure with reduced ejection fraction (HFrEF with an ejection fraction <40%), and those with preserved ejection fraction (HFpEF with an ejection fraction >50%). However, the most recent Guidelines on Acute and Chronic Heart Failure from the European Society of Cardiology (ESC) introduced a new category, namely HFmrEF or heart failure with mid-range ejection fraction (HFmrEF with and ejection fraction ± 50%).[9] More than 6.5 million people have been diagnosed with HF, and the percentage of patients with HFmrEF accounts for 15% of the total population affected. Instead, HFpEF patients account for 39%. In the last instance, the proportion of patients with HFrEF is at 47%.[10] These classifications help guide diagnostics and treatment for HF. Prognosis in patients with HFpEF is highly influenced by comorbidities, therefore the therapies rely on a combination of pharmacological and non-pharmacological approaches to reduce the risk of aggravation. On the other hand, HFrEF heavily relies on pharmacological therapy and in severe cases, device therapy is implemented to reduce the risk of sudden death. The therapy adopted with Patients with HFmrE is determined case by case and to date there is no standardization, however, often clinicians tend to apply similar strategies as for patients with HFpEF. Regardless of the major effort in developing new strategies to treat HF, most of the present therapies aim to minimise the mortality rate rather than completely reverting the ailing heart back into a healthy heart. In addition, not all the current therapies showed improvements, especially in HFrEF.[10] Non-pharmacological approaches aim to control risk factors and seek to treat conditions that can lead to HF, such as diabetes, hypertension, and others[11]. Conversely, pharmacological therapy uses mainly drugs such as angiotensin-converting enzyme inhibitors (commonly called ACEI) and Angiotensin II Receptor Blockers (ARBs). They have proven to be very effective in the treatment of heart failure caused by systolic dysfunction. ACEI produces vasodilation

by inhibiting the formation of angiotensin II due to the functional block of the Angiotensin-Converting Enzyme (ACE) which converts angiotensin I to angiotensin II[12]. The result on the heart is an enhancement in ventricular stroke volume and an improvement of ejection fraction (EF). Moreover, the vasodilation helps to improve the supply of oxygenated blood to the heart and reduces blood pressure. Overall, ACE inhibitor therapy has proven efficient in several trials with a reduction in mortality of nearly 25% in those treated with ACEI against controls [13]. Sadly, not all the patients seem to display similar favourable effects, especially in HFrEF. In these cases, therapies based on ARBs or Beta-blockers are used. Beta-blockers are drugs that bind to beta-adrenergic receptors and thereby block the binding of norepinephrine and epinephrine in the heart. The main concentration of adrenaline receptors is in the sinoatrial node, and the activation of these receptors stimulates heart rate and contractility. Furthermore, Beta-Blockers can antagonise the alpha-1 receptors, these receptors induce vasoconstriction and increased cardiac chronotropy; antagonism at the alpha-1 receptor leads to vasodilation, which leads to lower blood pressure and decreases heart rate. Therefore, upon administration of Beta-Blockers the heart is subjected to a lower level of stress and can recover. When added together with ACEI and diuretics (such as Aldosterone antagonists), beta-blockers result in a reduction in mortality in patients with severe HF.[14] Aldosterone Antagonists are drugs which antagonize the action of aldosterone. Aldosterone is a mineralocorticoid synthesized in the adrenal glands that can bind the mineralocorticoid receptors with the subsequent regulation of the sodium/potassium ratio in the cells. The change of this factor causes the cells to release liquid and increases the blood pressure. Antagonists block this effect by decreasing the blood pressure and bringing less stress to the heart.[15] At present, standard pharmacological HF therapy for patients in HFrEF includes ACEI/ARB and beta-blockers. The result is a significant 30% decrease in the risk of death in those patients[15]. Despite these treatments, the morbidity and mortality in patients with HF are still high, with a poor life quality. An alternative approach, applied when Pharmacological treatment does not appear to have effects, or in patients with high probability of sudden cardiac death (SCD), relies on the use of devices such as Implantable Cardioverter-Defibrillators (ICD) or Cardiac resynchronization therapy (CRTD). The ICD is a small battery-operated device implanted under the skin connected to the heart, capable of monitoring it to perform cardioversion, defibrillation, and pacing of the heart.[16]. On the other hand, CRTD is an implantable device, but it not only intervenes in the event of a stroke but actively regularizes the heartbeat. After cardiac remodeling or in HF due to decreased functionality, the heart also slowly loses the synchronism of contractions, further increasing stress. The logic of CRTD is to

restore the mechanical synchrony of the heart by electrical stimulation from the device. This device has proven very effective in cases and trials, especially in patients with milder HF symptoms. Despite HFrEF and HFpEF sharing many risk factors, and being treated with similar pharmacological therapies, their effect on the heart diverges significantly. For instance, HF is typically preceded by a compensatory response of the heart named cardiac hypertrophy. This phenomenon occurs under chronic cardiac stress or injury to maintain cardiac function. During the process of cardiac hypertrophy, heart muscle cells undergo enlargement, accompanied by various biochemical, molecular, structural, and metabolic alterations.

However, the type of hypertrophy the heart is subject to changes according to the subtype of HF, typically HFrEF leads to eccentric hypertrophy. In this type of hypertrophy, the cardiomyocyte increases in size by the addition of sarcomeres in series, and this make the cardiomyocyte longer. Therefore, the heart gets thinner walls and enlarged cavities with consequent stress for the heart subject to volume overload. On the other hand, HFpEF leads to concentric hypertrophy. The cardiomyocyte increases in size by the addition of sarcomeres in parallel, making the cardiomyocyte wider, consequently, the heart gets thicker walls and smaller cavities, which lead to pressure overload. The boundaries between different concentric and eccentric HF are not perfectly defined, and often with the worsening of the patient's HF, the hypertrophy can evolve from concentric to eccentric. Additionally, cardiac hypertrophy can further be divided into two other categories; physiological and pathological hypertrophy. The former is usually present in trained athletes and shows normal contractility as well as normal architecture and organization of the cardiac tissue. On the other hand, pathological hypertrophy is present in patients with any type of structural heart diseases, and it is characterized by structural remodeling and myocardial fibrosis [17]. Furthermore, maladaptive hypertrophy is associated with fibrotic remodeling, increased rates of cardiomyocyte death, dysregulation of Ca^{2+} handling proteins, altered sarcomere structure, insufficient angiogenesis, and thereby ventricular chamber dilatation mostly localized in the left ventricle [18] The ventricular chamber dilatation is a common trial to HFrEF due to eccentric hypertrophy. Instead HFpEF tend to have a reduction of the chamber due to concentric hypertrophy. Overall, all those modifications may lead to HF, and therefore, an improved understanding of the cellular and molecular processes involved in the development of cardiac hypertrophy is fundamental to create new therapies and drugs to treat HF. On the cellular level, cardiac hypertrophy involves the cardiomyocytes increasing in size through the addition of sarcomeres. Consequently, it becomes more difficult for the heart to contract correctly, and the demand for oxygen increases[19]. The inability to supply

oxygen leads to cardiac ischemia and cell death. On the molecular level, these changes occur due to a total variation in the transcriptional profile. The causes for these changes are not entirely clear. However, characteristic markers of maladaptive cardiac hypertrophy have been identified, such as the reactivation of fetal genes as well as a rearrangement of the metabolic profile. Fetal genes such as natriuretic peptide A (ANP), natriuretic peptide B (BNP), myosin heavy chain cardiac muscle β-isoform (MYHCβ), (or α- isoform in Humans) and skeletal muscle α- actin are commonly reactivated in hypertrophy.[20] Furthermore, following the development of hypertrophy, the metabolic profile of cardiomyocytes radically changes towards a glycolytic and anaerobic metabolism. The consequent oxidative stress induces the activation of apoptotic programs with lead to ischemia and cellular death. [21] It is clear that HF and cardiac hypertrophy are closely related, therefore to identify new therapeutic targets for the treatment of HF, many researchers have focused on the study of the molecular processes that control the course of cardiac remodeling. The full extension of the pathways involved in promoting maladaptive cardiac remodeling is not entirely charted yet. However, neuroendocrine hormones such as angiotensin II and endothelin 1 have been shown to stimulate cardiac myocyte hypertrophy through G-protein-coupled receptors (GPCRs) while also stimulating fetal gene transcription [22]. GPCRs are a family of transmembrane proteins that play a fundamental role in the regulation of cardiac function and cardiac hypertrophy. Those receptors are coupled to the heterotrimeric GTP-binding proteins, which are made up of three Gα subunits (Gαs Gαi, Gαq/Gα11) Gβ and Gγ. The different combinations between the activated GPCR and the stimulated G-protein determine a different pathway and cellular response. [23] Angiotensin II (AngII) can bind the α-adrenergic receptor AT1 and AT2, triggering the activation of the Gαq/Gα11 signaling. Severe cardiac hypertrophy has been reported in transgenic mice with AT1 overexpression after stimulation with AngII. Similarly, Endothelin 1 (ET-1) can bind the Endothelin receptors ET_A and ET_B. ET_A receptors can trigger the activation of the Gαq/Gα11 signaling, which contributes to the maintenance of Ca^{2+} handling in cardiomyocytes. When this pathway is activated, it stimulates the activity of the phospholipase C (PLC) which leads to the release of IP3. IP3 promotes the release of intracellular Ca2+ from the endoplasmic and sarcoplasmic reticulum through IP3 receptors, with the consequent activation of protein kinase Cα (PKCα) and the serine/ threonine kinase calcium/calmodulin dependent protein kinase type II (CaMKII). PKCα regulates cardiac contractility. Instead, CaMKII induces the nuclear exit of class II histone deacetylase 4 (HDAC4). In the nucleus, HDAC4 directly binds and represses MEF2 transcriptional factor, which has a major role in cardiac hypertrophy.[24] Interestingly, antagonists for ET-1 have

proven useful in reducing cardiac hypertrophy. [25] Another class of peptide which leads to cardiac hypertrophy are the catecholamines. Adrenalin and noradrenaline have been proven to be involved in stimulating hypertrophy by binding α-adrenergic receptor and β-adrenergic receptor, a subclass of GPCRs[26]. Adrenalin and noradrenaline can activate Gαs, the response is an increased heart rate and an increase of contractility[27]. Although several hormones can bind GPCRs, the downstream effectors of these G-proteins predominantly converge in activation of one or more members of the mitogen-activated protein kinases (MAPKs), such as the extracellular signal-regulated kinases (ERKs), the c-Jun amino-terminal kinase (JNKs), and the p38-MAPKs. MAPKs kinases have been proved to actively participate in the hypertrophy response with either enhancer or repressive role. Moreover, the activation of these G-proteins directly or indirectly induces an increase in the cytoplasmic level of calcium and the activation of the Calcineurin/ NFAT signaling. This signaling serves as a pivotal nodal control for the cardiac hypertrophic response. Cardiac-specific activation of calcineurin or downstream effector of NFAT are sufficient to induce cardiac hypertrophy in mice models. Similarly, genetic inhibition of calcineurin or NFAT highlighted that this pathway is necessary for a full hypertrophic response[28]. Moreover, studies in failing or hypertrophic human hearts, have shown elevations in calcineurin protein levels or phosphatase activity[29]. Several studies have been dedicated to elucidating the molecular mechanisms underlying the Calcineurin/ NFAT signaling. Although not all regulatory elements of this signaling pathway have been identified, the role of calcineurin and NFAT has been clarified. Calcineurin (CnA) is a calcium-dependent phosphatases, that was originally recognized as a hypertrophic signaling factor by employing transgenic mice with constitutional activation of CnA[30]. It is formed by 2 main subunits: calcineurin A which serves as catalytic subunit, and calcineurin B, involved in the regulatory mechanism of calmodulin, a calcium binding protein and partner of CnA[31]. Stimuli that increase the concentration of intracellular calcium led to calmodulin saturation, which promotes calcineurin activation. Once activated, CaN induces the translocation of cytoplasmic Nuclear factor of activated T-cells (NFAT) to the nucleus by dephosphorylating the serine-rich region (SRR) in the amino termini of NFAT, resulting in a conformational change, that exposes a nuclear localization signal which drives the nuclear translocation[32]. This transcriptional factor was first studied for its involvement in immune response, but lately has been found to play an important role in regulating the development of cardiac, skeletal, muscle, and nervous systems[33]. Once in the nucleus, NFAT participates in the transcriptional induction of various hypertrophic related gene, however, since NFAT proteins have weak DNA-binding capacity at the consensus sequence (A/TGGAAA)[34], they need to cooperate with

cofactors such as Gata4 and Mef2, to initiate and maintain the hypertrophic program. Interestingly, although NFAT is strongly involved in cardiac hypertrophy, in the adult mouse NFAT transcriptional activation was mainly observed during pathological hypertrophy, rather than physiological hypertrophy[35]. Nevertheless, the Calcineurin/ NFAT axis plays a fundamental role in the hypertrophic response of adult hearts. Consequently, several studies have focused on the identification of regulators of this signaling[36]. Recent studies have highlighted that CnA and CaMKII can be modulated by several proteins, namely the specificity tyrosine (Y) phosphorylation-regulated kinase 1A (DYRK1A)[37], and the Regulator of calcineurin (Rcan1)[37]. DYRK1A can antagonize calcineurin signaling by directly phosphorylating NFAT. Indeed, knockdown of DYRK1A induces the transcription of hypertrophic markers such as ANF and BNP, as well as an increase in the cardiomyocyte size. Moreover, further experiments proved that DYRK1A attenuates NFAT-activity in cardiomyocytes. Instead, the silencing of DYRK1A induce Calcineurin/ NFAT signaling stimulation[38]. Another important regulator of the Calcineurin/ NFAT axis is Rcan1 (previously known as MCIP/DSCR/calcipressin)[39]. The role of this protein in the stimulation or suppression of the hypertrophy response was controversial. Experiments with knockout mice for Rcan1 showed that calcineurin activity is decreased in response to cardiac hypertrophy induced by pressure overload.[40] On the other hand, the same experiments with knockout mice for Rcan1 and overexpression of CaN showed enhanced hypertrophy [41]. Therefore, Rcan1 is described as Regulator of calcineurin with bidirectional roles in the regulation of calcineurin-NFAT signaling. The balance between these two natures was speculated to be related to the amount of protein and level phosphorylation. Interestingly, Rcan1 has two promoters and two main isoforms called *Rcan1.1* and *Rcan1.4.* The difference between these isoforms is the presence or absence of the Exon 1. The switch between these two isoforms is caused by an alternative promoter adjacent to exon 4, this region was found to contain a remarkably dense cluster of consensuses NFAT binding motifs[42]. Moreover, *Rcan1.4,* has been found expressed in cardiac hypertrophy, suggesting the importance of this isoform. However, the mechanism that governs the selection between these two promoters is not clear yet. Finally, a recent study clarifies the bidirectional roles of this protein to blunt or enhance the calcineurin-NFAT signaling. Rcan1 is physically able to interact with TAB2, an adaptor protein, that mediates the activation of Transforming growth factor beta-activated kinase 1 (TAK1)[43]. This Complex is responding to TGF-beta signaling and can be modulated by CaN. Experiments with luciferase demonstrate that TAK1 is able to trigger the NFAT pathway and induce hypertrophy in cardiomyocyte through direct phosphorylation on Rcan1. Low TAK1 activity, leads to

dephosphorylated Rcan1 and inhibits calcineurin activity by binding to the regulatory site of calcineurin. At high TAK1 activity (different type of stimuli such TGF-beta or MAPK cascade) levels Rcan1 is predominantly phosphorylated, causing a change in its function towards calcineurin, now able to facilitate the activation of NFAT [44]. Regardless of the knowledge so far acquired about the molecular causes of hypertrophy, several mechanisms are still unclear. Therefore, to find new therapeutics that can be used to treat cardiac hypertrophy and HF, the scientific community has began to explore the transcriptome with greater attention. Up to 98% of the human genome encodes for RNAs[45], and the majority of those do not encode for proteins. Today it has been clarified that cellular processes are regulated not exclusively by proteins, but also by several classes of RNAs called non-coding RNAs. The main classes of non-coding RNAs that have been studied more carefully are microRNAs, circRNAs, which have recently drawn increasing interest, and LncRNAs. MicroRNAs (miRNAs) belong to a class of non-coding transcript with a length of ¬21-23 nucleotides. miRNAs are capable to physically bind messenger-RNA (mRNAs) and interfere with the process of translation by the action of the RISC complex. Over 100 microRNAs have been associated with cardiomyopathies[46], the role of each of them and the respective targets have not been identified. Taking into consideration, that a single microRNA can theoretically have hundreds of targets, the mechanism of action through which microRNA works is not always clear. For simplicity, microRNA in cardiac hypertrophy can be divided into two large classes: firstly, pro-hypertrophic, which is often upregulated during heart failure and cardiac hypertrophy, and secondly anti-hypertrophic, which has a protective role against pressure-overload or other hypertrophic stimuli and is often downregulated in hypertrophy and heart failure. Circular RNAs are a class of non-coding RNAs with a closed continuous loop that joins the 3′ and 5′ ends through back-splicing [47]. Compared with other classes of RNAs, circRNA are relatively unknown, due to the difficulties of identifying them during sequencing and a lack of knowledge about the molecular functions they can perform. CircRNAs are predominantly found in the cytoplasm and are highly stable. Moreover, they are evolutionarily conserved across eukaryotes [48]. As previously described, circRNAs are produced by alternative splicing (back-splicing) of coding genes and LncRNA genes. circRNAs have been demonstrated be involved in several molecular processes such as cardiomyocyte hypertrophy, fibrosis, autophagy, and apoptosis. Finally, Long non-coding RNAs (LncRNAs) belong to a class of non-coding transcripts with a length of over 200 nucleotides, which are often polyadenylated and are devoid of evident open reading frames (ORFs), however some LncRNA retains short open reading frames (sORF) that encode for small peptide or micro peptide[49]. The human

genome is estimated to contain >16,000 LncRNA genes [50]. Most of them are transcribed by RNA polymerase II, and only a small fraction of it has been investigated to date.[51] The study and analysis of this class of molecules are one of the challenges of modern molecular biology. The main reasons why their exploration is so challenging are low expression and poor evolutionary conservation. Additionally, the full extension of all mechanisms of action of this class of RNAs is not fully characterized .[52] Nevertheless, LncRNAs have emerged to be involved in numerous cellular processes, ranging from embryonic stem cell (ESC) pluripotency, cell-cycle regulation to diseases such as cancer or heart diseases. The role of LncRNA is particularly interesting in pathologies where current knowledge of molecular mechanisms is not enough to explain their development, such as in cardiac hypertrophy [53].

Aim of this thesis

The work described in this thesis attempts to increase our knowledge on the contribution of long non-coding RNA (LncRNAs) to the process of cardiac remodeling. **Chapter 2** reviews the most relevant observations in current literature on cardiac hypertrophy mechanisms by a number of individual LncRNAs. Additionally, we describe the hurdles to overcome to reach full therapeutic potential of these RNA molecules. **Chapter 3** reports our findings on a newly discovered LncRNA termed "*Bigheart*". Genomic locus analysis and experimental evidence demonstrated that this LncRNA is a direct target of the pro-hypertrophic calcineurin/NFAT signaling cascade. Furthermore, our studies revealed that *Bigheart* physically interacts with high mobility group box 1 (Hmgb1) and acts in trans-action to stimulate the transcription of Regulator of calcineurin 1 (*Rcan1),* a facilitator of calcineurin-NFAT signaling. Overall, our study reveals tha**t** *Bigheart* can modulate the local chromatin environment and *trans*-activate the target genes Rcan*1,* and therefore drive a positive feedforward loop in hypertrophic signaling. **In Chapter 4,** we further studied this LncRNA by generating a genome-wide chromatin-state map by employing Chromatin isolation by RNA purification (ChIRP), coupled with DNA sequencing (CHIRP-seq for *Bigheart* and chromatin immunoprecipitation (ChIP) coupled with DNA sequencing (ChIP-seq) for Hmgb1. Our results showed that *Bigheart* and Hmgb1 occupied various genomic regions related to cardiac hypertrophy and facilitated the expression of several genes involved in the mTOR pathway. Furthermore, we compared the expression of *Bigheart* throughout the development of the heart using Rna-seq and Chip-seq data of histonic markers of positive (H3K4me3, H3K27Ac) and repressed transcription (H3K27me3), confirming a precise transcriptional regulation of this LncRNA throughout human and mouse cardiogenesis. Additionally, we also highlighted a correlation between the activation status of the mTOR pathway and *Bigheart* expression in hIPSC-CMs and *in vivo.* Taken together, our experiments suggest that Bigheart can *trans*-activate several gene of the mTOR pathway and modulate the activation of this signaling both in cardiac hypertrophy and cardiogenesis. **Chapter 5** offers a general discussion and a summary of the major findings of the experimental work in this thesis. **Chapter 6** addresses the social impact of our findings and the economic value resulting from the identification of new targets in heart disease therapies.

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Chapter 2

Adapted from

Long non-coding RNAs in cardiac hypertrophy

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ABSTRACT

Heart disease represents one of the main challenges in modern medicine with insufficient treatment options. Whole genome sequencing allowed for the discovery of several classes of non-coding RNA (ncRNA) and widened our understanding of disease regulatory circuits. The intrinsic ability of long ncRNAs (LncRNAs) and circular RNAs (circRNAs) to regulate gene expression by a plethora of mechanisms make them candidates for conceptually new treatment options. However, important questions remain to be addressed before we can fully exploit the therapeutic potential of these molecules. Increasing our knowledge of their mechanisms of action and

refining the approaches for modulating LncRNAs expression are just a few of the challenges we face. The accurate identification of novel LncRNAs is hampered by their relatively poor cross-species sequence conservation and their low and context-dependent expression pattern. Nevertheless, progress has been made in their annotation in recent years, while a few experimental studies have confirmed the value of LncRNAs as new mechanisms in the development of cardiac hypertrophy and other cardiovascular diseases. Here, we explore cardiac LncRNA biology and the evidence that this class of molecules has therapeutic benefit to treat cardiac hypertrophy.

INTRODUCTION

Heart failure (HF) is a highly prevalent disease and a leading cause of hospitalization and death that affects 23 million patients worldwide [1]. From a clinical point of view, HF is defined as the state of deterioration of the heart where it can no longer supply sufficient blood to meet the circulatory demands of the organism. Heart transplantations are still the only genuine curative interventions for patients with advanced forms of HF, as contemporary pharmacotherapy is largely palliative and merely aimed to slow the progression of the disease [1]. The disease is typically preceded by structural remodeling of the heart, where heart muscle cells undergo maladaptive growth without an increase in cell numbe,r in response to sustained stress or injury, such as pressure- or volume overload, to temporally sustain cardiac output, resulting in a measurable thickening of heart muscle walls. However, cardiac hypertrophy is also accompanied by a plethora of biochemical, molecular, metabolic and extracellular changes that provoke a decrease of pump function over time, resulting in overt heart failure and a propensity for the occurrence of lethal arrhythmias [2]. Accordingly, a better understanding of the molecular underpinnings of cardiac hypertrophy will help to clarify the maladaptive nature of this disease and may open new therapeutic targets for future treatment of hypertrophic heart diseases, to reduce the number of HF patients.

The Encyclopedia of DNA Elements (ENCODE) and the Functional Annotation Of Mouse (FANTOM) consortiums reveal that there are much more transcripts than originally predicted. Over 80% of the genome is transcribed in various classes of RNA and, surprisingly, coding transcripts account for just up to 3% of the genome, while the vast majority of other transcripts have no coding ability (Figure 1) [3]. For a long time, the presence of a large number of noncoding transcripts was dismissed as evidence for junk DNA or transcriptional noise, but more recent research reveals that a

substantial proportion of these noncoding transcripts are functionally active RNA molecules that can be subdivided into small noncoding RNAs (< 200 nt), such as microRNAs (miRs), transfer RNAs, and small nucleolar RNAs, on the one hand, and longer noncoding RNAs (> 200 nt) that include ribosomal RNAs, natural antisense transcripts and other long noncoding RNAs (LncRNAs) [4; 5; 6]. While our knowledge of LncRNAs is still in its infancy, here we will summarize examples of LncRNAs that are involved in cardiac hypertrophy as it may provide useful insights how LncRNAs are functionally involved in the heart.

CLASSIFICATION SYSTEMS OF LNCRNAS

LncRNAs are often polyadenylated and frequently devoid of evident open reading frames (ORFs). The human genome is estimated to contain 16,000 LncRNA genes [7], mostly transcribed by RNA polymerase II and only a small fraction experimentally investigated [8]. Unfortunately, studying LncRNAs represents a challenge for molecular biology, as they are evolutionarily poorly conserved, show a relatively low expression level that – interestingly - is more restricted in a tissue- and time-specific manner, and mechanisms of action that remain incompletely understood [9].

Unlike other classes of RNA, a universally accepted classification system is lacking for LncRNAs. Using purely the genomic location as input information allows a classification into 5 main categories: sense, antisense, bidirectional, intronic, and intergenic. This classification mainly considers the position and orientation compared to the nearest gene or genomic features (promoters, enhancers) [10]. Similarly, based on genomic position and features, alternative classifications have also been proposed. LncRNAs that are encoded at the promoter region or originate near the transcription start site (uaRNAs) represent up to 60% of all LncRNAs [11], while those encoded from enhancer regions (eRNAs) amount to ~ 20% [12]. The remainder derive from coding genes or gene bodies, that can be subdivided into sense (gsRNAs) or antisense (gaRNAs), and amount to ~ 5% of all LncRNAs [13], while those encoded in intragenic spaces are referred to as iRNAs or lincRNAs [14]. Another widely used system is based on the cellular localization of LncRNAs, where they are subdivided into cytoplasmatic- or nuclear LncRNAs. Unfortunately, this system is not fully reliable either because only 30% of LncRNAs are found exclusively in the nucleus, 15% are found exclusively in the cytoplasm, while the remainder ~50% show both nuclear and cytoplasmic localization [15]. To make matters worse, some LncRNAs can translocate between the nucleus and cytoplasm after stress or stimulation [16].

In terms of mode of action, LncRNAs can interact with other RNAs (e.g. mRNAs, miRNAs, circRNAs, and rRNAs), with proteins (mainly but not exclusively transcriptional factors and chromatin-remodeling complexes) or perform their function by pervasive transcription [17]. One characteristic of LncRNAs is their ability to interact with proteins and is determined by their architecture and 3-dimensional folding. LncRNAs are single-strand RNAs that will try to reach a folded state with the lowest or most stable energy state. The form it takes will be determined from the primary sequence but also from its length, the electrical charges, and interaction with molecular chaperones [18]. Indeed, the folding and 3D shape seems an evolutionary conserved characteristic, since LncRNAs are less evolutionary conserved on the primary sequence, but more often maintain their 3D structure throughout species [19]. Specific RNA motifs have been shown to interact with proteins, allowing LncRNA to sequester or interact with various proteins simultaneously. Finally, by being RNA molecules, LncRNAs can create RNA:RNA duplex which allow them to interact with other RNA classes (e.g. microRNAs) or in other circumstances they can form RNA:DNA duplexes [20], which allow them to interact to specific target sequences on the DNA .

Based on these molecular specifics, an alternative and comprehensive classification divides LncRNAs into 4 classes. The first class is represented as **decoy LncRNAs** that can either be located cytoplasmatic or nuclear and interact physically with transcription factors, to block their interaction with their cofactors or inhibit their function [21]. LncRNA *ROR* is an example of a decoy LncRNA, which activates the TESC promoter by repelling the histone G9A methyltransferase [22]. Additionally, decoy LncRNAs can also sequester microRNAs such as *linc-MD1* that sponges miR-133 and miR-135 to regulate the expression of MAML1 and MEF2C, two transcription factors that activate muscle-specific gene expression [23].

Signal LncRNAs can also be localized either in the cytoplasm or nucleus and regulate gene expression in a time- and space dependent manner in response to cellular stimuli and often are only expressed at specific times during development [17]. Their task is to modulate gene expression of key genes by reshaping the chromatin in a specific locus as, for instance, *KCNQ1OT1* is expressed only during early development where it interacts with chromatin-modifying enzymes to trigger lineage-specific transcriptional profiles [24].

Guide LncRNAs are predominantly nuclear and can recruit chromatin-modifying complexes to specific loci either near the gene encoding the LncRNA (cis-action), or distant target genes from the production site of the LncRNA (trans-action). *HOTAIR* is an example of a guide LncRNA that functions in trans to direct the chromatin modifier Polycomb Repressive Complex 2 (PRC2) to the developmental

HOXD locus [25]. Instead, *HOTTIP* is a guide LncRNA encoded in the HOXA locus that binds WDR5 and the histone methyltransferase protein MLL and directs the WDR5/MLL complex towards activation of the HOXA locus [26].

Finally, **scaffold LncRNAs** can be either in the nucleus or cytoplasm and facilitate the assembling of multiple proteins, which may act on chromatin, affecting histone modifications in the nucleus or facilitate the assembling of ribonucleoproteins. Telomerase RNA *TERRA* is a classic example of an RNA scaffold that assembles the telomerase complex to maintain the ends of telomeres [27].

Even this classification system is not always adequate to unequivocally differentiate classes of LncRNA, as some can simultaneously belong to multiple classes. For example, LncRNA *KCNQ1OT1* functions both as a scaffold and guide LncRNA at the same time. For this reason, some authors have decreased the above classification system to only three: guides, decoy and a combined class called a dynamic scaffold. And yet, even with this adaptation, not all LncRNAs would fall into a specific category. Some LncRNAs for instance can influence proximal genes (in cis) just by the effect of their own transcription in a process called transcriptional interference. Indeed, *Airn* can modulate the Igf2r promoter as long as only a small fraction of *Airn* is transcribed as this LncRNA is antisense to Igf2r and its transcription influences the state of chromatin in the surrounding area with an inability of RNA Polymerase II to interact freely with the promoter of Igf2r [28].

Finally, although it is not often included in LncRNA classification systems, several studies revealed that a proportion of LncRNAs contain short open reading frames (sORFs) that encode for small proteins or micropeptides with largely overlooked but fundamental biological importance [29; 30; 31; 32; 33; 34; 35; 36]. For example, *Myoregulin* (MLN) is a micropeptide encoded by *LINC00948,* an important regulator of skeletal muscle physiology. This micropeptide peptide controls calcium re-uptake in the sarcoplasmic reticulum (SR) by Serca2a inhibition [29]. Recently, van Heesch and colleagues [37] used ribosome profiling to capture ribosomal footprints on human cardiac transcripts and inferred actively translated small open reading frames (sORFs) that encode previously unknown microproteins in over 169 LncRNAs and 40 circular RNAs that are expressed in the heart. Dozens of microproteins are expressed even from previously non-coding roles for LncRNAs, such as *DANCR* (also known as *ANCR* [38]), *TUG1* [39], *JPX* [40], *Myheart* [41], and *UPPERHAND* [42]. This finding suggests that a number of translated LncRNAs that could be separately classified as **sORF LncRNAs**, likely have dual coding and noncoding roles and this duality should be considered when deciphering the function of this class of “non-coding” transcripts.

Next-generation sequencing (NGS) techniques such as deep RNA-seq or CAGE-seq and 3P-seq have greatly aided to provide genome-wide identification of relatively low abundantly expressed transcripts such as LncRNAs [27]. The results of these profiling experiments are now incorporated in databases with collections of hundreds of LncRNAs with possible differential expression in pathological conditions in a variety of species (**Table 1**) [28].

PROHYPERTROPHIC CARDIAC LNCRNAS

H19 is a LncRNA that was first associated with genomic imprinting of the H19/Igf2 locus [43]. Subsequent studied demonstrated that *H19* is upregulated in pathological forms of cardiac hypertrophy and heart failure. Interestingly, *H19* also encodes for the small ncRNA miR-675 that is embedded in the *H19* locus and that can target Ca^{2+}/calmodulin-dependent protein kinase IIδ (CaMKIIδ), a powerful inducer of cardiac hypertrophy [44]. **CTBP1 Antisense RNA 2** (***CTBP1-AS2)*** is a novel LncRNA that can trigger the hypertrophic response as it is upregulated in hearts that undergo hypertrophy after transverse aortic constriction (TAC) surgery in mice, while, conversely, silencing of *CTBP1-AS2* attenuates hypertrophy in AngII agonist stimulation of cardiomyocytes in culture. Mechanistically, this LncRNA can stabilize the mRNA encoding Toll-like receptor 4 by recruiting the RNA-binding protein FUS/TLS, thereby triggering cardiac inflammation, a commonly observed phenomenon associated with cardiac hypertrophy [45]. **Cardiac Hypertrophy-Related Factor (*CHRF*)** is upregulated in both hypertrophic mouse hearts and human biopsies of patients with heart failure and functions as a sponge for miR-489, regulating Myeloid differentiation primary response gene 88 (Myd88) as downstream target of miR-489. Myd88, a signal transduction adaptor involved in immune modulation, was demonstrated to suppress cardiomyocyte hypertrophy. Other studies have shown that mir-489 is not the only target of *CHRF* [46], as miR-93 can also bind CHRF and influence AKT3 activation status [47]. LncRNA ***ROR*** acts as a decoy LncRNA that can trap miR-133, thereby influencing RhoA and Cdc42 downstream of Gαq/Gα11 signaling [48]. **Cardiac Hypertrophy-Associated Transcript** (***CHAST***) is a signal LncRNA that show temporal regulation of expression in cardiac hypertrophy in mice and silencing of *Chast* with a GapmeR *in vivo* attenuated the hypertrophic response. Conversely, *Chast* overexpression triggers cardiomyocyte hypertrophy. Further studies revealed that *Chast* negatively regulates Plekhm1, a multivalent endocytic adaptor involved in controlling selective and nonselective autophagy pathways resulting in adverse cardiac remodeling [49].

ANTI-HYPERTROPHIC CARDIAC LNCRNAS

Myosin Heavy Chain Associated RNA Transcripts (***MHRT****)* was first identified as the antisense transcript of Myosin heavy chain 7 (Myh7). *MHRT* is highly expressed in the mouse and human adult heart and *MHRT* lentiviral overexpression reduced hypertrophic stress markers. RNA immunoprecipitation (RIP) revealed that *MHRT* interacts with Brahma-related gene 1 (Brg1) to remodel chromatin and regulates gene expression such as *Myh6* [41] and can influence the acetylation of the Myocardin protein in an HDAC5-dependent fashion [50; 51]. LncRNA ***TINCR*** is downregulated in hypertrophic hearts and lentiviral TINCR overexpression attenuates cardiac hypertrophy by interacting with EZH2, a functional enzymatic component of the Polycomb Repressive Complex 2 (PRC2). Chromatin-immunoprecipitation (ChIP) pull-down assays against EZH2 demonstrated that the *TINCR*-EZH2 complex binds directly to the CaMKII promoter and induces H3K27me3 histone modification [52]. ***Lnc-Plscr4*** is a LncRNA that is significantly increased in hearts from mice that underwent pressure overload-induced cardiac hypertrophy, where it reduces the expression and activity of miR-214, a prohypertrophic microRNA [53; 54; 55]. The responsible downstream target of miR-214 is Mitofusin 2 (Mfn2), a protein that regulates mitochondrial fusion [56]. Accordingly, reduced expression of Mfn2 attenuates protein synthesis in cardiac hypertrophy [57]. ***HOTAIR*** is a LncRNA that has been extensively studied in embryonic development and cancer, but recently it was observed that it is substantially decreased in cardiac hypertrophy. Overexpression of *HOTAIR* suppresses angiotensin II-stimulated cardiomyocyte hypertrophy and following pressure overload surgery in mice. Mechanistically, *HOTAIR* was proposed to act as a ceRNA for miR-19, where miR-19 regulates Phosphatase and tensin homolog (PTEN) expression and indirectly regulates hypertrophy by influencing the activation status of PI3K/phospho-Akt signaling cascade in cardiomyocytes [58; 59].

CircRNAs

Circular RNAs are a class of non-coding RNAs with a continuous closed loop where the 3′ and 5′ ends of exons of protein-coding genes or LncRNA genes are joined through back-splicing [60]. CircRNAs are predominantly found in the cytoplasm and are highly stable. Moreover, they are evolutionarily conserved across the eukaryotic [61]. Based on their origin, circRNAs can be divided into 4 main classes: exonic circRNAs (ecircRNA), produced with the removal of each intron and generated from the back-spliced exons, while in exon-intron circRNAs (EIciRNA) the black-splicing takes place by including the intron inside the mature circRNA. The circularization of

only introns produces circular intron RNAs (ciRNAs), while intergenic circRNAs are produced when two fragments from different genomic regions called intergenic circRNA fragments or ICFs are merged together and circularized [62]. CircRNAs can interact with proteins, or they can serve as decoys for microRNAs. For example, ***Circ-Foxo3*** inhibits proliferation by direct interaction with with p21 and CDK2 and subsequent interference of the cell cycle [63]. ***CircACTA2*** instead acts as a microRNAs sponge in vascular smooth muscle cell where it interacts with miR-548f-5p, which in turn modulates the expression of smooth muscle α-actin (α-SMA) [64].

Similarly, ***circRNA_000203*** can modulate the expression in Gata4 by binding microRNA miR-26b-5p and miR-140-3p.[65] Occasionally, circRNAs can encode for micropeptides by sORFs, an example is ***Circ-ZNF609*** that controls myoblast proliferation the production of a new small peptide generated by the back-splicing event [66]. **Heart-related circRNA** (***HRCR***) is a circRNAs that regulates cardiac homeostasis by modulating the expression of miR-223 in *vivo*, a well-known microRNA that induces cardiac hypertrophy [67]. Conversely, ***CircSlc8a1*** is highly expressed in heart failure, and can bind miR-133 to induce cardiac hypertrophy [67; 68]. On the other hand, cirRNAs can act as early biomarkers of cardiac hypertrophy, ***circTMEM56*** and ***circDNAJC6*** could serve as indicators of disease severity in patients with hypertrophic obstructive cardiomyopathy [69]

EXPERIMENTAL AND THERAPEUTIC CONSIDERATIONS

One widely employed strategy to silence endogenous LncRNAs involves the introduction of double or single stranded antisense oligonucleotides in the form of siRNAs, or antisense locked nucleic acid (LNA) containing oligos (GapmeRs), respectively. For short hairpins RNA or siRNAs, it is observed that those molecules seem more suitable for cytoplasmic LncRNAs rather than nuclear LncRNAs [70], although this might depend on various other parameters in siRNA design as well. GapmeRs are very potent antisense oligonucleotides with two LNA-modified linkers at 3’ and 5’ end of the molecule to resist exo-and endonucleases once introduced in the cell or in the extracellular space. Once bound with the LncRNA by full base pair complementarity, the dsRNA complex is degraded in an RNAse H dependent manner [71]. These molecules are highly efficient in silencing both cytoplasmatic and nuclear LncRNAs (**Figure 2**).

To achieve the opposite, i.e. supplementing or overexpressing a LncRNA, viral-mediated gene delivery remains the most reliable system (**Figure 2**). Viral vectors enhance cardiac delivery of polyanions, such as RNA, across cell membranes. One frequently used viral vector is the non-pathogenic human parvovirus adeno-associated virus (AAV) because of its genomic simplicity, possibility of generating high-titre vector preparations, and capacity to deliver genes into postmitotic cells. The specific tropism of AAV serotypes drives their selectively for different tissues in *vivo*, where AAV9 stands as the most cardiotropic serotype in gene transfer studies in rodents. A limitation of AAV vectors is their packaging limit (~4.5 kb), which may be exceeded by certain LncRNAs. Another viral vector is based on human adenovirus (HAdV). The best studied member of the HAdV species is serotype 5 (HAdV-5). HAdV-5 infects many cell types, including low-replicative or quiescent cell populations such as cardiomyocytes. The HAdV-5 genome is easy to engineer with large foreign DNA cloning capacity and can be produced on an industrial scale. All these attributes make HAdV-5 vectors the most preferred vector type used to date in vaccine, cancer, and gene therapy trials [72].

Other systems of delivery are engineered nanoparticles and extracellular vesicles such as exosomes (**Figure 2**). Nanoparticles have demonstrated their potential in the oncological field as delivery systems for microRNAs, very efficiently and in general with relatively low toxicity. Furthermore, effective cardiac-specific nanoparticle delivery systems for *in vivo* use remain to be developed. In contrast, exosomes are a specialized subgroup of extracellular vesicles ranging from 50-150 nm and released from cells to the extracellular microenvironment where they can be found in various extracellular fluids including the systemic circulation. Naturally occurring exosomes carry a variable composition of proteins, lipids, RNA (mRNA, LncRNA, microRNA, and circRNA), and DNA, where they influence cell migration, angiogenesis, the immune response and tumor cell growth [73]. Engineered extracellular vesicles could be used as engineered vehicles to temporally deliver LncRNAs. Proof of principle exist in cancer research, where exosomes are used tom carry different LncRNAs to influence tumor development. *MALAT-1*, *BCAR4*, and *LncRNA-p21* are LncRNAs found in exosomes that travelled in the bloodstream derived from different types of cancers. Previous work has demonstrated the efficacy of exosomes as delivery vehicle for microRNAs in cardiac pathologies. Exosomes from cardiac progenitor cells enriched with miR-451/144 promote cardiomyocyte survival in a myocardial ischemia/reperfusion model. To tackle the problem of cellular tropism, recently, exosomes derived from cardiosphere-derived cells (CDCs) engineered to express Lamp2b, an exosomal membrane protein, fused to a cardiomyocyte-specific

peptide (CMP), have been used and resulted in an increased efficiency of exosomal uptake by cardiomyocytes [74].

CONCLUSIONS

The development of whole genome sequencing technologies allowed for the discovery of several classes of ncRNA and has strongly influenced our understanding of disease-regulatory circuits. LncRNAs represent one of the classes of ncRNA which has aroused more interest in the last decade. Several LncRNAs and circRNAs have been linked to cardiovascular physiology, providing new targets for the treatment of cardiovascular diseases. Additionally, the intrinsic ability of LncRNAs and circRNAs to regulate gene expression through various mechanisms makes them one of the best candidates for the development of more accurate and efficient therapies. However, important questions remain to be addressed before we can fully exploit the therapeutic potential of these molecules. Increasing our knowledge of their mechanisms of action and refining the approaches for modulating LncRNAs expression in *vivo* are two of the main challenges we still must face. Another aspect, that should be addressed, is the identification of novel LncRNAs involved in cardiovascular disease. Although the action of these RNAs is remarkably strong in the regulation of several molecular aspects, the low and time-related expression combined with a poor sequence conservation trough species makes the identification of novel LncRNAs difficult. Nevertheless, significant progress has been made in recent years, confirming the value of ncRNA as a new tool for the treatment of numerous diseases, including cardiac hypertrophy.

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CONFLICT OF INTEREST

L.D.W. is co-founder and stockholder of Mirabilis Therapeutics BV.

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FIGURE LEGENDS

Figure 1 | **Classification systems for LncRNAs (a)** LncRNA classification using purely their genomic location as input information allows a classification into 6 main categories: sense, antisense, bidirectional, intronic, intergenic and circRNAs. This classification mainly considers the position and orientation compared to the nearest gene or genomic features (promoters, enhancers). **(b)** An alternative classification system takes LncRNA mode of action into account, where LncRNAs have the ability to interact with DNA, other RNAs, with proteins or perform their function by pervasive transcription. Based on structural motifs within LncRNAs, an alternative classification divides LncRNAs into 5 classes, including short open reading frame containing **sORF LncRNAs** that often encode functional, small peptide sequences; **decoy LncRNAs** that can either be located cytoplasmatic or nuclear and interact physically with RNAs or transcription factors to block their interaction with their cofactors or inhibit their function; **signal LncRNAs** that regulate gene expression in a time- and space dependent manner in response to cellular stimuli; **guide LncRNAs** that are predominantly nuclear and recruit chromatin-modifying complexes to specific loci either near the gene encoding the LncRNA; **scaffold LncRNAs** that can be either in the nucleus or cytoplasm and facilitate the assembling of multiple proteins, affecting histone modifications in the nucleus or that facilitate the assembling of ribonucleoproteins.

Figure 2 | **Conceptual approaches to manipulate LncRNAs expression for therapeutic applications. (a)** Gapmer oligonucleotides have two LNA-modified linkers at the 3’ and 5’ end of the molecule to resist exo-and endonucleases once introduced into the cell or inside the extracellular space. Once bound to the LncRNA by full base pair complementarity, the dsRNA complex is degraded in an RNAse H dependent manner. **(b)** Adeno-associated viruses (AAV) have a packaging limit of 4.5 kb and can be engineered to transcribe LncRNAs or circRNAs. Exosomes are a specialized subgroup of extracellular vesicles ranging from 50-150 nm capable of carrying a variable composition of RNAs (LncRNA, microRNA, and circRNA),

Table 1: List of Open-Source databases with information on LncRNAs

Database	Species	# LncRNAs reported	Reference
LNCipedia	Human	118.777	[75]
NONCODE	16 species including human	487.164	[76]
LncRNADisease v2	4 species including human	19.166	[77]
ChIPBase v2	10 species including human	10.200	[78]
deepBase	14 species including human	191.547	[79]
GENCODE	human, mouse	42.302	[80]
LincSNP	Human	244.545	[81]
LncRNAdb v2.0	Human	295	[82]
FANTOM5	Human	19.175	[83]
HDncRNA	Human, mouse, rat	2.304	[84]

Table 2: LncRNAs with pro-hypertrophic functions

Name	Target	Function	Cell type	Reference
H19	miR-675	Regulation of CaMKIIδ	CM	[44]
MEG3	miR-361-5p	Regulation of STAT3	CM/CF	[85; 86]
Lnc-ROR	mir-133	Regulation of RhoA and Cdc42	CM	[48]
CHAST	Plekhm1	Regulation of Autophagy	CM	[49]
CAS15	miR-432-5p	Regulation of TLR4	CM	[87]
MIAT	miR-150	Regulation of CaMKIIδ	CF	(85)
CTBP1-AS2	FUS/TLS	Stabilize mRNA of TLR4	CM	[45]
SYNE1-AS1	miR-525-5p	Regulation of SP1	CM	[88; 89]
Chear	PCR2	Chromatin remodeling	CM/VM	[90]

CM, Cardiomyocytes; CF, cardiac fibroblast; VM, ventricular myocytes

Table 3: LncRNAs with anti-hypertrophic functions

Name	Target	Function	Cell type	Reference
Mhrt	Bgr1	Chromatin remodeling	CM	[41]
MAGI1-IT1	miR-302e	Regulation of HDAC9	CM	[59]
Plscr4	miR-214	Regulator of Mfn2	CM	[53]
HOTAIR	miR-19	Regulation of PTEN	CM/CF	[58]
XIST	miR-101/miR-330-3p/miR-130	Regulator of S100B	CM	[91]
TINCR	EZH2	Chromatin remodeling	CM	[52]
CYTOR	miR-155	Regulator of IKBKE	CM	[92]

CM, Cardiomyocytes; CF, cardiac fibroblast

Table 4: CircRNA involved in cardiac hypertrophy

Name	Target	Function	Cell type	Reference
Circ-Foxo3	p21 and Cdk2	Inhibit proliferation	CM/CF	[63]
Circ-ACTA2	miR-548f-5p	Regulation of α-SMA	VSMC	[64]
Circ-ZNF609		Produce a micropeptide	CM	[66]
HRCR	miR-223	Regulation of ARC	CM	[67]
CircSlc8a1	miR-133	Regulation of SRF	CM	[93]
CircRNA_000203	miR-26b-5p and miR-140-3p	Regulation of Gata4	CM	[65]
Circ-wwp1	miR-23a	Unknown	CM	[94]
CircTmem56	Unknown	biomarker	Plasma	[69]
CircDNAJc6	Unknown	biomarker	Plasma	[69]

CM, Cardiomyocytes; CF, cardiac fibroblast; VSMC, vascular smooth muscle cell

Figure 1

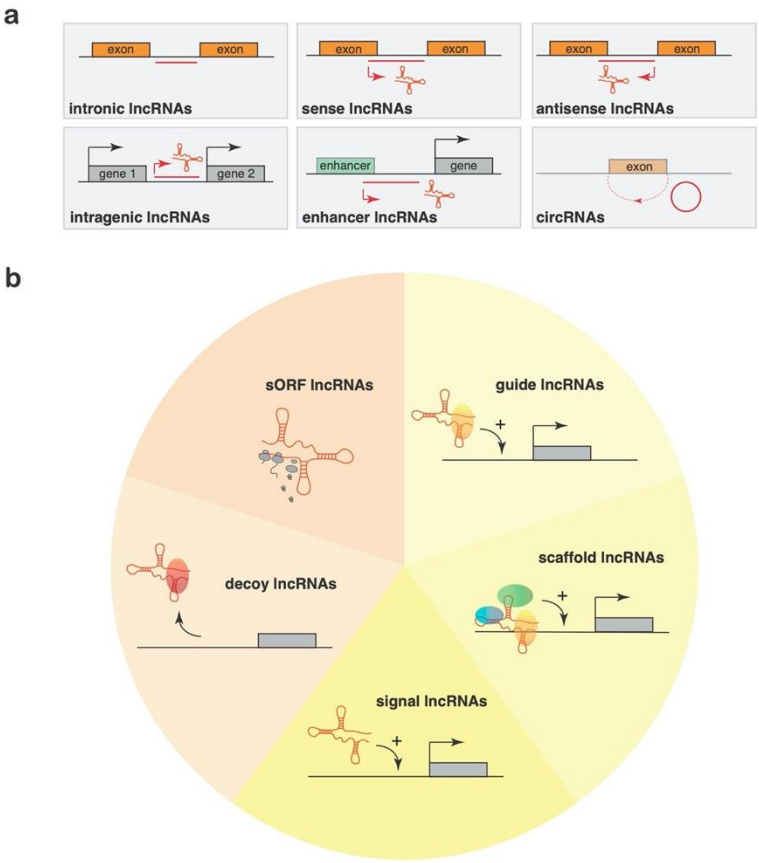
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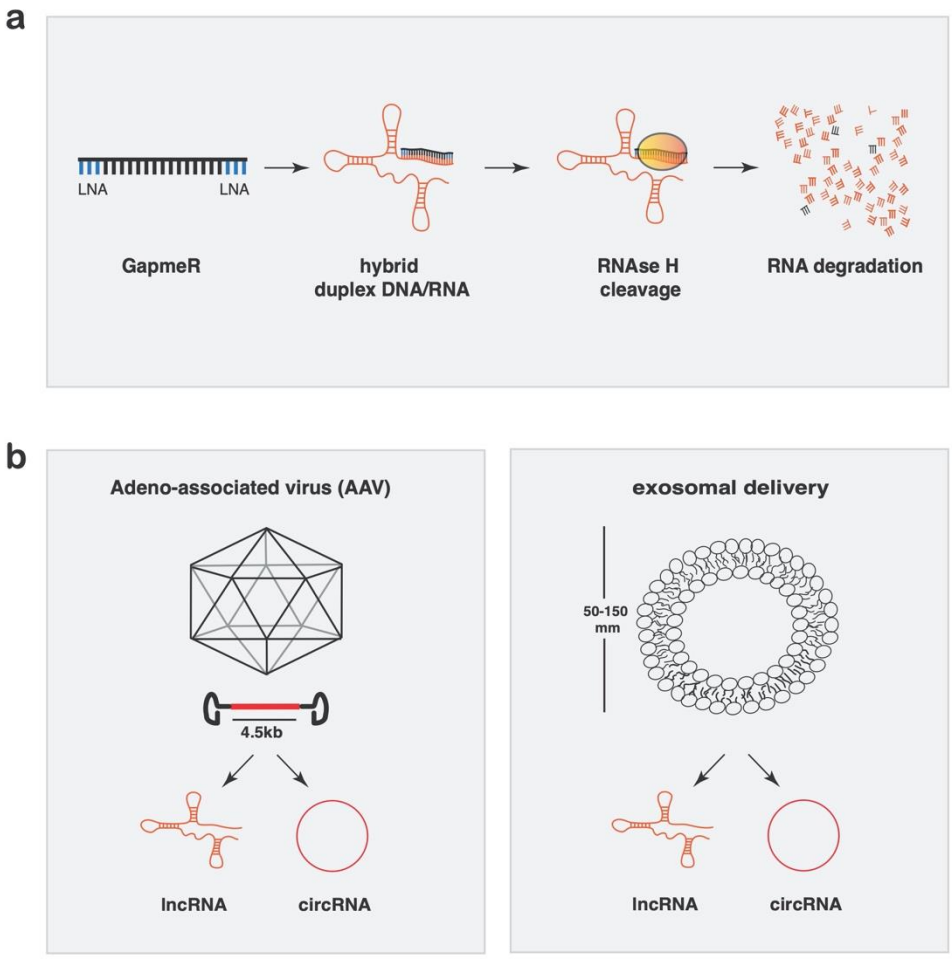
Figure 2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Chapter 3

Adapted from

LncRNA Bigheart trans-activates gene expression in a feed forward mechanism that facilitates calcineurin-NFAT signaling in myocardial hypertrophy

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ABSTRACT

Terminally differentiated cardiomyocytes exhibit hypertrophy as a default response to injury by translating biomechanical stress into a complex network of intracellular signaling events. The molecular intricacies how calcium-dependent signaling engage molecular circuits and epigenetic modifications to activate deleterious gene programs remain enigmatic. Here we report on the re-activation of the evolutionarily conserved lncRNA "*Bigheart*", which is repressed in the postnatal myocardium and quickly re-activated in a calcineurin-NFAT-dependent fashion in the diseased myocardium in man and mouse. In line, AAV9-mediated overexpression of lncRNA *Bigheart* in otherwise healthy primary cardiomyocytes or mouse hearts suffices to drive maladapative hypertrophy. Conversely, mice receiving a "Gapmer" antisense oligonucleotide designed to specifically silence endogenous lncRNA *Bigheart* display resistance to biomechanical stress-induced myocardial remodeling, indicating its requirement in left ventricular hypertrophy. Mechanistically, lncRNA *Bigheart* recruits the RNA binding proteins hnRNP-F1 and HMGB1 to modulate the local chromatin environment and *trans*-activate *Bigheart* target genes including *Rcan1* to stimulate calcineurin-NFAT coupling. Our observations confirm that human heart failure arises from specific susceptibilities in gene regulatory circuits that are amenable for therapeutic intervention using RNA-based therapeutics.

INTRODUCTION

Calcineurin (protein phosphatase 2B), the calcium-calmodulin-activated, Ser/Thr protein phosphatase that dephosphorylates NFAT transcription factors,[1,2] is essential for a wide range of biological activities, including T lymphocyte reactivity, neurological and vascular system development, bone growth, skeletal muscle fiber type switching, heart valve development, and pathological cardiac hypertrophy.[1-4] Cardiac hypertrophy entails the maladaptive growth of heart muscle cells without an increase in cell number that occurs in response to sustained stress or injury such as pressure- or volume overload to temporally sustain cardiac output, resulting in a measurable thickening of heart muscle walls.[5] However, cardiac hypertrophy is also accompanied by a plethora of biochemical, molecular, metabolic and extracellular changes that provoke a decrease of pump function over time rather than preserving it, predisposing the individual patient to lethal arrhythmias and overt heart failure, a serious clinical disorder that represents a primary cause of morbidity and hospitalization.[6-8]

Many signaling cascades that are necessary for myocardial hypertrophy engage molecular circuits that also control growth and gene expression in the embryonic heart, leading to the re-activation of so-called “fetal” gene programs in disease.[7] Long ncRNAs perform a variety of regulatory roles and can recruit chromatin-modifying complexes to specific loci either near the gene encoding the lncRNA (*cis*-action), or distant target genes from the production site of the lncRNA (*trans*-action). [9-11] Elucidating the mechanisms how stress-responsive lncRNAs act downstream of signaling events and convert this information to epigenetic gene regulation in terminally differentiated heart muscle may unlock new convergence points for therapeutic intervention in heart failure.

RESULTS

Bigheart is an NFAT-responsive lncRNA in hypertrophic heart disease

To profile the mammalian heart for differentially expressed lncRNAs in a genome-wide fashion with lncRNA microarrays, we utilized two established mouse models with early onset cardiac hypertrophy and heart failure, one subjected to surgically induced pressure overload by transverse aortic constriction (TAC)[12,13] and the other the calcineurin transgenic model (*Myh6*-CnA).[4] Each model displayed substantial cardiac enlargement, fibrosis, myocyte hypertrophy and strong dysregulation of the fetal stress genes *Nppa*, *Acta1* and *Myh7* (**Fig.1a,b; Supplementary Fig.1a**). Volcano plots visualized differentially expressed lncRNAs with high statistical significance versus a robust magnitude of change in expression, yielding the surprisingly high numbers of 6,107 and 7,025 differentially expressed lncRNAs in TAC or *Myh6*-CnA hearts, respectively, compared to their wild-type controls (**Fig.1c**). A large proportion of lncRNAs (>750) were commonly down- or upregulated between both models (**Fig.1d; Supplementary Fig.1b,c**), providing more support that these lncRNAs constitute a common response of the mammalian heart to pathological growth independent of the experimental model employed.

We uncovered a robustly dysregulated transcript in both models annotated as 4833412C05Rik-201 or ENSMUST00000181235.3 in *Mus musculus* genome assembly GRCm39, encoded on murine chromosome 7qC, and also known as “*Bigheart”* (**Fig.1e; Supplementary Fig.1b,c**). The human orthologue of the *Bigheart* lncRNA gene is encoded on human chromosome 15q11.2 and annotated as SYNM antisense RNA 1 or ENSG00000259475 in human genome assembly GRCh38. Interestingly, mouse and human lncRNA *Bigheart* show evolutionary synteny and in each case are embedded in an intragenic region between *Insulin-like growth factor 1 receptor* (*Igfr1*) and *Synemin* (*Synm*) (**Fig.1e-j**).

The conservation level on the primary structure between human lncRNA BIGHEART and murine Bigheart was relatively low (> 43%, Clustal2.1). Next, we used Minimum Free Energy (MFE) of the human and murine lncRNAs to establish secondary structure prediction using RNAfold, which revealed improved evolutionary conserved folding (**Fig.1f,i**). Although computational prediction of secondary structures is widely used, the accuracy is suboptimal and decreases for longer sequences.[14] After successfully establishing SHAPE (selective 2-hydroxyl acylation by primer extension)-seq[15] for the lncRNA H19 with the wildtype and the minor allele of SNP rs217727,[16] we performed SHAPE-seq for the 588 nucleotides long sequence of human lncRNA BIGHEART, and obtained 3.6 to 4.4 million paired-end reads with an average coverage of 236,703 , which were then incorporated during structure prediction to validate the length and secondary structure of human BIGHEART (**Supplementary Fig.1f,g**).

In humans, the BIGHEART transcript is primarily expressed in heart and brain, while in mice the expression pattern for the major isoform is more broadly present in various tissues (**Fig.1g,j**). In the heart, lncRNA Bigheart is more enriched in heart muscle cells rather than non-myocytes (**Fig.1k**). In human induced pluripotent stem cells (hiPSC), human BIGHEART is more abundantly expressed in the earlier stages of differentiation towards hiPSC-derived cardiomyocytes (hiPSC-CMs) compared to later stages of hiPSC-CM maturation (**Fig.1l**). In contrast and in line with the lncRNA profiling studies, Bigheart is re-expressed upon conditions of pathological hypertrophy (**Fig.1m; Supplementary Fig.1e**), reminiscent of “fetal” stress markers that are quickly re-activated in cardiac hypertrophy. Taken together, these data demonstrate that the evolutionary conserved and cardiomyocyte-enriched lncRNA *Bigheart* is higher expressed in early stages of cardiac development compared to the healthy adult myocardium and becomes strongly re-activated in response to pro-hypertrophic stimuli in the adult heart.

LncRNA *Bigheart* is sufficient and required to induce cardiomyocyte hypertrophy

To evaluate the biological ramifications of the induction of the *Bigheart* lncRNA on the cardiomyocyte phenotype, we first considered whether the gene encoding for *Bigheart* was a direct target of the calcineurin/NFAT pathway *in vivo*, since hearts from mice with transgenic activation of calcineurin signaling displayed very strong induction of the lncRNA transcript (**Fig.1m**). A 2 kb region upstream of *Bigheart* was scanned for evolutionary conserved cis elements representing potential

NFAT-binding sites (**Supplementary Fig.2a**). Luciferase activity experiments with a *Bigheart* promoter construct harboring the NFAT consensus binding sites revealed the existence of an evolutionary conserved and functional NFAT site centered around -0.7 kb upstream of the *Bigheart* gene (**Fig.2a,b**),[17] providing a mechanistic basis for the observed calcineurin/NFAT responsiveness of *Bigheart* in the postnatal myocardium.

Next, we made use of the high cardiac tropism and prolonged expression of serotype 9 adeno-associated viral (AAV9) vectors[18] to evoke overexpression of the lncRNA. AAV9 vectors expressing either human lncRNA *BIGHEART*, or a control vector expressing luciferase were used to infect cultures of primary neonatal rat cardiomyocytes (**Fig.2c,d**). We performed a fluorescence-microscopy-based cell surface analysis in cardiomyocytes infected with the AAV9 vectors or stimulated with the pro-hypertrophic agonists phenylephrine and isoproterenol. At 48 h, cells were stained for sarcomeric α-actinin to distinguish cardiomyocytes from non-myocytes and measured individual cell surface areas. The data show that lncRNA Bigheart overexpression suffices to evoke a cardiomyocyte hypertrophy response, albeit slightly less efficient than agonist-stimulated cardiomyocytes as measured by cell size analysis and induction of the fetal gene markers Nppb and Myh7 (**Fig.2e-g**).

Conversely, we assessed the requirement of lncRNA Bigheart expression for agonist-stimulated cardiomyocytes to induce a full hypertrophic response. To this end, we first tested different siRNAs to provoke targeted knockdown of endogenous lncRNA Bigheart by RNAi *in vitro* (**Fig.2h,i**). Next, cardiomyocytes were pretreated with scrambled siRNA or siRNA against Bigheart. Treatment with the prohypertrophic a1-adrenergic agonist phenylephrine (PE) after scrambled siRNA transfection resulted in a robust hypertrophic response, as shown by a significant increase in cell size (**Fig. 2j,k**). RNAi to lncRNA *Bigheart* abrogated the classical hypertrophic phenotype induced by PE treatment (**Fig. 2j,k**), suggesting that *Bigheart* is required to provoke a full hypertrophic response of cardiomyocytes *in vitro*. Taken together, these data indicate that lncRNA *Bigheart* is a direct calcineurin/NFAT target gene that is both sufficient and required for cardiomyocytes to mount a full hypertrophic response.

LncRNA *Bigheart* reactivation is required for cardiac remodeling *in vivo*

To evaluate whether a gain-of-function approach of *Bigheart* would also enhance cardiomyocyte hypertrophy *in vivo*, we injected AAV9-*BIGHEART* intraperitoneally in neonatal mice at p1 to elevate the expression and analyzed the

hearts at p12 (**Fig.3a,b**). The hearts of p12 mice injected with AAV9-*BIGHEART* were significantly enlarged compared to those from mice injected with the control AAV9, increased cardiac fibrosis content and cardiomyocyte size (**Fig.3c**). Postmortem quantification further indicated that heart weight corrected for body weight and the "fetal" stress markers *Nppa*, *Nppb* and *Myh7* as hallmarks of cardiac remodeling were significantly increased in hearts of animals injected with *BIGHEART* overexpression compared to hearts of animals injected with the control AAV9-luciferase (**Fig.3d-g**).

Finally, to assess the overall requirement of this lncRNA in experimental heart failure in the mouse, we silenced endogenous *Bigheart in vivo* with a specific Gapmer antisense oligonucleotide. We optimized dosing to weekly Gapmer administrations at a concentration of 25 mg/kg in line with previous reports to avoid toxicity effects while inducing specific and efficient loss of *Bigheart* transcripts in the heart as confirmed by real-time PCR on heart tissue (**Fig.3h,i**).[19] We then tested the requirement of *Bigheart* in the development of cardiac disease in transverse aortic constriction (TAC) pressure overloaded hearts. To this end, wild-type mice were treated weekly with either a Gapmer antisense oligonucleotide to silence endogenous *Bigheart* or vehicle and 2 days later randomized to be subjected to TAC pressure overload for four weeks (**Fig.3h**). Cardiac size, myocyte disarray, interstitial and replacement fibrosis and cardiomyocyte size were significantly increased four weeks after TAC in vehicle-treated, control mice, but *Bigheart* silencing attenuated all these parameters of histopathological remodeling (**Fig.3j**). Postmortem quantification further indicated that *Bigheart* silencing reduced left ventricular mass, myocyte cell size and fibrosis (**Fig.3k,l**) and attenuated re-expression of "fetal" cardiac genes *Nppa*, *Nppb*, *Acta1* and *Myh7* (**Fig.2m**). Taken together, these data demonstrate that lncRNA *Bigheart* is both sufficient and required for the adult heart to mount the maladaptive cardiac remodeling process following biomechanical stress.

LncRNA *Bigheart* recruits RNA binding proteins to *trans*-activate gene expression

To understand how lncRNA *Bigheart* can evoke cardiac hypertrophy, we first performed a meta-analysis of genomic variants in the human lncRNA locus. Cardiovascular diseases display a high proportion of genome-wide association studies (GWAS) associations in non-coding genome regions. For instance, transcript levels of the lncRNA *ANRIL* on chromosome 9p21 are directly correlated with the severity of atherosclerosis,[20] while the lncRNA *SRA1* at chromosome 6p21 is significantly associated in dilated cardiomyopathy (DCM).[21,22] DCM GWAS p-values

were replotted for the region that surrounds human *BIGHEART* (RP11-654A16.3), but no significant association with human DCM was observed (**Supplementary Fig.4a**). A proportion of lncRNAs contain short open reading frames (sORFs) that encode for small proteins or micropeptides with largely overlooked but fundamental biological importance. We analyzed the murine and human *Bigheart* sequences with ORFfinder to verify that *Bigheart* does not code for evolutionary conserved micropeptides (data not shown). Based upon recent classification systems, guide lncRNAs show a predominant nuclear localization and are able to recruit chromatin-modifying complexes to specific loci either near the lncRNA (*cis*-action), or distant target genes from the production site of the lncRNA (*trans*-action). We observed no induction of expression of the adjacent genes *Igfr1* and *Symn* in pressure overloaded hearts where *Bigheart* expression is strongly induced, excluding the possibility that *Bigheart* displays *cis*-action (**Supplementary Fig.4b**).

Next, to explore a possible *trans*-action for *Bigheart*, we silenced endogenous *Bigheart in vivo* with a specific Gapmer antisense oligonucleotide,[19] induced pressure overload in mouse hearts and performed RNA-seq, which revealed a defined number of differentially expressed transcripts that were sensitive to the expression of lncRNA *Bigheart* (**Fig.4c**, **Supplementary Fig.c,d**). Among the strongest induced, *Bigheart*-stimulated genes were the basic helix-loop-helix (bHLH) family member *Clock*, the z-disk located titin-assembling gene *Tcap* and the regulator of calcineurin signaling *Rcan1*. *Rcan1*, a member of a larger family that includes *Rcan2* and *Rcan3*,[23,24] acts in yeast and higher organisms as an enhancer of calcineurin-NFAT signaling by virtue of its phosphorylation status and interaction with calcineurin.[25] Given the observation that lncRNA *Bigheart* itself is also an NFAT-responsive target gene, we pursued the surprising contention that lncRNA *Bigheart* may constitute a new auto-amplification loop that stimulates calcineurin-dependent, pathological cardiac growth.

First, we could verify that both Rcan1 isoforms increase in mouse hearts following *Bigheart* overexpression with the AAV9 vector (**Fig.4d,e**). Since *Bigheart* works in *trans* to regulate distantly located genes such as *Rcan1*, we set out to verify the exact locations of its binding sites at higher resolution using chromatin immunoprecipitation by RNA immunoprecipitation (ChIRP)[26] to allow high-throughput discovery of *Bigheart*-bound DNA and proteins (**Fig.4f**). In brief, mouse cardiomyocytes were transfected with biotinylated complementary oligonucleotides that tile *Bigheart*, isolated using magnetic streptavidin beads and copurified chromatin was eluted for RNA, gDNA and protein analysis. First, we verified that with both the odd- and even set of tiling oligonucleotides, we could retrieve >80% of endogenous *Bigheart* transcripts in a specific manner without contaminating

unrelated RNA species (**Fig.4g**). Next, we examined whether lncRNA-associated chromatin and proteins could be copurified. *Bigheart* ChIRP “even” and “odd” probes specifically retrieved both promoter regions of the *Rcan1* locus but not the *Gapdh* locus, demonstrating the specificity of the *Bigheart* ChIRP approach and validating *Rcan1* as a *Bigheart*-responsive target gene (**Fig.4h**). We verified by running samples on silver-stained gels and Western blotting that *Bigheart*-ChIRP efficiently purified specific proteins from mouse cardiomyocytes (**Supplementary Fig.4e,f**). Using *Bigheart* ChIRP, we interrogated by an unbiased proteomics screen what proteins are associated to *Bigheart* when bound to chromatin (**Fig.4j**). Accordingly, we detected 128 proteins in total, many of which representing various members of heterogeneous nuclear ribonucleoproteins (hnRNPs) that harbor RNA binding motifs, are predominantly expressed in the nucleus and aid in the regulation of gene expression.[27] *Bigheart*-ChIRP also pulled down High-mobility group box 1 (Hmgb1), a non-histone DNA-binding nuclear protein and architectural chromatin-binding factor that bends the DNA and supports transcription by loosening nucleosome structure and interacting with transcription factors.[28] Coimmunoprecipitation assays revealed that there was a direct interaction between hnRNP-F1 and Hmgb1 with *Bigheart* in mouse cardiomyocytes, but not between *Bigheart* and Gapdh, hnRNP-k, hnRNP-A2/B1 or hnRNP-C1/C2 (**Fig.4k, Supplementary Fig.4g**). Finally, to simultaneously assess lncRNA Bigheart occupancy and profile chromatin status in a genome-wide fashion, we performed a combined ChIRP-seq on endogenous Bigheart and ChIP-seq for H3K27me3, H3K4me3, H3K27Ac, RNA Pol2, and Hmgb1 in mouse cardiomyocytes and analyzed the murine *Rcan1* locus. Distribution analysis of H3K27Ac around the *Bigheart* peaks revealed Hmgb1 presence, chromatin activation and transcriptional activity at the two *Bigheart*-bound promoter regions of *Rcan1* in mouse cardiomyocytes (**Fig.4m, Supplementary Fig.4n**).

Conclusively, lncRNA *Bigheart*, which is expressed at lower levels in the myocardium during late gestation and under healthy conditions postnatally, becomes quickly re-activated upon biomechanical stress as a direct calcineurin/NFAT target gene, where it acts in *trans* by recruiting chromatin remodeling complexes to regulate distantly located genes such as *Rcan1*, thereby serving as integrative platform for auto-amplification of calcineurin-dependent signaling that drives the cardiac hypertrophy response (**Fig.4o**). Exploiting the function of this endogenous regulator of pathological growth by antisense oligonucleotide technology has therapeutic effects on cardiac remodeling in pressure overload conditions.

DISCUSSION

Cardiomyocytes enter cell cycle arrest shortly after birth, become terminally differentiated,[29] and exhibit hypertrophy as a default reaction to internal or external injuries by translating biomechanical stress into an intricate network of intracellular signaling events.[5-7] Because increased ventricular mass is an independent risk factor for the development of overt heart failure and sudden cardiac death from lethal arrhythmias in humans,[30,31] slowing hypertrophic growth may be beneficial to function or prognosis, as evidenced by studies of genetically modified mice that disrupt particular hypertrophic pathways.[12,32,33] The stress-responsive intracellular signal transducers calcineurin and calcium/calmodulin-dependent protein kinases engage molecular circuits and epigenetic modifications such as histone or DNA modifications to re-activate gene programs that often result in cardiac remodeling as a precursor to heart failure,[34] a serious clinical condition that is the leading cause of morbidity and hospitalization in Western societies.[6,8]

Here we describe the dynamic cardiac expression of the evolutionarily conserved lncRNA *Bigheart*, which is more abundantly expressed during embryonic development, repressed in the postnatal stage and re-activated upon calcineurin/NFAT-dependent stimulation in the human and murine adult diseased heart. Accordingly, AAV9-mediated Bigheart overexpression was sufficient to induce a maladapative hypertrophic response upon agonist stimulation *in vitro* or pressure overload *in vivo*. On the other hand, treatment with “Gapmer” antisense oligonucleotide specific for Bigheart conferred resistance to biomechanical stress-induced myocardial remodeling *in vivo*, indicating the requirement of this lncRNA in the process.

By combining a wide range of sequencing approaches and biochemical techniques, we elucidated the structural characteristics, interacting partners and molecular function of LncRNA *Bigheart* in heart muscle cells. First, computational structure prediction after selective 2-hydroxyl acylation by primer extension (SHAPE)-sequencing allowed both an experimental validation of the primary sequence as well as the secondary folding structure of this heart muscle-enriched LncRNA. Next, we could not find statistically significant associations between the presence of possible genomic variants in the *Bigheart* locus from previously executed genome-wide association studies (GWAS) in patients with dilated cardiomyopathy, suggesting that the genomic response rather than genetic variants in LncRNA *Bigheart* are likely underlying hypertrophic heart diseases. We used bulk RNA-seq in combination with Gapmer-based antisense oligonucleotide silencing of the LncRNA in the murine heart in vivo and mapped the downstream genes such as *Rcan1* that are uniquely sensitive to fluctuating expression levels of *Bigheart*. Finally, we used chromatin

immunoprecipitation by RNA immunoprecipitation (ChIRP) followed by unbiased mass spec proteomics to reveal heterogeneous nuclear ribonucleoproteins (hnRNPs) and High-mobility group box 1 (Hmgb1) as preferential interacting binding partners that aid in remodeling the chromatin structure and *trans*-activation of Bigheart target genes. The functional interaction between Bigheart, Hmgb1 and hnRNPs caused an active and open chromatin structure at the two *Rcan1* promoters in heart muscle cells as experimentally revealed by a combination of genome-wide ChIRP- and chromatin immunoprecipitation (ChIP)-sequencing.

An emerging concept revolves around the ability of lncRNAs to recruit a wide variety of RNA-binding proteins (RBPs) to regulate gene expression to determine the fate of stem cells or how organs respond to internal or external cues.[35,36] For example, hnRNPs are multifunctional RNA-binding proteins that can provoke gene expression by their interaction with lncRNAs. For example, the lncRNA *THRIL* (TNFα and hnRNP L related immunoregulatory lincRNA) complexes with hnRNP L and binds the TNFα promoter following innate activation of human macrophages,[37] whereas the lincRNA-p21 physically interacts with hnRNP K as a coactivator for p53-dependent p21 transcription in mouse embryonic fibroblasts and various tumors.[38] Likewise, brown fat lncRNA-1 (Blnc1) and brown adipose tissue enriched long non-coding RNA 1 (lnc-BATE1) engage in an interaction with hnRNP U to stimulate thermogenic genes and adipogenesis in brown adipocytes.[39] Hmgb1 is an architectural non-histone chromatin-binding protein regulating transcription, DNA replication and repair, and nucleosome structure and number.[40] Hmgb1 elicits seemingly dichotomous effects on the heart depending on its origin, subcellular localization and relative redox state, where it can mediate pressure overload-induced hypertrophy and simultaneously protect the heart muscle from excessive DNA damage caused by hypertrophic conditions.[41,42] Interestingly, an emerging role for Hmgb1 as RNA binding protein is being recognized where in the cortex and cerebellum, Hmgb1 can associate with *brain specific DNA damage related lncRNA1* (*BS-DRL1*), where the interaction guides it to sites of DNA damage to facilitate repair in the control of motor function and purkinje cell degeneration,[43] whereas in multiple myeloma the interaction of Hmgb1 with LncRNA *MALAT-1* controls its degradation rate and the extent of autophagic flux and tumor remission.[44]

The combined findings in this study suggest a model whereby lncRNA *Bigheart* recruits two RBPs, hnRNP-F1 and Hmgb1, to modulate the local chromatin environment and higher order chromosomal organization with simultaneously increased tri-methylation at lysine 4 and acetylation of lysine 27 in histone H3 as active enhancer marks in *Bigheart* target genes including *Rcan1* in control of the

hypertrophic gene program. Given that targeted deletion of the *Rcan1/2* genes results in a loss of calcineurin signaling strength in vivo,[45,46] the overall net function of Rcan proteins is to facilitate NFAT activity downstream of calcineurin through enhanced calcineurin-NFAT coupling in response to their increased expression as well as their posttranscriptional phosphorylation status by TGFb-induced TAK1–TAB1–TAB2 signaling.[25] Here, we demonstrate that re-activation of the NFAT target lncRNA *Bigheart* acts, in fact, as an RNA-based auto-amplification loop that enhances calcineurin-NFAT signaling in heart muscle cells. Previously, we identified a separate posttranscriptional feed forward mechanism whereby a calcineurin/NFAT-responsive small non-coding microRNA gene, *miR-199b*, directly targets the nuclear NFAT kinase dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1a (Dyrk1a), effectively reducing the rephosphorylation (inactivation) of NFAT transcription factors, leading to acceleration of calcineurin-NFAT signaling strength and hypertrophic gene expression.[47] A concept emerges where calcineurin signaling cascades integrates with a variety of small and long ncRNA species to autoregulate kinases and accessory proteins that regulate its signaling strength in the postnatal mammalian myocardium with impact on hypertrophic gene programs. Our findings also show that human heart failure is caused by unique susceptibilities in genetic regulatory circuits, and that a better knowledge and exploitation of these circuits will aid in the prediction of future therapeutic intervention sites for RNA-based treatments.

METHODS

Mouse models. We used 3-6 month old calcineurin transgenic male mice in a B6SV129F1 background, which expressing an activated mutant of calcineurin in the postnatal heart under control of the 5.5 kb murine *Myh6* promoter (*Myh6*-CnA).[4] Other mice used in this study were male and female B6SV129F1 wild-type mice of postnatal (p) day 0 or of 3-6 months of age, as well as C57Bl6/N male mice of 7-10 weeks of age (Charles River Laboratories). All animal studies were performed in accordance with local institutional guidelines and regulations and were approved by the animal review committee of Medanex Inc. and the University of Porto (0421/000/000/2018). Sample size was determined by a power calculation based upon an echocardiographic effect size. Randomization of subjects to experimental groups was based on a single sequence of random assignments. Animal caretakers blinded investigators to group allocation during the experiment and/or when assessing the outcome.

Production of recombinant AAV vectors. Human bigheart was synthesized as MiniGene™ Synthetic Gene in pUC IDT plasmid by Integrated DNA Technologies Inc., (Leuven, Belgium) using the human reference sequence of human GRCh37 assembly. The full cDNA sequence of the lncRNA was then amplified with forward primer: 5'-GTATCATAAGGATCCCTTTCCACTGCTCTGGTGAG-3' and reverse primer: 5'- GTATCATAAGTCGACCTCACCTAGCTGTCTGTCC-3' and cloned into pAAV-MCS (Cat#: VPK-410, Cell Biolabs Inc.) using the restriction enzymes BamH I and HindIII sites. Recombinant AAV serotype 9 vectors were produced, purified, and titrated by real-time PCR on vector genomes at the AAV Vector Unit of the German Center for Cardiovascular Research (DZHK), Partner Site Hamburg/Kiel/Lübeck, Kiel (Germany) as described previously.[48] B6SV129F1 mice at postnatal day 0 were intraperitoneally injected with a control AAV9 vector (AAV9-luciferase) or AAV9-BIGHEART at a dose of 1 x 10^{11} viral genome particles per animal, using an insulin syringe with 30-gauge needle. 12-15 days after injection, the hearts were collected for histological analysis.

Aortic banding, Gapmer treatment and histological analysis. Transverse aortic constriction (TAC) or sham surgery was performed in 2-6 month-old B6SV129F1 mice by subjecting the aorta to a defined 27 gauge constriction between the first and second truncus of the aortic arch as described previously.[47,49] All animals were randomized to receive either weekly a Gapmer antisense oligonucleotide to silence endogenous Bigheart or vehicle (phosphate buffered saline, PBS). The Gapmer specific to murine *Bigheart* was purchased at Qiagen Inc (Hilden, Germany).[19] Treatment of vehicle or Gapmer-*Bigheart* started at 2-3 days after sham or aortic banding surgery by IP injections every 7 days (0.1 ml PBS or Gapmer-*Bigheart* at 25 mg/kg body weight/day) for a period of 5 weeks over a total of 6 weeks of pressure overload, ensuring that all animals received the last dose of vehicle or Gapmer 5-7 days before euthanasia. Six-eight weeks after surgery, animals were euthanized under deep anesthesia (sevofluorane 8%) and the hearts were removed, rinsed in ice-cold PBS, atria removed, snap-frozen in liquid nitrogen and stored in liquid nitrogen until use. A subset of hearts was arrested in diastole, perfusion fixed with 4% paraformaldehyde/PBS solution, embedded in paraffin and sectioned at 4 μm. Paraffin sections were stained with hematoxylin and eosine (H&E) for routine histological analysis; Sirius Red for the detection of fibrillar collagen; and FITC-labelled rabbit polyclonal antibody against wheat-germ-agglutinin (WGA) to visualize and quantify the myocyte cross-sectional area (1:100, Sigma Aldrich T4144). Cell surface areas and fibrotic areas were determined using ImageJ imaging software

(http://rsb.info.nih.gov/ij/).

Primary cardiomyocyte cultures, cell lines and transfections. 3T3-L1 fibroblasts (ATCC, cat# CL-173) and HL-1 cells (Sigma-Aldrich, cat# SCC065) were purchased from respective vendors. 3T3-L1 were cultured in DMEM supplemented with 10% FBS, 100 units/ml penicillin/streptomycin and 2 mmol/liter L-glutamine (Thermo Fischer). HL-1 cells were cultured in Claycomb Medium (Sigma-Aldrich, cat# 51800C) supplemented with 10% FBS, 100 units/ml penicillin/streptomycin, 0.1 mmol/liter of Norepinephrine and 2 mmol/liter L-glutamine (Thermo Fischer). Cardiomyocyte cultures were isolated by enzymatic dissociation of 1 day-old neonatal rat hearts and processed for immunofluorescence as described previously.[47] Neonatal cardiomyocytes were seeded in Corning® Primaria™ 6-well plates (for microscopy) or in Corning® Primaria™ 10 cm dishes (for RNA isolation) and one day later, cardiomyocytes were transfected with inhibitors (Dicer Substrate Duplex RNAs, Integrated DNA Technologies Inc., Leuven, Belgium) of specific lncRNA transcripts at a final concentration of 10 nM using Oligofectamine (Invitrogen). Twenty-four hours after transfection, culture medium was replaced by fresh medium; cardiomyocyte hypertrophy was induced by phenylephrine (PE, 10 μM) for an additional 24 hrs as described previously.[47] For visualization of cardiomyocyte size and sarcomeric organization, cells were washed with ice cold PBS, fixed with 4% PFA (for 10 min) and stained with an anti-sarcomeric alpha actinin antibody [EA-53] (1:500; Abcam, cat#ab9465) followed by a phalloidin Texas Red- conjugated antibody (1:800; Molecular Probes). Nuclear staining was performed with VECTASHIELD Mounting Medium containing 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories).

Human induced pluripotent stem cell (hiPSC) derivation, maintenance and differentiation to cardiomyocytes. Wildtype BXS0116 hiPSCs, derived from bone marrow CD34+ cells obtained from a healthy Caucasian female donor were purchased from ATCC (ATCC® ACS-1030™). Cells were maintained in Matrigel-coated plates (Corning®), passaged with Versene solution (Thermo Fisher Scientific) and cultured in StemMACS™ iPS-Brew XF medium (Miltenyi Biotec) supplemented on the first day after passaging with 5μM ROCK inhibitor (Stemolecule Y27632). Differentiation of hiPSCs to cardiomyocytes was performed by modulation of the Wnt signaling pathway. Briefly, differentiation was started on cells cultured on Matrigel-coated plates when reaching 80-90% confluence. Cells were initially cultured for 3 days in mesodermal induction medium, consisting of RPMI 1640 medium (Thermo

Fisher) supplemented with 1% 100X glutaMAX (Thermo Fisher), 1% 100X sodium pyruvate (Invitrogen), 1% 100X penicillin/streptomycin (Invitrogen), 2% 50X B-27® Serum-Free Supplement (Invitrogen), 200uM L-ascorbic acid 2 phosphate sesquimagnesium salt hydrate (Sigma), 1uM CHIR99021 (Stemgent), 5 ng/ml Recombinant human BMP4 (R&D Systems), 9 ng/ml Recombinant Human/Mouse/rat Activin A (R&D Systems) and 5 ng/ml human FGF-2 (Miltenyi Biotec). Then, cells were cultured for 7 days in cardiac differentiation medium, consisting of RPMI 1640 medium (Thermo Fisher) supplemented with 1% 100X glutaMAX (Thermo Fisher), 1% 100X sodium pyruvate (Invitrogen), 1% 100X penicillin/streptomycin (Invitrogen), 2% 50X B-27® Serum-Free Supplement (Invitrogen), 200uM L-ascorbic acid 2 phosphate sesquimagnesium salt hydrate (Sigma) and 5uM IWP-4 (Stemgent). HiPSC-CMs were finally subjected to a 4-day metabolic selection in RPMI 1640 without glucose, without HEPES (Thermo Fisher), 1% 100X penicillin/streptomycin (Invitrogen), 2.2mM 50% sodium lactate (Sigma) and 0.1 mM 2-mercaptoethanol (Invitrogen). HiPSC-CMs were cultured for up to 25 days from the beginning of the differentiation protocol.

Microarray analysis of long non-coding RNAs and mRNAs expression. Total RNA from each heart (n=3 for each group) was quantified by the NanoDrop ND-1000 and RNA integrity was assessed by standard denaturing agarose gel electrophoresis. For microarray analysis, Agilent Array platform was employed. The sample preparation and microarray hybridization were performed based on the manufacturer's standard protocols with minor modifications. Briefly, mRNA was purified from total RNA after removal of rRNA (mRNA-ONLY™ Eukaryotic mRNA Isolation Kit, Epicentre). Then, each sample was amplified and transcribed into fluorescent cRNA along the entire length of the transcripts without 3' bias utilizing a random priming method. The labeled cRNAs were hybridized onto the Mouse lncRNA Array v2.0 (8 x 60K, Arraystar). After having washed the slides, the arrays were scanned by the Agilent Scanner G2505C. Agilent Feature Extraction software (version 11.0.1.1) was used to analyze the acquired array images.

Western blot analysis. Whole tissue or cell lysates were produced in RIPA buffer (Sigma-Aldrich) supplemented with PhosSTOP- and Protease inhibitor cocktail (Roche Applied Science). Samples were boiled in 1x Leammli buffer, including 2% β-mercaptoethanol, for 5 minutes at 95°C. SDS-PAGE and Western blotting were performed using the Mini-PROTEAN 3 system (Biorad). Blotted membranes were blocked in 5% BSA / TBS-Tween. Primary antibody labeling was performed overnight

at 4°C in blocking buffer. The following rabbit polyclonal antibodies were used at a 1:500 dilution: anti-HNRNPF (Sigma-Aldrich, cat# HPA069667); anti-HNRNPK (Sigma-Aldrich, cat# HPA044105); anti-HNRNPA2/B1 (Sigma-Aldrich, cat# HPA001666); anti-HNRNPC (Sigma-Aldrich, HPA051075); anti-DSCR1 (Sigma-Aldrich, cat# D6694); anti-HMGB1 (Abcam, cat# ab18256). Other antibodies applied were mouse monoclonal anti-GAPDH (1:5000, Millipore, cat# MAB374 clone 6C5), mouse monoclonal anti-alpha-Tubulin (1:5000, Sigma-Aldrich, cat# T6074) rabbit polyclonal anti-Histone H3 (1:5000, Cell Signaling Technolog, cat# 9715S) and the secondary polyclonal swine anti-rabbit immunoglobulins/HRP (1:10.000, DAKO P0399) and polyclonal rabbit anti-mouse immunoglobulins/HRP (1:10.000, DAKO P0161). Secondary HRP conjugated antibodies were applied for 1 hour at room temperature. Following antibody incubation, blots were washed for 3x10 minutes in TBS-Tween. Images were generated using the Western Lighting Ultra (Perkin Elmer) chemiluminescent detection kit and the LAS-3000 documentation system (FujiFilm Life Science). Stripping was performed using a buffer according to Abcam's medium stripping formulation (1,5 % w/v Glycine, 0,1 % v/w SDS, 1,0% v/v Tween-20, set to pH 2,2). Output intensities were normalized for loading.

RNA isolation. Total RNA was extracted from cultured cells or myocardial tissue of mice euthanized at the timepoints reported above using the Direct-zol RNA purification Kit (ZYMO Research) following the manufacturer's protocol.

cDNA synthesis. Reverse transcription of the total RNA was performed using M-MLV and RNAsin (Promega) following manufacturer's protocol, complemented with both Oligo(dT)15 and random hexamer primers (IDT).

Quantitative PCR. Transcriptional expression was assessed in a CFX optical thermal cycler (Biorad) from 2x SYBR Green master mix (Biorad) reactions, according to manufacturer's instructions. Expression was normalized to expression levels of 5S rRNA. For mRNA-based reverse transcription, total RNA was reverse transcribed using hexameric random primers. The housekeeping gene ribosomal protein L7 (RPL7) was used for normalization. Fold changes were determined using the $2^{-\Delta\Delta CT}$ method. Real-time PCR primer sequences used in the study are listed in **Supplementary Table 1**.

Luciferase-reporter assays. A construct bearing 2 kb of the murine Bigheart promoter (pGL3-B.HTSS2KB) was subcloned as a HindIII and SacI fragment into the

pGL3-Basic vector (Promega). pGL3-Intron3-DSCR1 harbors the complete (875 bp) of Rcan1 as described previously.[50] Low-passage 3T3-L1 cells were grown in DMEM (Invitrogen) supplemented with 10% FCS and seeded (2,5 x 10^4) in 48-well plates. For cotransfection assays, 150 µg/well of either pGL3-B.HTSS2KB or pGL3-Intron3-DSCR1 reporter construct was transfected with 150 µg/well of pCDNA3-ΔCnA,and 300 µg/well pEF-BOS-hNFATp and/or 150 until 600 µg/well of pEGFP-VIVIT.[7,17]. The total amount of DNA per well was adjusted to 0 to 450 µg using pcDNA3 empty vector. The cells were washed 24 hours after transfection with phosphate-buffered saline and lysed with 100 µl of Reporter Lysis Buffer (Roche), and lysates were assayed for luciferase activity using Bright-Glo™ (Promega) on a 96-well using The VICTOR3™ Multilabel Plate Reader (PerkinElmer LAS Germany GmbH)

Chromatin isolation by RNA purification (ChIRP). ChiRP was performed as described previously with minor modifications. Briefly, a series of 10 antisense DNA probes with 3'-Biotin-TEG modification were designed against the murine Bigheart full-length sequence (**Supplementary Table 2**) using LGC Biosearch Technologies software at https://www.biosearchtech.com/support/tools/design-software. 50 million HL-1 cells were cultured in Corning® Primaria™ 10-cm dishes and cross-linked in 1% glutaraldehyde for 30 min, followed by 0.125 M glycine quenching for 5 min. Next, the pellets were decanted with subsequent centrifugation and washing steps in PBS, pellets were resuspended in lysis buffer with fresh PMFS and PhosSTOP and protease inhibitor cocktail (Roche), kept on ice for 10 minutes and sonicated until the gDNA was in the size range of 100–500 bp. At this point, chromatin was diluted in hybridization buffer (500mM NaCl, 1%SDS, 100mM Tris 7.0, 10mM EDTA, 15% Formamide, add DTT, PMSF, P.I, and Superase-in fresh).), and probes were added at a final concentration of 100 pmol per 100 µL of chromatin for 24 hrs. Then 100 µL of streptavidin magnetic C1 beads per 100 pmol of probes were added and allowed to hybridize for 1 hr. Beads were then divided from the solution with a magnetic stand and washed 5 times with 1 ml wash buffer (2x SSC, 0.5% SDS, add DTT and PMSF) and processed for elution of RNA, DNA, or proteins. For the RNA extraction, beads were resuspended in 50 µL 10× RNA elution buffer (Tris 7.0, 1% SDS) and boiled for 15 min, and RNA extracted with the Direct-zol RNA purification Kit (ZYMO Research) and RNA quality assessed with controlled with nanodrop. For DNA elution, the beads were resuspended in 150 µL 3× volume of DNA elution buffer (50 mM NaHCO3, 1% SDS, 200 mM NaCl), and DNA eluted with a cocktail of 100 ug/ml RNase A and 0.1 units/microliter RNase H and samples purified with The Quick-DNA™ Miniprep Plus Kit (Qiagen). For protein elution, beads were resuspended in 3x original volume of

DNase buffer (100 mM NaCl and 0.1% NP-40), and protein was eluted with a cocktail of 100 ug/ml RNase A (Sigma-Aldrich) and 0.1 units/ µL RNase H (Epicenter), and 100 U/ml DNase I (Invitrogen) at 37 °C for 30 min.

Silver staining

Samples were lysate in RIPA Lysis and Extraction Buffer (thermos fish) and PhoSSTOP and protease inhibitor cocktail (roche). Then samples were boiled in 4x leammli buffer with 2% β-mercaptoethanol for 5 minutes at 95^{o} C. then, the sample has been run in SDS-PAGE gel. Next, the gel was incubated in a Fixer solution (40% ethanol, 10% acetic acid, 50% H2O) for 1 hour. Sensitized with a solution of 0.02% sodium thiosulfate for 1 minute and incubated for 30 min with a Staining solution (0.1% AgNO3,H2O, 0.02% formaldehyde). Finally, the gel was developed in a solution of 3% sodium Carbonate and stabilized in a solution of 5% acetic acid.

Proteomics analysis. ChiRP proteins eluate were kept -20 °C. The gel bands were subjected to in-gel digestion with trypsin and tryptic peptides were separated on a nanoflow LC system (Dionex UltiMate 3000), eluted with a 40-min gradient, and the column (Dionex PepMap C18) coupled to a nanospray source (Picoview). Spectra were collected from an ion trap mass analyzer (LTQ-Orbitrap XL). MS/MS was performed on the top six ions in each MS scan using the data-dependent acquisition mode with dynamic exclusion enabled. MS spectra were separately analyzed in MaxQuant Software. To construct a MS/MS peak list file, up to top eight peaks per 100 Da window were extracted and submitted to search against a concatenated forward and reverse version of the UniProtKB/Swiss-Prot mouse database. A principal component analysis was performed using the normalised high/low ratios of all proteins from all samples. Significant differences were identified using the BioConductor package Limma[51] and Bayesian statistics to moderate variance across proteins and calculate a p-value.

RNA sequencing. All hearts from vehicle- or Gapmer-Bigheart treated mice were prepared simultaneously during all steps of this analysis in order to exclude introduction of technical variability. Quality control of total RNA was performed using the RNA 6000 Pico Kit (Agilent Bioanalyzer) yielding in RIN values of 6.3 and higher. Removal of rRNA was carried out by use of the NEBNext rRNA Depletion Kit Human/Mouse/Rat (NEB) followed by strand-specific cDNA NGS library preparation (NEBNext Ultra II Directional RNA Library Prep Kit for Illumina, NEB). The size of the

resulting library was controlled by use of a D1000 ScreenTape (Agilent 2200 TapeStation) and quantified using the NEBNext Library Quant Kit for Illumina (NEB). Equimolar pooled libraries were sequenced in a paired end mode (75 cycles) on the NextSeq 500 System (Illumina) using v2 chemistry yielding in an average QScore distribution of 84% >= Q30 score.

Secondary structure prediction and SHAPE-Seq

We used the Vienna package RNAfold[52] was used to predict the MFE structures for both murine and human Bigheart lncRNA. Next, we performed SHAPE-Seq for human Bigheart to determine the secondary structure. The RNA transcripts were generated in vitro from a ThermoFisher Gene Synthesis Plasmid with the T7 RNA polymerase. For this the T7 promoter and a sequence specific restriction site was attached to the sequence. For practical reasons, we restricted our approach to RNAs that are no longer than 1000 nucleotides. In vitro SHAPE-Seq was performed according to the protocol by Watters and Lucks[15] using the 1M7 reagent, which can achieve a single nucleotide resolution. The samples were later sequenced using the Illumina NextSeq500 system and v2 chemistry in paired-end mode. The resulting reads were analyzed with Spats (v1.0.2) for generating a reactivity profile. These length-normalized reactivities were then incorporated as an additional constraint during secondary structure prediction. The SHAPE-probing information guided sampling of the structural ensemble was performed through RNAStructure[53] pipeline consisting of Rsample, stochastic and RsampleCluster.

Bioinformatics analyses: For analysis of the microarray datasets, quantile normalization and differential expression analysis were performed using the GeneSpring GX v12.0 software package (Agilent Technologies). After quantile normalization of the raw data, lncRNAs and mRNAs that at least 6 out of 9 samples have flags in Present or Marginal (“All Targets Value”) were chosen for further data analysis. Differentially expressed lncRNAs and mRNAs with statistical significance between two groups were identified by filtering for adjusted p-value < 0.05 and fold change >=2 or <= 0.5. Pathway analysis and GO analysis were applied to determine the roles of these differentially expressed mRNAs played in these biological pathways or GO terms, using the top 100 differentially expressed genes as it is implemented in clusterProfiler package version 3.8.1.[54] Finally, Hierarchical Clustering on the top50 differentially expressed features was performed to show the distinguishable lncRNAs and mRNAs expression pattern among samples. For RNA-seq analysis, after quality control with FastQC v0.11.2 raw sequencing reads were

trimmed for Illumina adapter sequences and quality score using trimmomatic v0.36 and aligned to the mouse reference genome GRCm38 (ensemble release 90) with hisat2 v2.1.0. Overall alignment rate was observed between 70-84% for all samples. Gene assembly was done using stringtie v1.3.4d 33 with set '-e' parameter to produce read count matrices for reference genes and transcripts. Differential expression analysis of read counts was done using the DESeq2 package in R programming language. Genes are considered significant if fold changes are >= 1.5 or <= 2/3 and obtained p-values are below 0.05 after adjustment for false discovery rate (fdr). Sets of significant up-regulated and down-regulated genes were analyzed for over-represented gene ontology terms (GO) and KEGG pathways as implemented in the R-package clusterProfiler applying a significance threshold of $p < 0.05$ after adjustment for fdr. The complete microarray analysis and RNA-seq data sets were deposited at the Gene Expression Omnibus (GEO) with references xx and yy in MINSEQ-compliant data submission format under restricted release date and will become publicly available pending acceptance of the current manuscript.

Statistics and Reproducibility. Results shown for images or blots were repeated independently at least once with similar results. The results are presented as mean ± standard error of the mean (SEM). Statistical approaches for bioinformatics analyses are described above. All other statistical analyses were performed using Prism software (GraphPad Software Inc.), and consisted of One-way ANOVA followed by Dunnet's multiple comparison test when group differences were detected at the 5% significance level, or Student's *t*-test when comparing two experimental groups. Differences were considered significant when $P<0.05$.

Data availability. The data that support the findings in this study are available within the article and its supplementary information files. Raw and processed microarray or RNAseq data generated in this study have been deposited at the GEO database under accession codes: xx, yy and zz. Published resources evaluated included HomoloGene NCBI database (https://www.ncbi.nlm.nih.gov/homologene) and STRING (https://string-db.org/). Source data are provided with this paper. Any remaining raw data will be available from the corresponding author upon reasonable request.

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Competing interests:
P.D.C.M. and L.D.W. are co-founders and stockholders of Mirabilis Therapeutics BV. The remaining authors declare no competing interests.

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FIGURE LEGENDS

Figure 1 | **LncRNA profiling identifies "*Bigheart*" as a calcineurin/NFAT regulated non-coding RNA target gene. (a)** Workflow of the experiment. **(b)** Representative images of whole hearts (top panels), haematoxylin & eosin (H&E)-stained sections of four-chamber view (second panel), high magnification H&E sections (third panel), Sirius Red stained sections (fourth panel) and wheat germ agglutinin (WGA)-stained (fifth panel) histological sections. **(c)** Volcano plots reporting the differentially expressed lncRNAs in hearts of pressure-overloaded mice compared to control animals (left panel) or in hearts of calcineurin transgenic mice compared to wild-type control animals (right panel). In blue and yellow are down- and up-regulated genes, respectively. **(d)** VENN diagrams showing the number of significantly downregulated (left panel in blue) or upregulated lncRNAs (right panel in yellow) in each heart failure model resulting from the comparison to control animals. Schematic representation of the **(e)** murine *"Bigheart"* genomic locus. **(f)** Consensus secondary structure of murine Bigheart derived from LocARNA. **(g)** RT-PCR analysis of lncRNA Bigheart expression in different murine tissues with Gapdh as loading control. **(h)** Schematic representation of the human "*BIGHEART"* genomic locus. **(i)** Consensus secondary structure of human BIGHEART derived from LocARNA. **(j)** RT-PCR analysis of lncRNA BIGHEART expression in different human tissues with GAPDH as loading control. **(k)** Real-time PCR analysis of lncRNA Bigheart in primary cardiomyocytes or fibroblasts isolated from the adult mouse heart, *n* refers to number of hearts. **(l)** Real-time PCR analysis of lncRNA Bigheart in human induced-pluripotent stem cells (hiPSCs) differentiated into mesoderm and cardiomyocytes (CMs), *n* refers to number of independent differentiations. **(m)** Real-time PCR analysis of lncRNA Bigheart in hearts from mice subjected to transverse aortic constriction (TAC) compared to sham surgery (left panel) or calcineurin transgenic mice compared to wild-type controls (right panel), *n* refers to number of hearts. Source data are provided as a Source Data file.

Figure 2 | **LncRNA Bigheart regulates hypertrophy in cultured primary cardiomyocytes. (a)** Schematic representation of the Bigheart luciferase reporter with location of an evolutionary conserved NFAT site. **(b)** Activity assay of the Bigheart luciferase reporter construct showing the sensitivity to calcineurin/NFAT signaling, *n* refers to number of transfection experiments. **(c)** Schematic representation of serotype 9 recombinant adeno-associated vectors (AAV9). **(d)** Workflow of the experiment. **(e)** Confocal microscopy images of neonatal rat

cardiomyocytes infected with AAV9-luciferase, AAV9-BIGHEART or agonist-stimulated with phenylephrine (PE) and isoproterenol (ISO); nuclei visualized with Hoechst (blue) and stained with an antibody against α-actinin (red). **(f)** Quantification of cell surface area of conditions in **(e)**, *n* refers to number of cells analyzed. **(g)** Real-time PCR analysis of fetal marker gene expression in conditions in **(e)**. **(h)** Workflow of the experiment. **(i)** Real-time PCR validation of siRNA-mediated silencing of endogenous Bigheart in primary rat cardiomyocytes, *n* refers to number of experiments. **(j)** Confocal microscopy images of neonatal rat cardiomyocytes transfected with indicated siRNAs and agonist-stimulated with phenylephrine (PE); nuclei visualized with Hoechst (blue) and stained with an antibody against α-actinin (red). **(k)** Quantification of cell surface area in conditions in (j), *n* refers to number of cells analyzed. **P < 0.05* vs corresponding control group; #*P < 0.05* vs corresponding treatment (error bars are s.e.m.). Statistical analysis consisted of a two-tailed Student's t-test **(i)** or One-way ANOVA followed by Dunnett multiple comparison test **(f,g,k)**. Source data are provided as a Source Data file.

Figure 3 | **LncRNA *Bigheart* is sufficient and required to provoke cardiac hypertrophy *in vivo*. (a)** Workflow of the experiment and representation of recombinant AAV9 vectors used. **(b)** Real-time PCR analysis of lncRNA *Bigheart* expression in mouse hearts infected with AAV9-luciferase or AAV9-BIGHEART. **(c)** Representative images of whole hearts (top panels), haematoxylin & eosin (H&E)-stained sections of four-chamber view (second panel), high magnification H&E sections (third panel), Sirius Red stained sections (fourth panel) and wheat germ agglutinin (WGA)-stained (fifth panel) histological sections. **(d)** Quantification of heart weight (HW)/body weight (BW) ratio, *n* refers to number of animals. Real-time PCR analysis of **(e)** *Nppa*, **(f)** *Nppb* and **(g)** *Myh7*, *n* refers to number of animals. **(h)** Workflow of the experiment. **(i)** Real-time PCR analysis of lncRNA *Bigheart* in hearts subjected to sham or TAC surgery and treated with vehicle or Gapmer. **(j)** Representative images of high magnification H&E sections (first panel), Sirius Red stained sections (second panel) and wheat germ agglutinin (WGA)-stained (third panel) histological sections. **(k)** Quantification of heart weight (HW)/body weight (BW) ratio, *n* refers to number of animals. **(l)** Histological analysis of cardiomyocyte areas and % fibrosis in hearts from (j). (m) Real-time PCR analysis of *Nppb* and *Acta1* transcript abundance in hearts subjected to sham or TAC surgery and treated with vehicle or Gapmer.**P < 0.05* vs corresponding control group; #*P < 0.05* vs corresponding treatment (error bars are s.e.m.). Statistical analysis consisted of a

two-tailed Student's t-test **(b, d-g)** or One-way ANOVA followed by Tukey's multiple comparison test **(i, k-m)**. Source data are provided as a Source Data file.

Figure 4 | LncRNA *Bigheart* Chromatin Isolation by RNA Purification (ChIRP) reveals binding partners and chromatin targets. (a) Workflow of the study. **(b)** Quantification of heart weight (HW)/body weight (BW) ratio, *n* refers to number of animals. **(c)** Heatmap representation of myocardial transcripts differentially expressed by Gapmer-Bigheart. **(d)** Western blot analysis of endogenous Rcan1 isoforms and Gapdh as a loading control in hearts from mice receiving AAV9-luciferase or AAV9-BIGHEART, *n* refers to number of hearts. **(e)** Quantification of relative Rcan1 isoforms from (d). **(f)** Design of antisense DNA-tiling probes, grouped into ''even'' and ''odd'' sets based on their positions along mouse lncRNA Bigheart and workflow of the study. **(g)** Complementary DNA-tiling oligonucleotides efficiently and specifically retrieve lncRNA Bigheart from chromatin, *n* refers to number of independent pull-down experiments. **(h)** ChIRP-qPCR validation of peaks from Bigheart ChIRP signals in *Rcan1* promoter regions, *Gapdh* served as negative control, *n* refers to number of independent pull-down experiments. **(i)** Tabular representation of proteins detected by mass spectrometry after ChIRP retrieval of lncRNA Bigheart from chromatin. **(j)** Bigheart ChIRP retrieves hnRNP-F and Hmgb1 proteins. As a negative control, Gapdh was not detected after Bigheart ChIRP. **(k)** Worfkflow of the experiment. **(l)** ChIP-seq profiles for H3K27me3, H3K4me3, H3K27Ac, RNA Pol2, Hmgb1 and ChIRP-seq profiles for lncRNA Bigheart carried out in cardiomyocytes on the murine *Rcan1* gene. **(m)** Schematic representation of the model. *$*P < 0.05$* vs corresponding control group; *#P < 0.05* vs corresponding treatment (error bars are s.e.m.). Statistical analysis consisted of a One-way ANOVA followed by Tukey's multiple comparison test **(b, e)**. Source data are provided as a Source Data file.

Supplementary Figure legend

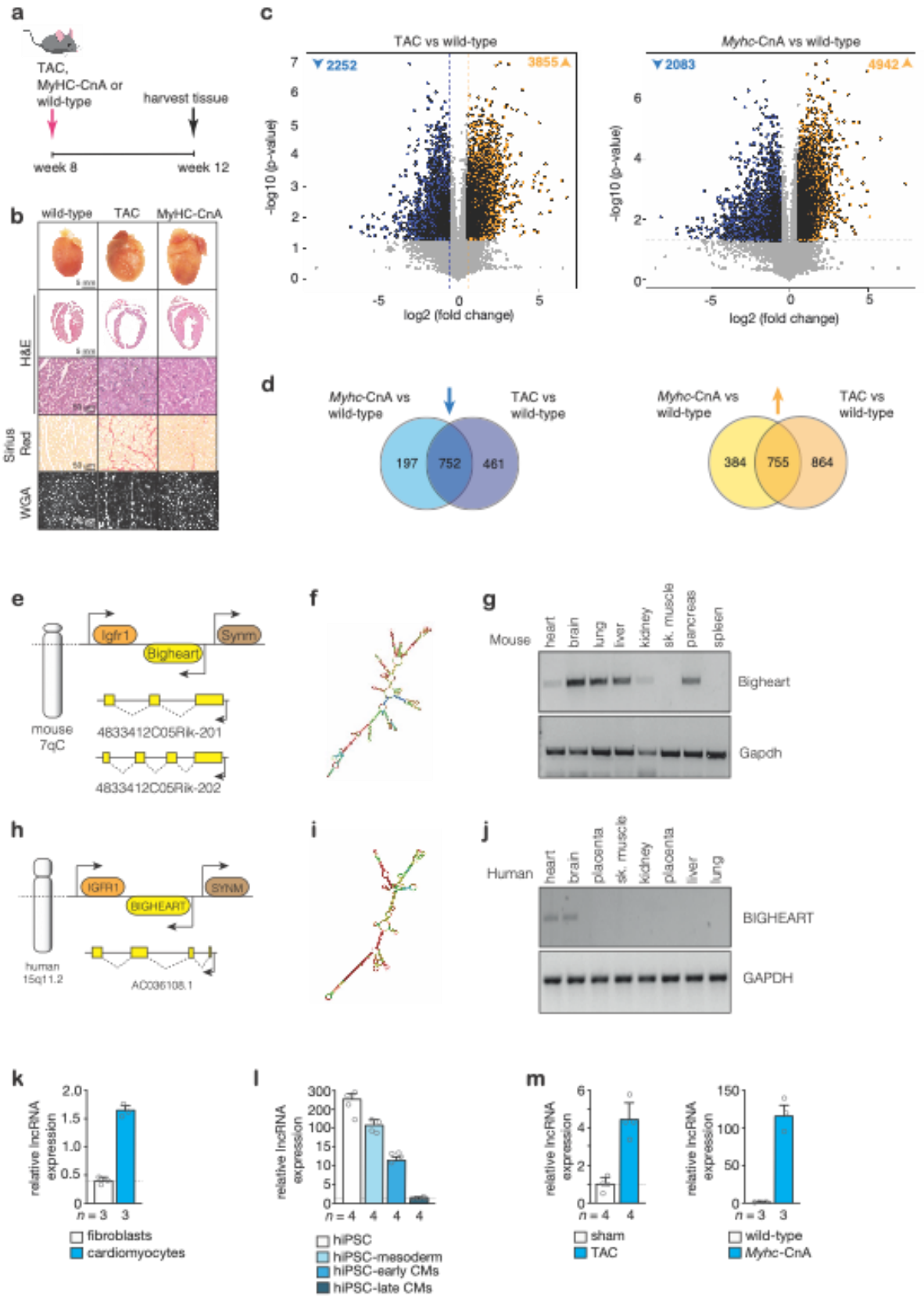
Supplementary Figure 1 | **LncRNA *Bigheart* characteristics**. **(a)** Real-time PCR analysis of fetal marker gene transcript abundance in mouse models of heart failure. **(b)** Heatmap representation of the top 50 myocardial lncRNA transcripts differentially expressed in hearts from mice subjected to sham surgery or transverse aortic constriction (TAC) for 4 weeks or **(c)** non-transgenic (nTg) or Myh6-CnA transgenic mice. (**d**) Schematic representation of the murine *Bigheart* genomic locus. **(e)** Real-

time PCR analysis of the expression of its minor isoform in mice subjected to sham surgery or TAC for 4 weeks, *n* refers to the number of hearts. **(f,g)** SHAPE-seq. Error bars are s.e.m.. Statistical analysis consisted of a two-tailed Student's t-test. Source data are provided as a Source Data file.

Supplementary Figure 2 | LncRNA *Bigheart* contains several consensus binding sites for NFAT transcription factors. Schematic representation of the murine *Bigheart* gene structure (exons represented as black squares) with the location of four evolutionary conserved NFAT sites (N1 through N4) represented as purple squares.

Supplementary Figure 3 | LncRNA *Bigheart* Chromatin Isolation by RNA Purification (ChIRP). **(a)** Absence of statistical associations between the presence of possible genomic variants in the *Bigheart* genomic locus in genome-wide association studies (GWAS) in patients with dilated cardiomyopathy. **(b)** Schematic representation of the murine *Bigheart* genomic locus (left panel) and real-time PCR analysis of the transcript abundance of the adjacent genes *Igfr1* and *Symn* in mice subjected to sham surgery or TAC for 4 weeks, *n* refers to the number of hearts. **(c)** PCA plots of the mRNAs data representing the expression profiles trends of hearts from mice subjected to transverse aortic constriction (TAC) and treated with vehicle or Gapmer for 4 weeks. The plots have been generated using the first two principal components, which together account for the 95% and 1% of the variance between samples, n=3 hearts for each mouse model. **(d)** Gene-Concept networks visualizing the individual protein-coding genes associated to the GO-terms found enriched for Gapmer-treated mice. (e) Silver-stained PAGE gel with visualization of total proteins before or after Bigheart-ChIRP. **(f)** Bigheart ChIRP does not retrieve Gapdh or **(g)** hnRNP-k, hnRNP-A2/B1 or hnRNP-C1/C2. As a negative control, Gapdh was not detected after Bigheart ChIRP. Error bars are s.e.m. Statistical analysis consisted of a two-tailed Student's t-test. Source data are provided as a Source Data file.

Figure 1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TAC, MyHC-CnA or wild-type
harvest tissue
week 8
week 12
b
wild-type
TAC
MyHC-CnA
H&E
Sirius Red
WGA
c
TAC vs wild-type
2252
3855
-log10 (p-value)
log2 (fold change)
Myhc-CnA vs wild-type
2083
4942
-log10 (p-value)
log2 (fold change)
d
Myhc-CnA vs wild-type
TAC vs wild-type
197
752
461
Myhc-CnA vs wild-type
TAC vs wild-type
384
755
864
e
Igfr1
Synm
Bigheart
mouse 7qC
4833412C05Rik-201
4833412C05Rik-202
f
g
Mouse
heart
brain
lung
liver
kidney
sk. muscle
pancreas
spleen
Bigheart
Gapdh
h
IGFR1
SYNM
BIGHEART
human 15q11.2
AC036108.1
i
j
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heart
brain
placenta
sk. muscle
kidney
placenta
liver
lung
BIGHEART
GAPDH
k
relative lncRNA expression
n = 3 3
fibroblasts
cardiomyocytes
l
relative lncRNA expression
n = 4 4 4 4
hiPSC
hiPSC-mesoderm
hiPSC-early CMs
hiPSC-late CMs
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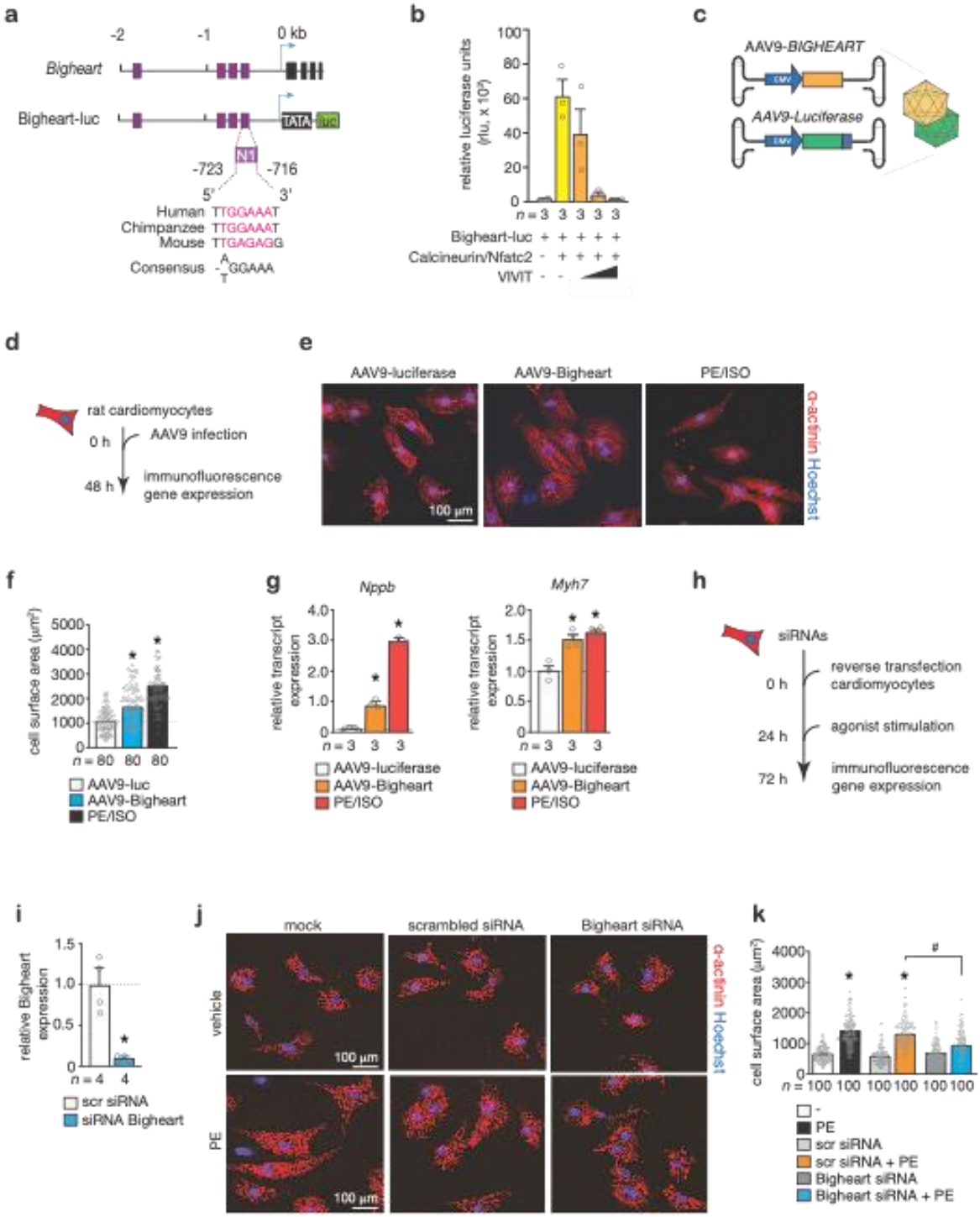
sham
TAC
relative lncRNA expression
n = 3 3
wild-type
Myhc-CnA

Figure 2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-1
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Bigheart
Bigheart-luc
TATA
luc
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-723
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5'
3'
Human TTGGAAAT
Chimpanzee TTGGAAAT
Mouse TTGAGAGG
Consensus -A/TGGAAA
b
relative luciferase units (rlu, x 10²)
n = 3 3 3 3 3
Bigheart-luc + + + + +
Calcineurin/Nfatc2 - + + + +
VIVIT - -
c
AAV9-BIGHEART
AAV9-Luciferase
CMV
d
rat cardiomyocytes
0 h
AAV9 infection
48 h
immunofluorescence
gene expression
e
AAV9-luciferase
AAV9-Bigheart
PE/ISO
100 µm
α-actinin Hoechst
f
cell surface area (µm²)
n = 80 80 80
AAV9-luc
AAV9-Bigheart
PE/ISO
g
Nppb
Myh7
relative transcript expression
n = 3 3 3
AAV9-luciferase
AAV9-Bigheart
PE/ISO
h
siRNAs
0 h
reverse transfection
cardiomyocytes
24 h
agonist stimulation
72 h
immunofluorescence
gene expression
i
relative Bigheart expression
n = 4 4
scr siRNA
siRNA Bigheart
j
mock
scrambled siRNA
Bigheart siRNA
vehicle
PE
100 µm
α-actinin Hoechst
k
cell surface area (µm²)
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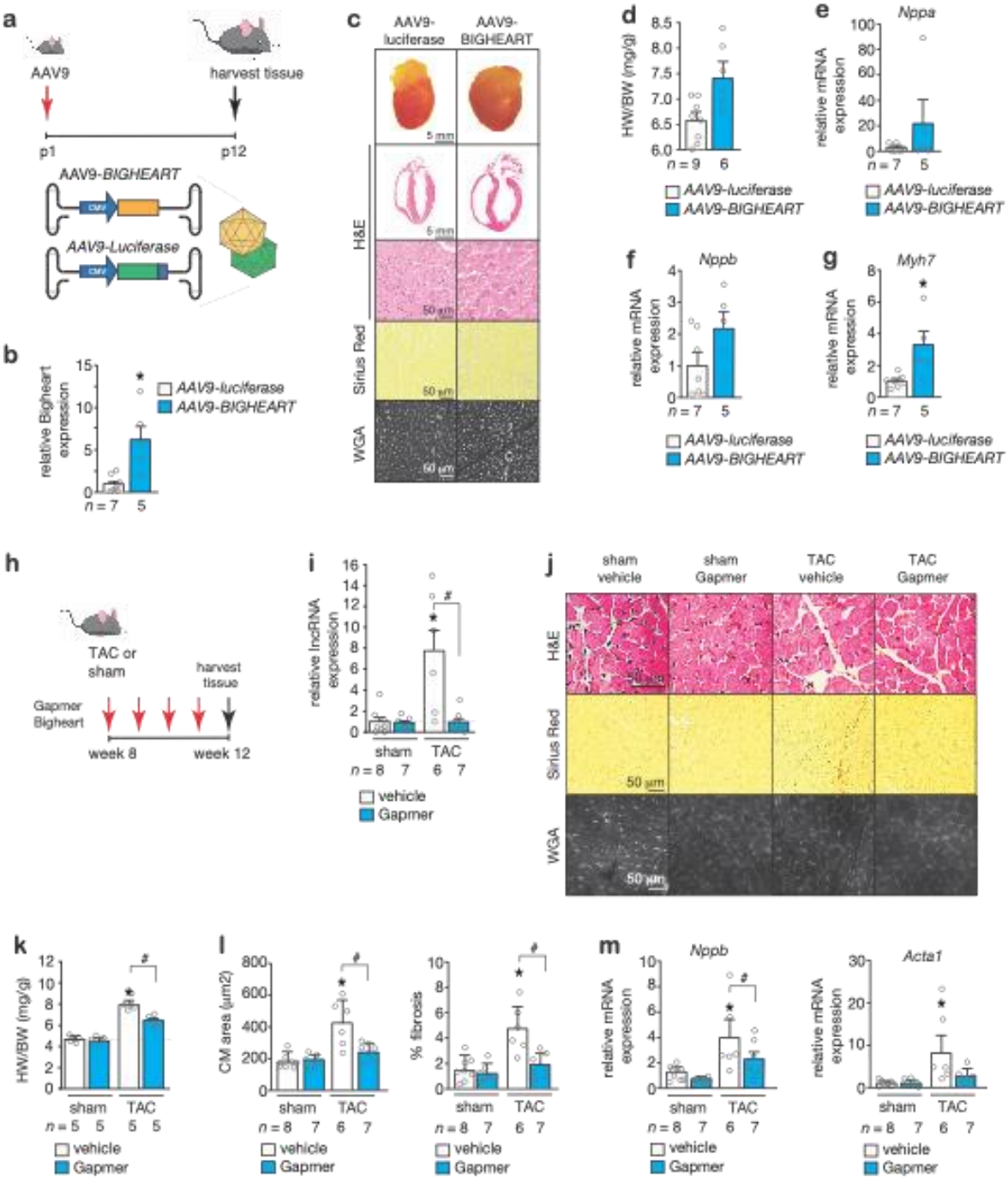
PE
scr siRNA
scr siRNA + PE
Bigheart siRNA
Bigheart siRNA + PE

Figure 3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AAV9
harvest tissue
p1
p12
AAV9-BIGHEART
AAV9-Luciferase
b
relative Bigheart expression
AAV9-luciferase
AAV9-BIGHEART
n = 7 5
c
AAV9-luciferase
AAV9-BIGHEART
H&E
Sirius Red
WGA
d
HW/BW (mg/g)
n = 9 6
e
Nppa
relative mRNA expression
n = 7 5
f
Nppb
g
Myh7
h
TAC or sham
harvest tissue
Gapmer Bigheart
week 8
week 12
i
relative lncRNA expression
sham TAC
n = 8 7 6 7
vehicle
Gapmer
j
sham vehicle
sham Gapmer
TAC vehicle
TAC Gapmer
50 µm
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HW/BW (mg/g)
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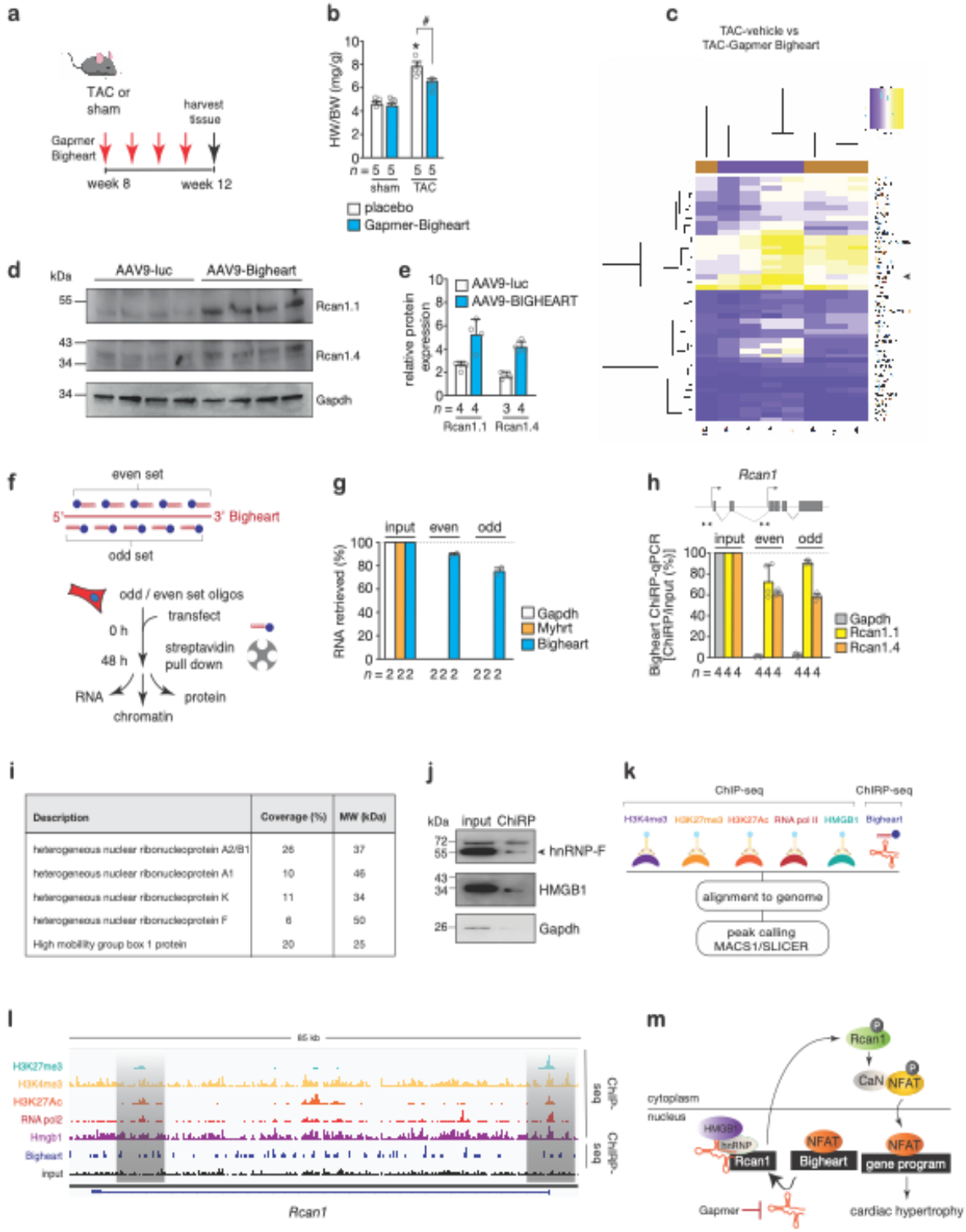
CM area (µm2)
% fibrosis
m
Nppb
Acta1

Figure 4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TAC or sham
harvest tissue
Gapmer Bigheart
week 8
week 12
b
HW/BW (mg/g)
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sham
TAC
placebo
Gapmer-Bigheart
c
TAC-vehicle vs TAC-Gapmer Bigheart
d
kDa
AAV9-luc
AAV9-Bigheart
55
Rcan1.1
43
34
Rcan1.4
34
Gapdh
e
AAV9-luc
AAV9-BIGHEART
relative protein expression
n = 4 4 3 4
Rcan1.1
Rcan1.4
f
even set
5'
3' Bigheart
odd set
odd / even set oligos
transfect
0 h
48 h
streptavidin pull down
RNA
protein
chromatin
g
input
even
odd
RNA retrieved (%)
n = 2 2 2 2 2 2
Gapdh
Myhrt
Bigheart
h
Rcan1
input
even
odd
Bigheart ChIRP-qPCR [ChIRP/input (%)]
n = 4 4 4 4 4 4
Gapdh
Rcan1.1
Rcan1.4
i
Description
Coverage (%)
MW (kDa)
heterogeneous nuclear ribonucleoprotein A2/B1
26
37
heterogeneous nuclear ribonucleoprotein A1
10
46
heterogeneous nuclear ribonucleoprotein K
11
34
heterogeneous nuclear ribonucleoprotein F
6
50
High mobility group box 1 protein
20
25
j
kDa
input
ChIRP
72
55
hnRNP-F
43
34
HMGB1
26
Gapdh
k
ChIP-seq
ChIRP-seq
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H3K27me3
H3K27Ac
RNA pol II
HMGB1
Bigheart
alignment to genome
peak calling MACS1/SLICER
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H3K4me3
H3K27Ac
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Hmgb1
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input
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Rcan1
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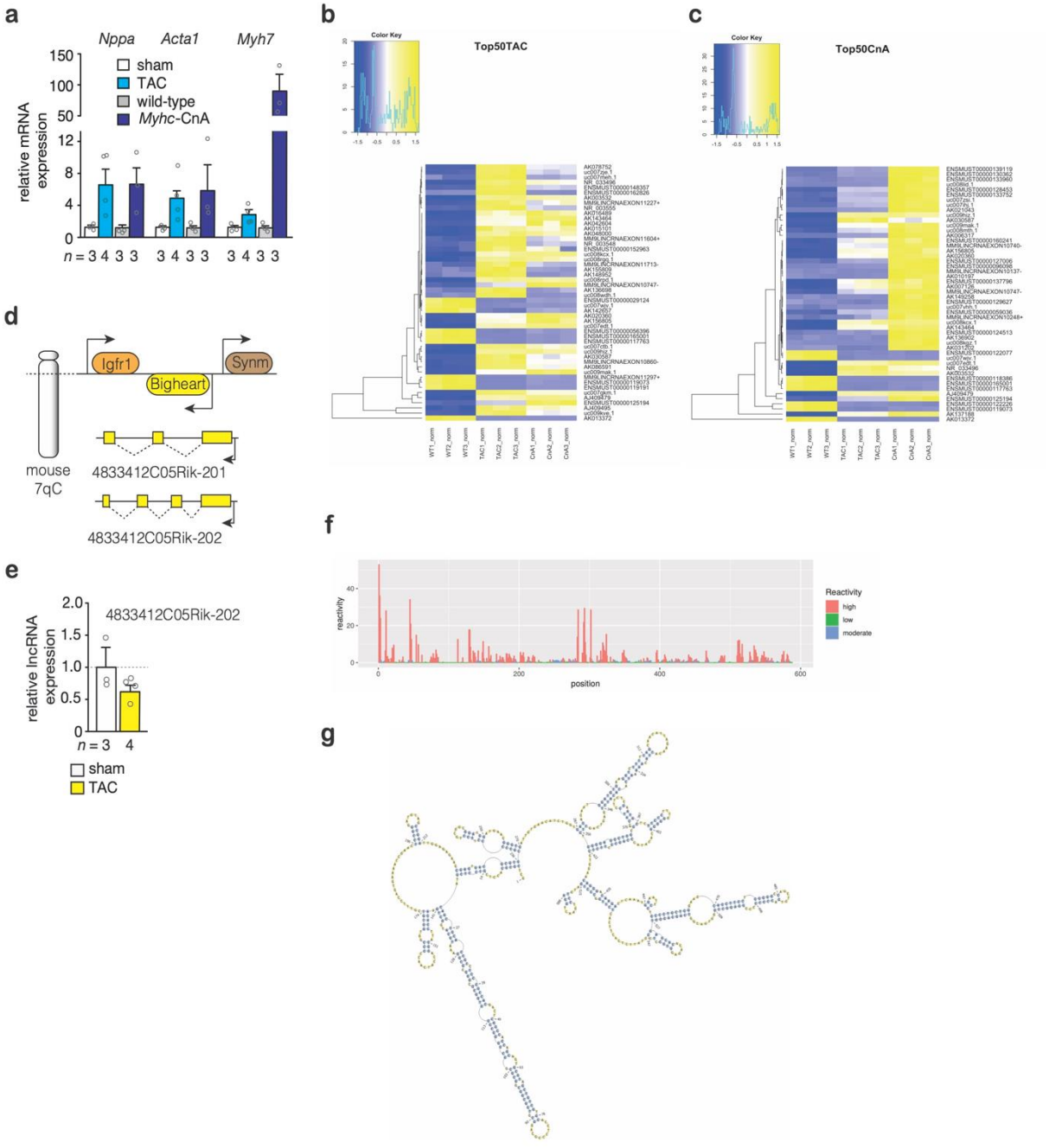
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Top50TAC
c
Color Key
Top50CnA
d
Igfr1
Bigheart
Synm
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reactivity
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low
moderate
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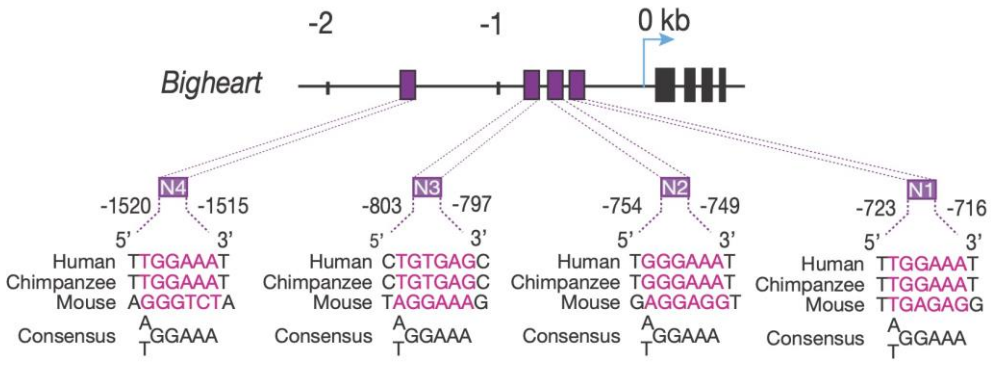
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3'
Human CTGTGAGC
Chimpanzee CTGTGAGC
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5'
3'
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Chimpanzee TGGGAAAT
Mouse GAGGAGGT
Consensus A/T GGAAA
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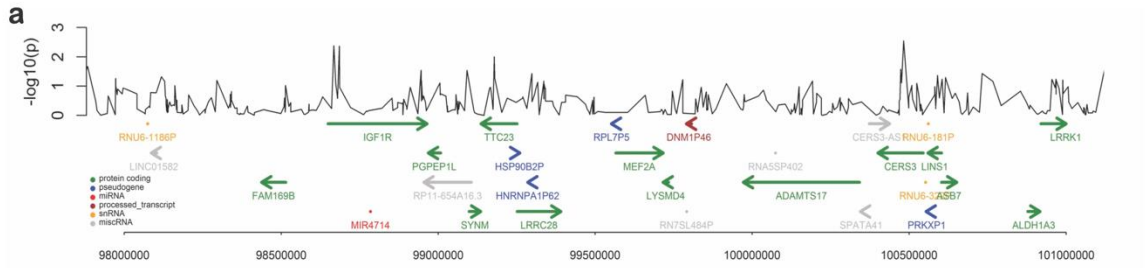
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LRRK1
LINC01582
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MEF2A
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CERS3
LINS1
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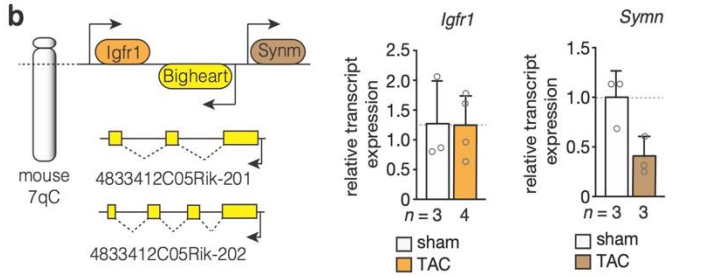
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Synm
Bigheart
mouse
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2.0
1.5
1.0
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sham
TAC
Symn
relative transcript expression
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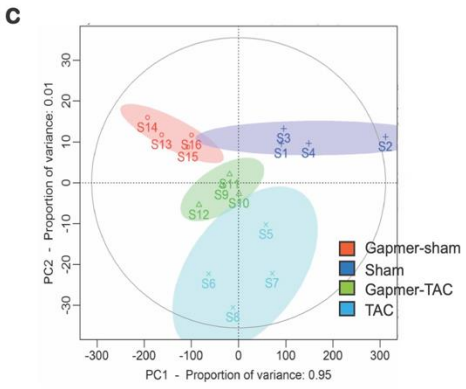
1.0
0.5
0
n = 3 3
sham
TAC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S14
S13
S16
S15
S3
S1
S4
S2
S11
S9
S10
S12
S5
S6
S7
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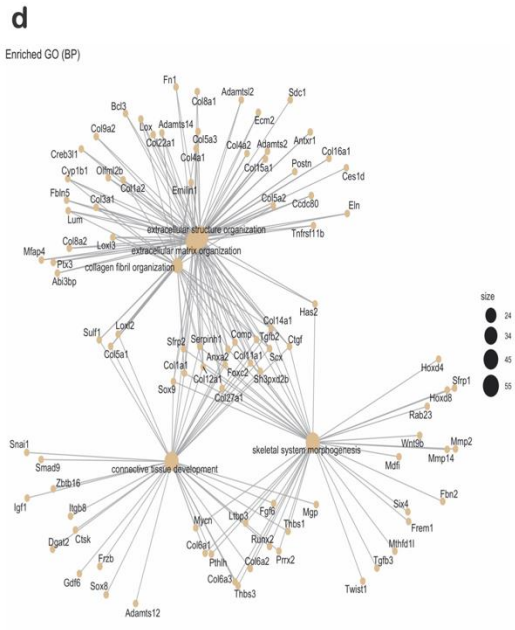
Gapmer-sham
Sham
Gapmer-TAC
TAC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extracellular matrix organization
collagen fibril organization
skeletal system morphogenesis
connective tissue development
size
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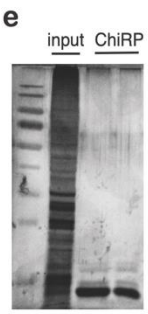
34
45
55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ChiRP


f
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ChiRP
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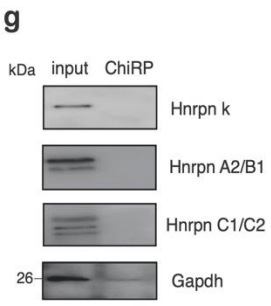
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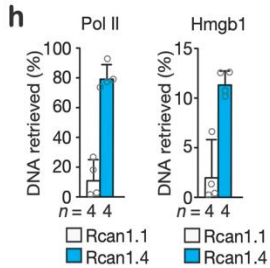
Hnrpn A2/B1
Hnrpn C1/C2
26
Gapdh


h
Pol II
Hmgb1
DNA retrieved (%)
100
80
60
40
20
0
n = 4 4
Rcan1.1
Rcan1.4
DNA retrieved (%)
15
10
5
0
n = 4 4
Rcan1.1
Rcan1.4

Supplementary Table 1. real-time PCR primers used in the study

Name	Primer Forward	Primer Reverse
L7	GAAGCTCATCTATGAGAAGGC	AAGACGAAGGAGCTGCAGAAC
Bigheart isoform 1 (rat/mouse)	GCATGAAGAGCAAGGTGTATGG	ATCTCGAGTGGATCAGCCCT
Bigheart isoform 2 (rat/mouse)	AGTGAAAACCGAACTCCGGG	TCACCCGTTCATCCATCCG
BIGHEART (human)	GGTGGTGTCGTTTCCAGTGA	ATCACCCGTTCATCCATCCG
Nppa	ATTGACAGGATTGGAGCCCAGA	ACACACCACAAGGGCTTAGGAT
Nppb	TGAAGGTGCTGTCCCAGATGAT	TCCAGCAGCTTCTGCATCTTGA
Acta1	CCCTCTTACTGGGGACTAAATCCA	CACATGGTGTCTAGTTTCAGAGGC
Myh7	CCTGCGGAAGTCTGAGAAGG	CTCGGGACACGATCTTGGC
Igfr1	ACGAGTGGAGAAATCTGCGG	ACTCGGTAATGACCGTGAGC
Symn	GCAAGCGGCTAATCGAAAGG	GCGTAGAGTGCCTGATCCTC
Rcan1.1	AGGGCGGGCACTGGAA	CTCCCTTAGCTCCGCTCCA
Rcan 1.4	GCTTGACTGAGAGAGCGAGTC	CCACACAAGCAATCAGGGAGC
Gapdh	GGGTCCCAGCTTAGGTTCAT	CCCAATACGGCCAAATCCGT
Promoter Rcan1.4	GCT TTT TAA GCT GCG GCT GG	AAC GAA CGA CTC GCTCTCTC
Promoter Rcan 1.1	GGG CAC TGG AAG GCG G	CTT AGC TCC GCT CCA CTC G
Promoter Gapdh	GCAGTGGCAAAGTGGAGATT	AAGATGGTGATGGGCTTCC
Myhrt	TAGGAGATGCAGTGGAAGGA	ATTGCCTCTGTTTCCTTGG

All oligos are depicted in 5' --> 3' direction. FW, forward; RV, reverse.

Supplementary Table 2. antisense DNA-tiling probes, grouped into ‘‘even’’ and ‘‘odd’’ sets based on their positions along mouse lncRNA *Bigheart* used in this study for Chromatin Isolation by RNA Purification (ChIRP).

PROBE #	PROBE (5'-> 3')	PROBE POSITION
1	CACTAGTCTGCTGCTGCATG	25
2	AGCTGAAGAAAGCACACAGT	129
3	AGCTGAAGAAAGCACACAGT	229
4	AAATCTCGAGTGGATCAGCC	334
5	CAGGAAAGATGGCATTTCCA	435
6	TCTTTGTAGACTAGGCTGGC	545
7	CAGGAGGCCAAGACGTATAA	646
8	CGAGCAGCATTTTATCTCTG	770
9	TCAGCGATTTCCAAAGGGAT	888
10	GGTTCTTCGAGAAATCCATG	997

Chapter 4

Adapted from

Mapping Chromatin Occupancy of LncRNA Bigheart reveals major impact in the mTOR signaling cascade regulation in pathological remodeling and heart development.

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(in preparation)

Abstract

Cardiac hypertrophy leads to maladaptive remodeling of the heart and frequently causes heart failure and death. The process requires a profound change in the transcriptional profile of cells, including the reactivation of several genes involved in cardiac morphogenesis and fetal development. Long non-

coding RNAs (LncRNAs) have assumed a significant role in regulating the transcriptional profile of the cells, both in physiological and pathological conditions. The expression of the lncRNA *Bigheart* increases significantly upon cardiac stress and is related to cardiac hypertrophy. In the present study, we report a novel function of *Bigheart* in regulating gene expression through direct chromatin binding in the mouse cardiomyocyte-like cell line HL-1. We generated a genome-wide chromatin-state map of *Bigheart* and high mobility group box 1 Hmgb1, which occupy various genomic regions related to cardiac hypertrophy and facilitate the expression of several genes involved in the mTOR pathway. *Bigheart* overexpression using a serotype 9 adeno-associated virus (AAV) induced activation of the mTOR pathway, while silencing this lncRNA with specific siRNAs blunted mTOR signaling. Additionally, *in vivo* GapmeR silencing of *Bigheart* led to the deregulation of several developmental genes.

Finally, we compared the expression of *Bigheart* throughout cardiac development in human tissues and in hIPSc-CMs, hypothesising that this lncRNA could be involved in activating the mTOR signaling pathway, both in cardiac hypertrophy and cardiac development, and its reactivation upon cardiac stress is part of the fetal gene program (FGP) reactivation.

INTRODUCTION

Heart failure is typically preceded by cardiac hypertrophy, a crucial adaptive response of the heart, caused by chronic cardiac stress or injury, including pressure or volume overload. A prolonged period of hypertrophic growth can lead to maladaptive remodeling of the heart.[1] Typically, cardiac hypertrophy shows in a thickening of the heart's walls due to the cardiomyocytes increasing size. Over time it becomes more difficult for the heart to contract correctly, and the demand for oxygen increases. The inability to meet the oxygen demand leads to cardiac ischemia and cell death. This process causes a significant change in the transcriptional profile[2]. One of the most evident changes is the reactivation of the fetal gene program (FGP). Fetal genes, such as natriuretic peptide A (ANP), natriuretic peptide B (BNP) myosin heavy chain, cardiac muscle β-isoform (MYHCβ or β-MHC) and skeletal muscle α- actin, are commonly reactivated in hypertrophy[3]. The reasons for this process are still unclear and the exact number of genes reactivated and the functions of these genes during cardiac remodeling remain to be elucidated.

Long non-coding RNAs (LncRNAs) are a class of RNAs widely studied for their involvement with different physiological and pathological conditions. The functional potential of this class of RNA ranges from interactions with other RNA and DNA to direct interaction with protein partners[4]. Interestingly, LncRNAs can regulate gene expression through direct interaction with different chromatin modifying enzymes[5]. This feature allows them to orchestrate different molecular processes, including biological processes, such as cardiac hypertrophy and heart development[6]. For example, the lncRNA myosin heavy-chain-associated RNA transcript (Mhrt) was downregulated in both cardiac development and hypertrophy. It has also been established that Mhrt can control the ratio of α-MHC (adult) to β-MHC (fetal) in failing hearts[7]. Similarly, it has been noted that the lncRNA H19 is downregulated in fetal and early postnatal growth[8]. However, H19 Is also strongly expressed in the early stage of pressure overload as a negative regulator of cardiomyocyte hypertrophy[9]. These findings point towards the involvement of LncRNAs in both developmental and pathological processes[10]. However, the full extent of the roles and functions of LncRNAs have not been explored yet.

The lncRNA Bigheart was first identified in cardiomyocytes defective for hnRNP U, a protein involved in the correct splicing of pre-mRNAs during postnatal cardiac development. *Bigheart* was practically absent in physiological conditions and was

highly expressed in cardiac hypertrophy and myocardial infarction.[11] Recently, our group showed that *Bigheart* and Hmgb1 (High Mobility Group 1) interact by *trans*-action with the promoters of *Rcan1.1* and *Rcan 1.4* and play an essential role in the calcineurin and NFAT (Nuclear factor of activated T-cells) pathway. However, whether *Bigheart* participates in regulating other pathways in heart disease by *trans*-action has not yet been established. Here, we generated a genome-wide chromatin-state map of *Bigheart* and Hmgb1 through a combination of Chromatin isolation by RNA Purification coupled with DNA sequencing (ChIRP-seq) and RNA-seq. Our results detail how *Bigheart* is expressed during the embryonic development of the heart and subsequently re-expressed in a failing heart. Furthermore, we show how this lncRNA is involved in cardiac remodeling by interacting with genes associated with cardiac hypertrophy and the mTOR pathway.

RESULTS

Bigheart binds 6,790 loci across the genome of HL-1 cells

To gain insight into how *Bigheart* is associated with the chromatin fraction and its influence on transcriptional modifications, we aimed to generate a genome-wide chromatin-state map of *Bigheart.* First, we used qPCR to establish the expression of *Bigheart* in HL-1, an immortalized mouse cardiomyocyte cell line that exhibits the morphology of differentiated cardiomyocytes [12]. *Bigheart* was previously reported to increase in expression upon cardiac injury, such as myocardial infraction (MI) or cardiac hypertrophy[11]. Compared to healthy cardiomyocytes, an increased expression of this lncRNA had been observed in cardiomyocytes subjected to pressure overload due to transverse aortic constriction (TAC). Interestingly, the expression of *Bigheart* in HL-1 was even higher than in TAC samples (**Supplementary Figure 1a).** Subsequently, we determined the regions that this lncRNA binds to in the chromatin by employing Chromatin isolation by RNA purification (ChIRP), coupled with DNA sequencing (CHIRP-seq)[13]. (**Figure 1a**). Additionally, we aimed to characterize the epigenomic profile in HL-1 and correlate the localization of *Bigheart* on the genome with active transcription through chromatin immunoprecipitation (ChIP) coupled with DNA sequencing (ChIP-seq). We selected three markers associated with active transcription[14], RNA pol II, H3K27ac, and H3K4me3, along with H3K27me3, which is associated with repressed regions[15]. We also took into account Hmgb1, which we previously proved to be a *Bigheart* interaction partner. We identified 6,790 peaks

corresponding to the genomic sites for *Bigheart* occupancy. Next, to minimize the bias and isolate significant peaks from background noise, we employed two algorithms, MACS1 (model-based analysis of ChIP-seq) using as cut-offs p-value $1e^{-7}$ for narrow peaks, and p-value $1e^{-1}$ for broad peaks[16]. We also used SICER (spatial clustering approach for the identification of ChIP-enriched regions) with a cut-off of FDR $1e^{-10}$ with gap parameter of 600 bp[15]. Furthermore, peak filtering was performed by removing false ChIP-Seq peaks, as defined by the ENCODE blacklist. This allowed us to identify peaks corresponding to the genomic sites for *Bigheart* occupancy. This strategy was applied to all the conditions, resulting in 23,039 peaks collectively for H3k27ac, 43,784 for H3K27me3 and 16,278 peaks for H3K4me3. (**Supplementary Table 1**). Peak distribution of RNA pol II, H3K27ac, H3K4me3 and H3K27me3 around transcription start sites (TSSs) (±5 kb) seem to confirm an enrichment in promoter areas as expected (**Supplementary Figure 1b).** Moreover, as anticipated, the Pearson correlation between *Bigheart* and Hmgb11 peaks was remarkably high (0.85), suggesting that the two are most likely interacting, as supported by experiments previously reported from our group. Additionally, we noticed a strong correlation between the localization of Hmgb1 and RNA pol II (0.80). (**Figure 1b**).

Bigheart showed similar consensus sequences to MyoD1

Occupancy peaks distribution showed that all chromosomes (except chr Y) were bound by *Bigheart,* with chromosome 6 and 1 having the most abundant peaks, at 25% and 14% respectively **(Figure 1c).** The same analysis also revealed that most of the peaks were in distant intragenic loci and introns **(Figure 1d).** We identified a total of 469 target genes for *Bigheart,* based on the intensity of the signal (fold change over control) and the location of the peaks (every peak 10 kb upstream or downstream of a gene was considered as being associated with that gene)[17,18] (**Supplementary Table 2**). Further analysis revealed that this lncRNA can directly bind to the promoter area of 43 genes (**Supplementary Table 3**), including among the top 10 (sorted by signal intensity) essential genes involved in the positive regulation of cardiac hypertrophy, such as Mitofilin (Immt), and Pyruvate Dehydrogenase Kinase 1 (Pdk1) [19-21]. From our ChiRP-seq data we confirmed that *Bigheart* physically interacts with their promoters **(Supplementary Figure 1 f-g).** Next, to further evaluate *Bigheart's* target, we employed Over representation analyses (ORA) using the database from the Kyoto Encyclopaedia of Genes and Genomes KEGG [22], and found a significant (set as p-value<0.05 and FDR<0.05) over-representation in genes related to the mTOR pathway (p-value $3.73e^{-04}$) and GnRH pathway (p-value $2.95e^{-05}$) **(Figure 1e).** The activation of the mTOR pathway promotes pathological hypertrophy during pressure overload[23].

The interaction of *Bigheart* with many genes related to cardiac hypertrophy is consistent with the previous observations on *Bigheart* as a pro-hypertrophic LncRNA. Consequently, we considered the possibility that *Bigheart* could regulate the expression of several genes from distal regulatory elements[24]. In fact, it has been shown in the past that lncRNAs and transcriptional factors can influence gene expression by interacting with distant regulatory elements or by influencing the three-dimensional structure of the chromatin[4,5]. In order to evaluate possible additional targets for Bigheart, we employed T-Gene to predict the regulatory targets of *Bigheart* based on its distance from a potential target TSS[25]. We predicted a list of 87 genes regulated by *Bigheart* **(Supplementary Table 4)** KEGG showed enrichment in the JAK-STAT signaling pathway (p-value=9.10e-^{04}) **(Supplementary Figure 1e)**. Although, the JAK-STAT signaling is connected to the response to cardiac hypertrophy[26], we did not further evaluate results from this list and focused on the direct 489 targets we defined for *Bigheart.* Next, to see whether it was possible to identify a motif for *Bigheart,* we employed De novo Motif discovery analysis by MEME-chip [27] and identified a palindromic consensus sequence of CTGCTG. Additionally, Motif comparison found that these motifs significantly resembled previous known motifs such as Myogenic Differentiation 1 MyoD1 (p-value=1.96e^{-05}), Bha15 (alias MIST1) (p-value=3.08e^{-04}) and ASLC1 (p-value= 1.35e^{-03}) consensus sequences. **(Figure 1f).** MyoD1 is a differentiation marker for myogenic precursors and a transcriptional factor pivotal for myocyte commitment[28]. Hmgb1 instead showed a strong similarity with several members of the Sp/KLF family of transcription factors, namely (SP5 and Sp1) and Early growth response protein 1 (EGR1) **(Supplementary Figure 1i).** These results suggested that *Bigheart* is strongly involved in regulating a group of genes associated with muscle differentiation and cardiac hypertrophy.

Bigheart is expressed in cardiac but not skeletal muscle

Next, we focused on the similarity between MyoD1 and *Bigheart.* Skeletal and cardiac muscle cells express overlapping sets of muscle-specific genes. The regulation of muscle-specific transcription in skeletal muscle is controlled by MyoD1[29]. Although many of the genes that are regulated by MyoD1 are also expressed in cardiac muscle, MyoD1 have never been detected in cardiac muscle[30]. This was confirmed by ChIP-seq data of markers of positive transcription (H3K27ac, and H3K4me3) and repressed transcription (H3K27me3) obtained from the Encyclopedia of DNA Elements (ENCODE) database. H3K27me3 clearly showed a strong transcriptional repression of MyoD1 locus throughout murine cardiac differentiation, moreover GO analysis on *Bigheart* targets showed an enrichment on genes involved in striated muscle

differentiation (p-value $7.124e^{-05}$) **(Figure 1g).** We noticed a similar trend for MyoD1 with GO Biological Processes (p-value $8.89e^{-08}$), as expected from the master gene for skeletal muscle differentiation (**Supplementary Figure 1j**). Therefore, we wanted to address whether *Bigheart* was expressed in myocytes. Semi-quantitative PCR showed that *Bigheart* is not expressed in mice skeletal muscle tissue but is detectable in heart tissue **(Figure 1i),** Comparably, ChIP-seq data from ENCODE on mouse myocytes showed a strong transcriptional repression in *Bigheart* locus, **(Figure 1j).** This observation suggests that *Bigheart* is specifically necessary to the cardiac muscles. Interestingly, evaluation of MyoD1 targets from ENCODE with KEGG showed an enrichment in mTOR signaling (p-value $9.88e^{-12}$) **(Supplementary Figure 1j),** suggesting the importance of this pathway during the differentiation. Therefore, we hypothesized that *Bigheart* could replace MyoD1 during cardiac differentiation, by interacting with similar targets. To study this point further, we compared CHIP-seq data from ENCODE for MyoD1 with our data of CHIRP-seq. However, comparison between ChIP-seq data of MyoD1 from ENCODE and our ChiRP-seq data with Pearson's correlation analysis showed no correlation between *Bigheart* and MyoD1 (figure1k). Nevertheless, we also considered the possibility that although MyoD1 and *Bigheart* do not bind the same genomic areas (for instance the promoter), they could bind and regulate the transcription of the same genes[31].To investigate this possibility, we downloaded the complete list of all MyoD1 targets from the ENCODE transcription factor targets dataset. We compared MyoD1 with the *Bigheart* target genes in HL-1 gained by the CHIRP-seq. Out of the 469 *Bigheart* targets, 113 were in common with the MyoD1 data set **(Supplementary Table 6**). To compute the significance of the overlap we applied the hypergeometric distribution (p-value= $8.12e^{-3}$) revealing a significant overlap **(Figure 1l).** Our findings therefore suggest that *Bigheart* could be involved in muscle differentiation and share a pool of targets with MyoD1. However, *Bigheart* expression is limited to the cardiac tissue, and we did not find evidence of *Bigheart* involvement in myocytes differentiation.

Bigheart binds 247 loci across the genome of HL-1 and adult cardiomyocytes

While HL-1 cells are widely accepted and used as a cardiac cell model[32], they are derived from a cardiomyocyte tumour lineage. Hence their transcriptional profiles might resemble, but not perfectly mimic the trend of healthy cardiomyocytes as recently reported[31]. Additionally, several studies proved that tumours activate the AKT and mTOR signaling pathways[33]. This is confirmed from our data too, as KEGG analysis of HL-1 transcription reveals the mTOR signaling pathway as highly enriched (p-value=$1.06e^{-16}$). (**Supplementary Figure 1K).** Consequently, we wanted to address

the role of *Bigheart* in HL-1 cells, to ascertain that our data could be translated in cardiomyocytes. To compare them, we downloaded the epigenomic profiles of markers of positive transcription (H3K27ac, H3K4me3) from CHIP-seq data on 8-week-old mice cardiomyocytes (CMs) (fold change over control) from the ENCODE database[34] and compared them against our results from CHIP-seq in HL-1. As expected, we noticed a very different pattern at the *Bigheart* locus. Adult cardiomyocytes did not show enrichment in markers of active transcription around the *Bigheart* locus. On the other hand, HL-1 cells showed a robust and clear enrichment in H3K27ac and H3K4me3 signal (fold change over control), suggesting an active and constant transcription **(Figure 4c).** Next, we compared the peaks distributions of the histones H3K27ac and H3K4me3 in HL-1 against the counterparts in CMs. The data showed a very similar distribution around transcription start sites (TSS) (± 2 kb) between HL-1 and CMs **(Figure 2a).** Pearson correlation showed a value of 0.78 and 0.72% between H3K27ac and H3K4me3 (**Figure 2d).** However, studying the non-common genes with active transcription annotated as genes with merged peaks for H3K27ac and H3K4me3, within 10kb upstream of the closest TSS[35], we isolated 589 genes expressed only in HL-1, and 928 genes specific to adult cardiomyocytes **(Supplementary Table 6**). KEGG revealed strong enrichment in genes associated with the cell cycle (p-value $1.02e^{-10}$) for the genes specific for HL-1 **(Figure 2b).** Instead, the 928 genes specific for adult cardiomyocyte reveal a strong enrichment in genes associated with angiogenesis and vasculature development (p-value $2{,}54e^{-10}$) **(Figure 2c).** Once we asserted the transcriptional profile of HL-1 and CMs, we focused our attention on the genes targeted by *Bigheart,* trying to establish how many of these genes were also expressed in adult cardiomyocytes or exclusively in HL-1. Therefore, we compared the overall transcriptional profile of adult cardiomyocytes (merged data from H3K27ac and H3K4me3) against the *Bigheart* targets. Out of 469 genes, only 247 are common between cardiomyocytes and *Bigheart* (hypergeometric distribution p-value=$1.8e^{-3}$) **(Figure 2e).** Therefore, we limited our focus on these loci. Next, we focused our attention on the molecular meaning of the genes regulated by *Bigheart.* Previously we employed KEEG analysis on the overall list of genes bound by this lncRNA and we found the mTOR pathway strongly enriched. Therefore, to confirm to accuracy of our study we verified if mTOR related genes bound by *Bigheart* were also expressed in adult CMs. Interestingly, all the genes we previously defined as part of the mTOR pathway are conserved in CMs. **(Supplementary table 6)**. The result included 9 genes pivotal to the mTOR pathway (**Supplementary figure 2a)**. Next, validation of these 9 genes by qPCR in mice that underwent Transverse aortic constriction (TAC) versus Sham revealed the upregulation of 5 genes upon cardiac stress: CAP-Gly Domain

Containing Linker Protein 1(Clip1)[36], Calcium Binding Protein 39(Cab39),[37] RAF proto-oncogene serine/threonine-protein kinase (Raf1)[38], Eukaryotic Translation Initiation Factor 2 Alpha Kinase 3 (Eif2ak3/Perk)[39], and Eukaryotic translation initiation factor 4E (Eif4e)[40] **(Figure 2f).** Visualization of ChiRP-seq and ChIP-seq data in HL-1 confirmed the presence of *Bigheart* peaks in these 5 genes, suggesting a possible *trans*-action for *Bigheart* on these genes during cardiac hypertrophy**. (Figure 2g)**

***Bigheart* influences activation of the mTOR signaling pathway in vivo and vitro**

To further evaluate the function of *Bigheart* in influencing genes related to the mTOR pathway, we measured the expression of *Bigheart* in neonatal rat cardiomyocytes that underwent hypertrophy, induced by treatment with phenylephrine (PE) and isoproterenol (iso)[41]. The data demonstrated that *Bigheart* was on average 2-fold higher than in untreated cells **(Figure 3b).** Similarly, Cab39, Raf1, Eif2ak3, and Eif4e increased by 3-fold upon Pe- Iso treatment **(Figure 3c).** Subsequently, following our previous observation on the high expression of *Bigheart* in HL-1 cells, we silenced *Bigheart* with siRNA against this LncRNA in HL-1 cells. The data showed that *Bigheart* downregulation in HL-1 by the siRNA **(Figure 3e)** was followed by dramatic repression of several genes associated with the mTOR pathway (**Figure 3f).** Next, to better address the effect of *Bigheart* on the mTOR pathway, we employed serotype 9 adeno-associated viral (AAV9) vectors. AAV9 vectors[42] expressing human *Bigheart* (AAV9-BH) or a control vector with luciferase (AAV9-luc) were injected intraperitoneally in neonatal mice at day 1 and tissue was harvested 3 weeks afterwards **(Figure 3i).** The data clearly showed a successful overexpression of *Bigheart* **(Figure 3i)** as well as a histological profile towards cardiac hypertrophy and remodeling **(Figure 3h).** Remarkably, in line with our previous experiments, we observed a significant overexpression of the mTOR related genes in vivo **(Figure 3i).** Consequently, we focused our attention on the activation of the mTOR signaling. Although *Bigheart* seem involved in regulating several genes associate to this pathway we did not establish yet whether *Bigheart* expression could directly influence the mTOR signaling. Subsequently, we evaluated the level of mTOR pathway activation upon *Bigheart* infection. Western blot analysis on mTOR 4ebp1 and p70s6k, two main downstream players in mTOR signaling [43], showed a strong activation in hearts infected with AAV9-BH **(Figure 3 k-l).** Taken together, these results showed that an elevated level of *Bigheart* expression *in vivo* or *in vitro* can significantly influence the mTOR pathway activation, suggesting the crucial role *Bigheart* plays in regulating the mTOR pathway in cardiac cells.

Bigheart is involved in cardiac development through the mTOR pathway

Next, we focused our attention on understanding the molecular function of *Bigheart.* We performed an RNA-seq on rats that underwent TAC and Sham surgery with and without Gapmer, to understand which genes were mostly affected by *Bigheart* downregulation. We compared the genes that significantly *(set as p-value<0.05 and adjp-value<0.05)* changed in expression between rats subjected to TAC surgery and Sham, rats treated with GapMer for Bigheart in mice that underwent TAC surgery **(Figure 4a).** By analysing the RNA-seq data we identified 2349 genes with a significant change in expression in TAC vs Sham We also noticed a 4-fold increase of *Bigheart* (4833412C05Rik) in TAC samples, consistent with our previous data (**Supplementary Table 5).** We then compared the two lists, (TAC mice treated with Gapmer BH vs TAC and TAC vs Sham) looking for genes with significant expression variations following the silencing of *Bigheart*. We identified 697 genes **(Figure 4b)** strongly affected by *Bigheart* depletion. Out of these genes, 459 were downregulated upon *Bigheart* depletion and 238 genes were upregulated **(Figure 4c).** Among the most significantly downregulated genes we found Regulator of calcineurin Rcan1. This is consistent with previous results from our laboratory, where we demonstrate that *Bigheart* can regulate Rcan1 transcription by trans-action. On the other hand, among the upregulated genes, we found Hepatic Leukemia Factor (HlF) and Thyrotroph embryonic factor TEF. Both genes are clock-controlled PAR bZip transcription factors and are involved in circadian rhythm gene regulation. Interestingly, Tef and Hlf knockout mice developed cardiac hypertrophy[44] .Therefore also regulation of those genes might have a specific proposes in regulating cardiac hypertrophy. To get insights into the molecular functions altered by Bigheart depletion we performed ORA analysis using KEGG. We noticed that overall Bigheart depletion influences gene related to ECM transition and regulation (p-value $1.39e^{-13}$), PIK3-AKT signaling pathway (p-value $6.20e^{-06}$) along with regulation of TGF-beta and calcium signaling pathway (p-value $4.97e^{-5}$), **(Figure 4d).** All these pathways are strongly involved in cardiac hypertrophy[45], reinforcing our previous observation that *Bigheart* is strongly associated with cardiac hypertrophy. Additionally, both PIK3-AKT cascade and ECM-receptors are strongly involved in regulating mTOR pathway[46,47] Subsequently, we performed a GO and network pathway analysis to understand the consequences of *Bigheart* silencing. GO biological process analysis revealed a clear trend; silencing of *Bigheart* strongly affected genes of skeletal system development and tissue remodeling (p-value $8.60e^{-10}$ and p-value $8.05e^{-10}$). A similar result was collected applying network analysis[48] in TAC mice treated with Gapmer BH vs TAC on RNA-seq data **(Supplemetary Figure 3a-b)** with a clear trend in Tissue

development ($1.36e^{-25}$). These data suggested a significant contribution of *Bigheart* to the cardiac development and they are consistent with our result from ChIRP-seq suggesting a strong correlation between Bigheart and muscle development. Furthermore, our RNA-seq, ChIP-seq and qPCR data collectively suggested that *Bigheart* is actively transcribed upon pressure overload**.** This is also consistent with literature[49], suggesting a role of *Bigheart* in cardiac Hypertrophy. However, our data RNA-seq, PCR and ChIP-seq from adult heart did not show a significant expression of *Bigheart* in a physiological situation **(Figure 4 g),** rather we observed a specific upregulation upon hypertrophic stimulus and abundant expression in HL-1. Since pressure overload can reactivate several fetal genes , such as nPPb and nPPa,[50] and both RNA-seq and ChIRP-seq suggested a role for this LncRNA in muscles development, we asked whether *Bigheart* was also expressed during cardiogenesis and reactivates upon stress. To investigate this hypothesis, we utilized published chromatin immunoprecipitation followed by sequencing (ChIP-seq) data of H3K4me3, H3k27ac and H3k27me3 in embryonic hearts from the ENCODE portal. We used embryotic hearts from day E11.5 since day 0, and as an adult tissue we used 8-week-old. We then compared the chromatin states of *Bigheart* locus (4833412C05Rik) throughout the development and noticed a strong enrichment of H3K4me3 and H3k27ac signals in the promoter of *Bigheart* during the embryogenesis, suggesting an active transcription **(Figure 4g).** However, the signal completely disappeared in adulthood, suggesting *Bigheart* plays a role in cardiac commitment but not in physiological cardiac expression. This data are in line with previously published data from the Kaessmann lab [51]. Furthermore, the same trend was observed also for the previously identified *Bigheart* human homolog (RP11-6545163, Ensembl ID AC036108.1**) (Figure 4e).** We therefore began to theorize that the role of *Bigheart* was to modulate the expression of some genes necessary for cardiac or muscle differentiation. We then further evaluated the role of *Bigheart* in cardiac differentiation in human induced pluripotent stem cells differentiated in cardiomyocytes (hIPCs-CMs). We evaluated the expression of *Bigheart* throughout the process of differentiation in cardiomyocytes from day 0 (undifferentiated), until day 25 (fully differentiated)[52] **(Figure 4h**). The data demonstrates that *Bigheart* was on average 300-fold higher expressed in the early phase of differentiation (D0 to D3) and slowly decreased in expression as cellular differentiation progressed, after which the expression remained low **(Figure. 4l).** Similarly, *Bigheart* expression in the differentiated cells was very low compared to not differentiated cells. The mTOR pathway is reported as a relevant player in embryonic heart development [53,54]. Therefore, we evaluated the levels of activation of mTOR in differentiating cells. The data clearly showed a reduction in

mTOR activation, consistent with the *Bigheart* trend during differentiation **(Figure 4J).** Taken together these data suggest that *Bigheart* could regulate the mTOR pathway throughout the differentiation, both in humans and mice.

DISCUSSION

Pathological cardiac hypertrophy and the following cardiac failure are characterized by a series of complex molecular processes that involve transcriptional, posttranscriptional, and epigenetic alterations. These events resemble those observed during fetal cardiac development[55]. Therefore, one of the hallmarks of cardiac hypertrophy is the reactivation of several genes involved in the embryonic development, collected under the name of fetal gene program or FGP[56]. The reasons for their reactivation and the function of many of these genes are still unknown. Reactivation of this class of genes can logically include any class of gene including non-coding genes. LncRNAs are a class of RNAs that play a fundamental role in governing cardiac hypertrophic processes. The LncRNA *Bigheart* was first described as an RNA highly expressed in cardiac hypertrophy and myocardial infarction. Following, we described *Bigheart* as a trans-acting regulator of the Rcan1 (Regulator of Calcineurin 1) and involved in positive feedback in the calcineurin NFAT pathway[57]. Based on these data, we hypothesized that *Bigheart* could regulate several genes involved in the development of cardiac hypertrophy. Hence, this study aimed to investigate the role of *Bigheart* in cardiac hypertrophy by creating a genome-wide chromatin-state map of *Bigheart*.

Bigheart is highly expressed in samples of mice subjected to TAC surgery. Interestingly, we found this lncRNA highly expressed in HL-1, a cardiomyocyte-like cell line. The HL-1 is a cell line derived from a tumour but maintains a cardiac phenotype[58]. *Bigheart* had only been identified in cells in cardiac hypertrophy previously, hence we tried to understand the reasons for its expression in HL-1. Comparing the markers of active transcription between cardiomyocytes and HL-1 mice, we noted a significant similarity between the two cell types. The largest discrepancies are associated with the cell cycle, so we established that although not identical, HL-1 could represent a good model to study *Bigheart* chromatin occupancy in a cardiac context. As Hmgb1 was previously described as a *Bigheart* partner, we also decided to study its interactions with chromatin. As described in the literature, Hmgb1 is involved in chromatin remodeling and is spread uniformly throughout the genome[59]. Surprisingly, it seems to be correlated with the position of the RNA Pol2, suggesting that Hmgb1 and RNA Pol2 are part of the same functional complex. On the other hand, *Bigheart*

appears to be associated with a few specific DNA regions, often in genes of the mTOR pathway. Indeed, in line with *Bigheart* upregulation, the mTOR activation promotes pathological hypertrophy[60]. Interestingly, mTOR activation is also required for cancer cells to proliferate and grow[61]. This observation could explain the high and stable expression of *Bigheart* in HL-1. In fact, tumorigenic cells tend to reactivate several fetal genes[62]. Moreover, *Bigheart* seems to be involved in several cardiac and immune system differentiation processes. Further evidence of its involvement in differentiation is the similarity with the transcription factor MyoD1, a key regulator of Cardiac and muscle commitment[63] and Aslc1, homolog of MyoD1 and master gene of neural differentiation.[64]

Next, since HL-1 appears to be a model not perfectly aligned with the cardiomyocytes epigenetic profile, we decided to observe which genes are influenced by *Bigheart* silencing in mice subjected to TAC surgery. *Bigheart* silencing leads to an important down-regulation of heart development genes alongside deregulation of the AKT pathway, which together with mTOR signaling is crucial to many aspects of cell growth and survival, in physiological as well as in pathological conditions[65], suggesting that *Bigheart* may be associated with fetal development and ATK/mTOR. We then verified our hypothesis in vivo by forcing overexpression of *Bigheart* with AAV9 in postnatal mice hearts. The data confirmed that several genes of the mTOR pathway are under direct transcriptional regulation of *Bigheart* and the activation of the mTOR pathway is severely affected upon upregulation or downregulation of this lncRNA.

Additionally, as mTOR is required for embryonic cardiovascular development[66] we speculated that *Bigheart* was involved in embryonic development by regulating activation of the mTOR signaling. We studied the *Bigheart* locus during embryonic development from day E11.5 till day 0 and observed how *Bigheart* is expressed in the embryonic period and repressed in adulthood (week 8), to be reactivated only due to cardiac hypertrophy or myocardial infarction. A similar trend can be observed both in humans and mice. Moreover, in human induced pluripotent stem cells under cardiac commitment (hiPSC-Cm) *Bigheart* and mTOR pathway are stably expressed and active only during cardiac differentiation and repressed afterwards (from day 11 to 25), alongside with mTOR activation. Thereby, our data suggest a novel role for *Bigheart* in HL-1 and in cardiac development. There are several examples of lncRNA involved simultaneously in cardiac disease and cardiac development. *MHRT*, *FENDRR* and *CARMEN* are three lncRNAs involved in cardiac specification and cardiac commitment [67], however, they are also reported to be related to cardiac hypertrophy,[68] suggesting

that many lncRNAs may have multiple roles depending on the context. Therefore, the trend identified for *Bigheart* corresponds to that identified for other lncRNAs with similar functions. Further studies are needed to identify all the molecular mechanisms involved in this lncRNA. Nevertheless, the genome-wide chromatin-state maps of *Bigheart* that we have extrapolated from HL-1 can be used to identify new possible candidates in cardiac development and in the study of cardiac hypertrophy.

METHODS

Animal models Hearts were removed, rinsed in ice-cold phosphate buffered saline, atria removed, snap-frozen and stored in liquid nitrogen until use. Adult BL6CBAF1/j mice (12–16-week-old) underwent transverse aortic constriction (TAC) by subjecting the aorta to a defined 27-gauge constriction between the first and second truncus of the aortic arch as described previously in detail.[69] At 4 weeks after TAC surgery, animals were sacrificed, hearts removed, rinsed in ice-cold phosphate buffered saline, atria removed, snap-frozen and stored in liquid nitrogen until use.

Cell cultures and reagents HL-1 cells (Sigma-Aldrich, cat# SCC065) were purchased from respective vendors. HL-1 were cultured with Claycomb Medium Medium (Sigma-Aldrich, cat# 51800C) supplemented with 10% FBS, 100 units/ml penicillin/streptomycin, 0.1 mmol/liter of Norepinephrine and 2 mmol/liter L-glutamine (Thermo Fisher) Humans RNAs samples were purchased from Takara (*Human MTC™ Panel I)* Lot Number. 636742.

Chromatin Immunoprecipitation (ChIP) Chip has been performed with ChIP-IT High Sensitivity Kit following manufacturer's instruction and ChIP-Seq library produced by Active Motif. 150 million HL-1 cells were cultured in Corning® Primaria™ 10-cm dishes and cross-linked as per manufacturer's instruction. Antibodies used are included in Supplementary Table 2.

Chromatin isolation by RNA purification (ChIRP). ChiRP was performed as described previously [13] with minor modifications. A series of 10 antisense DNA probes with 3'-Biotin-TEG modification were designed against the murine *Bigheart* full-length sequence (**Table 3**) using LGC Biosearch Technologies software (https://www.biosearchtech.com/support/tools/design-software). 50 million HL-1 cells were cultured in Corning® Primaria™ 10-cm dishes and cross-linked in 1% glutaraldehyde for 30 min, followed by 0.125 M glycine quenching for 5 min. Next, the pellets were decanted with subsequent centrifugation and washing steps in PBS, resuspended in lysis buffer with fresh PMFS and PhosSTOP and protease inhibitor cocktail (Roche), kept on ice for 10 minutes and sonicated until the gDNA was in the size range of 100–500 bp. At this point, chromatin was diluted in hybridization buffer (500mM NaCl, 1%SDS, 100mM Tris 7.0, 10mM EDTA, 15% Formamide, add DTT, PMSF, P.I, and Superase-in fresh), and probes were added at a final concentration of

100 pmol per 100 µL of chromatin for 24 hrs. Then 100 µL of streptavidin magnetic C1 beads per 100 pmol of probes were added and allowed to hybridize for 1 hr. Beads were then divided from the solution with a magnetic stand and washed 5 times with 1 ml wash buffer (2x SSC, 0.5% SDS, add DTT and PMSF) and processed for elution of RNA, DNA, or proteins. For the RNA extraction, beads were resuspended in 50 µL 10× RNA elution buffer (Tris 7.0, 1% SDS) and boiled for 15 min, and RNA extracted with the Direct-zol RNA purification Kit (ZYMO Research) and RNA quality assessed with Nanodrop. For DNA elution, the beads were resuspended in 150 µL 3× volume of DNA elution buffer (50 mM NaHCO3, 1% SDS, 200 mM NaCl), and DNA eluted with a cocktail of 100 ug/ml RNase A and 0.1 units/microliter RNase H and samples purified with the Quick-DNA™ Miniprep Plus Kit (Qiagen). For protein elution, beads were resuspended in 3x original volume of DNase buffer (100 mM NaCl and 0.1% NP-40), and protein was eluted with a cocktail of 100 ug/ml RNase A (Sigma-Aldrich) and 0.1 units/ µL RNase H (Epicenter), and 100 U/ml DNase I (Invitrogen) at 37 °C for 30 minutes.

ChIP-seq and ChIRP-seq. Analysis and peak calling was performed by Active Motif Sequence Analysis from 75-nt single-end sequence reads that were generated by Illumina sequencing using NextSeq500, then the sequences are mapped to the genome using the BWA aligner (“bwa aln/samse” with default settings). Alignment information for each read is stored in the BAM format. Only reads that pass Illumina’s purity filter, align with no more than 2 mismatches, and map uniquely to the genome are used in the subsequent analyses. In addition, duplicate reads (“PCR duplicates”) are removed. Next, the 5´-ends of the aligned reads (= “tags”) represent the end of ChIP/IP-fragments, the tags are extended in silico at their 3´ends to a length of 200 bp, which corresponds to the average fragment length in the size-selected library. To identify the density of fragments (extended tags) along the genome, the genome is divided into 32-nt bins and the number of fragments in each bin is determined. Peak calling was performed with MACS1.4.3 using as setup for identification of narrow peaks a p-value 1e-7 of and for broad peaks a p-value 1e-1. Instead SICER (v1.1) was set with a cut-off of FDR 1e-10 and with gap parameter of 600 bp. Finally, Peak filtering was performed by removing false ChIP-Seq peaks as defined within the ENCODE blacklist. Next, peaks were annotated using the ChIPseeker package in R, and Peak distribution was calculated by normalizing the total length of peaks per chromosome by the size of their respective chromosome.

The full list of programs used to perform the study are bcl2fastq2 (v2.20): processing of Illumina base-call data and demultiplexing. bwa (v0.7.12): alignment of reads to

reference genome. Samtools (v0.1.19): processing of BAM files. BEDtools (v2.25.0): processing of BED files. MACS (v1.4.3): peak calling; narrow peaks. SICER (v1.1): peak calling: broad peaks. wigToBigWig (v4): generation of bigWIG files.

Bioinformatics analyses: Heatmaps for comparison between H3K4me3 peaks in HL-1 and CMs were generated using the EaSeq analysis software from bigwig files previously obtained from either Active motif (described above) or from ENCODE processed accordingly with the information contained on the website.

The Pearson's correlations heatmaps was generated using GALAXY (https://usegalaxy.org/) with default parameters. NetworkAnalyst 3.0 was used to conduct overrepresentation analysis (ORA) by using KEGG database ranked with Welch's t-test, and Network analysis using Signor 2.0 as a reference database and applying default parameters[70] Instead, Gene Ontology analyses on selected genes were performed using GREAT version 4.04. Motif analyses was performed with MEME-CHIP (https://meme-suite.org),the parameters were used as such: The width of the expected motif was set between 6 and 50, The expected occurrence per sequence was set to 0 or 1, and the maximum number of motifs to search for was 5. The prediction of target genes was performed using the default parameters with T-GENE (https://meme-suite.org).

Primary neonatal rat cardiomyocytes cultures and siRNA transfections

Cardiomyocyte cultures were isolated by enzymatic dissociation of 1 day-old neonatal rat hearts as described previously in detail[71]. Cells were seeded in 10-cm dishes for RNA isolation or in 6-well plates to perform immunocytochemistry. One day after plating, the neonatal rat cardiomyocytes were transfected with inhibitors (Dicer Substrate Duplex RNAs, IDT) of specific lncRNA transcripts (10nM) using Oligofectamin (Invitrogen). After 24 hours, cardiomyocyte hypertrophy was induced by phenylephrine (PE, 10 µM) stimulation for an additional 24h, as described previously[72].

Human induced pluripotent stem cell (hiPSC) derivation, maintenance, and differentiation to cardiomyocytes. Wildtype BXS0116 hiPSCs, derived from bone marrow CD34+ cells obtained from a healthy Caucasian female donor were purchased from ATCC (ATCC® ACS-1030™). Cells were maintained in Matrigel-coated plates (Corning®), passaged with Versene solution (Thermo Fisher Scientific) and cultured

in StemMACS™ iPS-Brew XF medium (Miltenyi Biotec) supplemented on the first day after passaging with 5μM ROCK inhibitor (Stemolecule Y27632). Differentiation of hiPSCs to cardiomyocytes was performed by modulation of the WNT signaling pathway. Briefly, differentiation was started on cells cultured on Matrigel-coated plates when reaching 80-90% confluence. Cells were initially cultured for 3 days in mesodermal induction medium, consisting of RPMI 1640 medium (Thermo Fisher) supplemented with 1% 100X glutaMAX (Thermo Fisher), 1% 100X sodium pyruvate (Invitrogen), 1% 100X penicillin/streptomycin (Invitrogen), 2% 50X B-27® Serum-Free Supplement (Invitrogen), 200uM L-ascorbic acid 2 phosphate sesquimagnesium salt hydrate (Sigma), 1uM CHIR99021 (Stemgent), 5 ng/ml Recombinant human BMP4 (R&D Systems), 9 ng/ml Recombinant Human/Mouse/rat Activin A (R&D Systems) and 5 ng/ml human FGF-2 (Miltenyi Biotec). Then, cells were cultured for 7 days in cardiac differentiation medium, consisting of RPMI 1640 medium (Thermo Fisher) supplemented with 1% 100X glutaMAX (Thermo Fisher), 1% 100X sodium pyruvate (Invitrogen), 1% 100X penicillin/streptomycin (Invitrogen), 2% 50X B-27® Serum-Free Supplement (Invitrogen), 200uM L-ascorbic acid 2 phosphate sesquimagnesium salt hydrate (Sigma) and 5uM IWP-4 (Stemgent). HiPSC-CMs were finally subjected to a 4-day metabolic selection in RPMI 1640 without glucose, without HEPES (Thermo Fisher), 1% 100X penicillin/streptomycin (Invitrogen), 2.2mM 50% sodium lactate (Sigma) and 0.1 mM 2-mercaptoethanol (Invitrogen). HiPSC-CMs were cultured for up to 25 days from the beginning of the differentiation protocol.

Quantitative PCR and PCR Total RNA (1ug) was extracted using Direct-zol RNA Kit (ZYMO Research) according to the manufacturer's guidelines. Then RNA was reverse transcribed using hexameric random primes and M-MLV Reverse Transcriptase (PROMEGA). Real-time PCR was performed with SYBR® green Supermix (BIO-RAD) according to manufacturer's instructions. The housekeeping gene ribosomal protein L7 was used for normalization. Fold changes were determined using the 2-ΔΔCT method. PCR was performed using LongAmp™ Taq DNA Polymerase. GAPDH and HRPT1 have been used as housekeeping genes. A full list of the primers used is present in **Table 1.**

Western blot Analysis Samples were lysed in RIPA Lysis and Extraction Buffer (Thermo Fisher) and PhoSSTOP and protease inhibitor cocktail (Roche). Then samples were boiled in 4x leammli buffer with 2% β-mercaptoethanol for 5 minutes at 95^{o}C. SDS-PAGE, and Western blotting were performed using Mini-PROTEAN 3

system. The gels were blocked with 5% milk/tbs TWEEN or 5% BSA/tbs TWEEN depending to the antibody. Primary antibody labelling was performed at 4^{o}C overnight, and HRP conjugated antibodies was applied for 2 hours at room temperature. After being washed 3 times with TBS/TWEEN for 10 minutes, each of the membranes was exposed to SuperSignal West Dura Extended Duration Substrate according to the manufacturer's protocol and the image Fujifilm LAS-3000 Imager. The stripping of the membrane has been performed with restore western blot stripping buffer (pierce). Output intensity was normalized for loading. A full list of the antibodies used is present in **Table 2.**

Production of recombinant AAV vectors. Human Bigheart was synthesized as MiniGene™ Synthetic Gene in pUC IDT plasmid by Integrated DNA Technologies Inc., (Leuven, Belgium) using the human reference sequence of human GRCh37 assembly. The full cDNA sequence of the lncRNA was then amplified with forward primer: 5'-GTATCATAAGGATCCCTTTCCACTGCTCTGGTGAG-3' and reverse primer: 5'-GTATCATAAGTCGACCTCACCTAGCTGTCTGTCC-3' and cloned into pAAV-MCS (Cat#: VPK-410, Cell Biolabs Inc.) using the restriction enzymes BamH I and HindIII sites. Recombinant AAV serotype 9 vectors were produced, purified, and titrated by real-time PCR on vector genomes at the AAV Vector Unit of the German Center for Cardiovascular Research (DZHK), Partner Site Hamburg/Kiel/Lübeck, Kiel (Germany) as described previously.[73] B6SV129F1 mice at postnatal day 0 were intraperitoneally injected with a control AAV9 vector (AAV9-luciferase) or AAV9-BIGHEART at a dose of 1 x 10^{11} viral genome particles per animal, using an insulin syringe with 30-gauge needle. 15 days after injection, the hearts were collected for histological analysis.

Aortic banding, Gapmer treatment Transverse aortic constriction (TAC) or sham surgery was performed in 2-6 month-old B6SV129F1 mice by subjecting the aorta to a defined 27 gauge constriction between the first and second truncus of the aortic arch as described previously[13] The Gapmer specific to murine Bigheart was purchased at Qiagen Inc (Hilden, Germany).[74] Treatment of phosphate buffered saline (vehicle) or Gapmer-Bigheart started at 1 day before sham or aortic banding surgery by IP injections every 7 days (0.1 ml PBS or Gapmer-Bigheart at 25 mg/kg body weight/day). Hearts were removed, rinsed in ice-cold phosphate buffered saline, atria removed, snap-frozen and stored in liquid nitrogen until use

RNA sequencing. All hearts from vehicle- or Gapmer-Bigheart treated mice were prepared simultaneously during all steps of this analysis to exclude introduction of technical variability. Quality control of total RNA was performed using the RNA 6000 Pico Kit (Agilent Bioanalyzer) yielding RIN values > 6. Removal of rRNA was carried out by the NEBNext rRNA Depletion Kit Human/Mouse/Rat (NEB) followed by strand-specific cDNA NGS library preparation (NEBNext Ultra II Directional RNA Library Prep Kit for Illumina, NEB). The size of the resulting library was controlled by use of a D1000 ScreenTape (Agilent 2200 TapeStation) and quantified using the NEBNext Library Quant Kit for Illumina (NEB). Equimolar pooled libraries were sequenced in a paired end mode (75 cycles) on the NextSeq 500 System (Illumina) using v2 chemistry yielding in an average QScore distribution of 84% >= Q30 score. After quality control with FastQC (v0.11.2)[75] raw sequencing data was read trimmed using trimmomatic v0.36 [31] and aligned to the mouse reference genome GRCm38 (ensemble release 90) with hisat2 v2.1.0. [76]Overall alignment rate was observed between 70-84% for all samples. Gene assembly was done using stringtie v1.3.4d [33] with set '-e' parameter to produce read count matrices for reference genes and transcripts.[77]

Statistics and Reproducibility. Results shown for images or blots were repeated independently at least once with similar results. The results are presented as mean ± standard error of the mean (SEM). All other statistical analyses were performed using Prism (version 8.2.1) (GraphPad Software Inc.) and consisted of One-way ANOVA followed by Dunnet's multiple comparison test when group differences were detected at the 5% significance level, or Student's *t*-test when comparing two experimental groups followed by a Bonferroni's multiple comparison test when differences were detected at the 5% significance level. Differences were considered significant when $P<0.05$ unless specified otherwise. KEGG results were considered significant when $P<0.05$ and to correct for multiple hypothesis testing, the false discovery rate (FDR) was separately used for each KEEG analysis with the threshold of FDR < 0.05 as 'high confidence' or 'statistical significance[78] same system was applied to network analysis. The *P*-values of the Venn diagrams were obtained by hypergeometric test using the whole set of Chip-seq genes as a background[79]. Additional information (Hits, FDR, E-value, Q-value) for KEGG and MEME-ChIP, are provide within **Supplementary Table 7**

Data availability Sequencing data generated from this work are available under the GEO accession numbers Xxxxxx Source data from previously published work are available under the following accession numbers. MyoD1 target gene were

downloaded by ENCODE Transcription Factor Targets external link (http://www.ncbi.nlm.nih.gov/gene/4654) Bigheart RNA-seq expression profile in human and mice we collected by (lncrnas.kaessmannlab.org). MyoD1 H3K4me3 and H3K27ac ChIP-Seq and input control from developing mouse hearts were downloaded from the ENCODE portal (https://www.encodeproject.org).

Conflict of interest statement

The authors declare a potential conflict of interest and state it below
L.D.W. is co-founder and stockholder of Mirabilis Therapeutics BV

Author contribution statement

NM wrote the manuscript and performed the experiments; CRG and RC performed surgery on mice treated with AAV9-BH, AR performed siRNA experiment in HL-1 and GS provided differentiated hIPSC-CMs, OM produced the virus, MS edited the manuscript and LDW obtained the funding.

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Figure legends

Figure 1| (**a)** Schematic representation of the pipeline used to process data from ChiRP-seq and ChIP-seq **(b)** Heatmap representing Pearson coefficient between the samples (H3K27me3, RNA pol 2, H3K27Ac and H3K4me3 and Bigheart), **(c)** Chromosomal distribution of the Bigheart peaks **(d**) Genomic distribution of the Bigheart peaks. **(e)** KEGG analyses of genes with Bigheart peaks validated with MACS1 **(f)** Motif comparison by MEME-CHIP for Bigheart peaks dataset. **(g)** ENCODE Tracks representing H3K27ac, H3K4me3, and H3K27me2 signals at different mouse embryonic stages from E11.5 to day 0 at Myod1 gene locus **(h)** GREAT GO enrichment biological process analysis in HL-1 cells from Bigheart ChiRP-seq data. **(i)**RT-PCR analysis of lncRNA expression in murine heart and skeletal muscle tissues with Gapdh as loading control.(J) ENCODE Tracks representing RNA pol II, H3K4me3, and H3K27me2 signals in Myocytes at Bigheart locus **(k)** Pearson correlation coefficient

between MyoD1 Hmgb1 and Bigheart (**l)**Venn Diagram of the gene target of Myod1 validated by Chip-seq data and Bigheart gene target of validated by ChiRP-seq.

Figure 2 | (a) Heatmaps of the peak distribution around the TSS (+/- 2 kb distances) of H3K27ac and H3K4me3 in HL-1 and CMs. **(b)** KEGG analyses of genes expressed only in HL-1. **(c)** KEGG enrichment analysis of genes expressed only in adult CMs. **(d)**Pearson correlation between CMs and HL-1 for H3K27ac and H3K4me3 **(e)**Venn Diagram of the gene expressed in adult cardiomyocytes and Bigheart gene targets of validated by ChiRP-seq. **(f)** Real-Time PCR analysis of the expression of several mTOR related genes in TAC and Sham samples. Values normalized against L7 n correspond to the number of repetitions. The error bars represent mean ±SEM and *$P < 0.05$ vs corresponding control group; **(g)** H3K27me3, H3K27ac, H3K4me3, RNA Pol2 and Bigheart ChIP-Seq tracks signals in HL-1 cells for Cab39, Perk,Raf1,Eif4e gene loci.

Figure 3 (a) Schematic representation of the setup used to evaluate the gene expression in isolated neonatal rat cardiomyocytes treated with phenylephrine/isoproterenol PE/ISO,**:** (**b)**Validation of Bigheart expression by Real-Time PCR in isolated neonatal rat cardiomyocytes treated with phenylephrine/isoproterenol PE/ISO, Values normalized against L7; n correspond to the number of repetitions. The error bars represent mean ±SEM and*$P < 0.05$ vs corresponding control group; **(c)** Real-Time PCR analysis of the expression of Raf1, Cab39, clip1, Perk and eif4e in isolated neonatal rat cardiomyocytes treated with phenylephrine/isoproterenol PE/ISO, Values normalized against L7 n correspond to the number of repetitions. The error bars represent mean ±SEM **and** *$P < 0.05$ vs corresponding control group; **(d)** Schematic representation of silencing experiment. **(e)** Validation of Bigheart silencing (4833412C05RIK-001) by Real-Time PCR in HL-1 untreated and HL-1 treated with siRNA for Bigheart. Values normalized against L7 n correspond to the number of repetitions. The error bars represent mean ±SEM and *$P < 0.05$ vs corresponding control group; **(f)** Real-Time PCR analysis of the expression of Raf1, Cab39, clip1 eif2ak3 and eif4e in HL-1 untreated and treated with siRNA for Bigheart. Values normalized against L7 n correspond to the number of repetitions. The error bars represent mean ±SEM and *$P < 0.05$ vs corresponding control group; **(g)** Schematic representation of overexpression experiment with AAV9-luciferase and AAV9-Bigheart in postnatal mice **(h)** Histological sections of hearts infected with AAV9-luciferase and AAV9-Bigheart representatives' images of Haematoxylin & Eonsin H&E

stained hearts (top panel) and high magnification sections (botton panel) (**i)** Validation of Bigheart overexpression by semi-quantitative PCR of Bigheart (4833412C05RIK-001) in mice infected with AAV9-luciferase and AAV9-Bigheart. normalized against mouse GAPHD. **(j)** Real-Time PCR analysis of the expression of Raf1, Cab39, Clip1 Eif2ak3 and Eif4e in in mice infected with AAV9-luciferase, AAV9-Bigheart, values normalized against L7; n correspond to the number of repetitions. The error bars represent mean ±SEM and *$P < 0.05$ vs corresponding control group; **(k)** Western blot for mTOR-p mTOR, 4eBP1, 4eBP1-p p70s6k p70s6k-p following AAV-mediated overexpression of Bigheart. **(l)** Quantification of the Phosphorylation levels in figure k

Figure 4 | **(a)** Schematic representation of the *in vivo* silencing experiment of Bigheart with GapMer in mice that underwent TAC surgery. **(b)** Venn Diagram of the gene in commen between RNA-seq data for GapMer bH vs TAC and TAC vs Sham**(c)** Heatmap of the 697 gene from figure b divided by expression **(d)** KEGG analysis and Gene Ontology (GO) enrichment biological process analysis of the mRNAs differentially expressed from the *in vivo* silencing of Bigheart with a GapMer in mice that underwent TAC surgery. **(e)** RNA-seq data from Kaessmann lab on Bigheart expression throughout cardiac differentiation in mice and human (value expressed in RPKKM)

(G) ENCODE and HL-1 H3K27me3 H3K27ac and H3K4me3 ChIP-Seq tracks at the Bigheart gene locus. Tracks representing H3K27ac and H3K4me3 signals at different embryonic stages are indicated **(e-f) Mouse and Human Bigheart expression during cardiac differentiation, data collected from** Kaessmann paper. **(h)** Schematic representation of hIPSCs differentiation protocol**. (i)** Real-Time PCR analysis of Bigheart expression in wildtype hIPSCs at day 0,3,10,17,25 of the differentiation protocol and human adult heart values normalized against L7 n correspond to the number or repetitions. The error bars represent mean ±SEM and *$P < 0.05$ vs corresponding control group. **(j)** Western blot for mTOR-p mTOR, 4eBP1, 4eBP1-p p70s6k p70s6k-p in wildtype hiPSCs at day 0,3,10, of the differentiation protocol

Supplementary Figure legend

Supplementary Figure 1 (a) qPCR of Bigheart in TAC, Sham and HL-1 samples**,** values normalized against L7. n correspond to the number of repetitions. The error bars represent mean ±SEM and *$P < 0.05$ vs corresponding control group.

(b) Tag distributions (using bigWIG metrics) across the transcription start sites (TSS; +/- 5 kb**) (c)** KEGG enrichment analysis of genes expressed in HL-1 **(d)** Genomic distribution of the sample's peaks. **(e)** KEGG enrichment analysis of bigheart's distant targets accordingly with T-Gene **(f-g)** HL-1 H3K27me3 H3K27ac and H3K4me3 ChIP-Seq tracks at the Immt and Pdk1 genomic loci. **(h)** GREAT GO enrichment biological process analysis in HL-1 cells from Hmgb1 ChiRP-seq data. **(i)** Motif comparison by MEME-CHIP for Hmgb1 peaks dataset **(k)** KEGG enrichment analysis of all the MyoD1 targets from ENCODE targets database (**j)** GREAT GO enrichment biological process analysis from MyoD1 ChiP-seq data from ENCODE.

Supplementary Figure 2| (a) HL-1 H3K27me3 H3K27ac and H3K4me3 ChIP-Seq tracks at several gens related to the mTOR pathway

Supplementary Figure 3| (a) Pathway network analysis of RNA-seq data form GapMerBH vs TAC, nodes related to the 3 main GO biological term are labelled by the most significant group term in, orange, blue and green. **(b)** GO term for biological process of all the nodes.

Supplementary tables legend

Supplementary table 1

Summary of the alignment and peak calling statistics.

Supplementary table 2

Gene-centric presentation of all the 469 Bigheart's targets, including position and peak values

Supplementary table 3

List of the genes targeted by Bigheart in the promoter area (upstream <10kb) , including position and peak values

Supplementary table 4

List of the distant genes targeted by Bigheart accordingly with T-gene including p-value

Supplementary table 5

RNA-seq result from the GapMer experiment including p-value

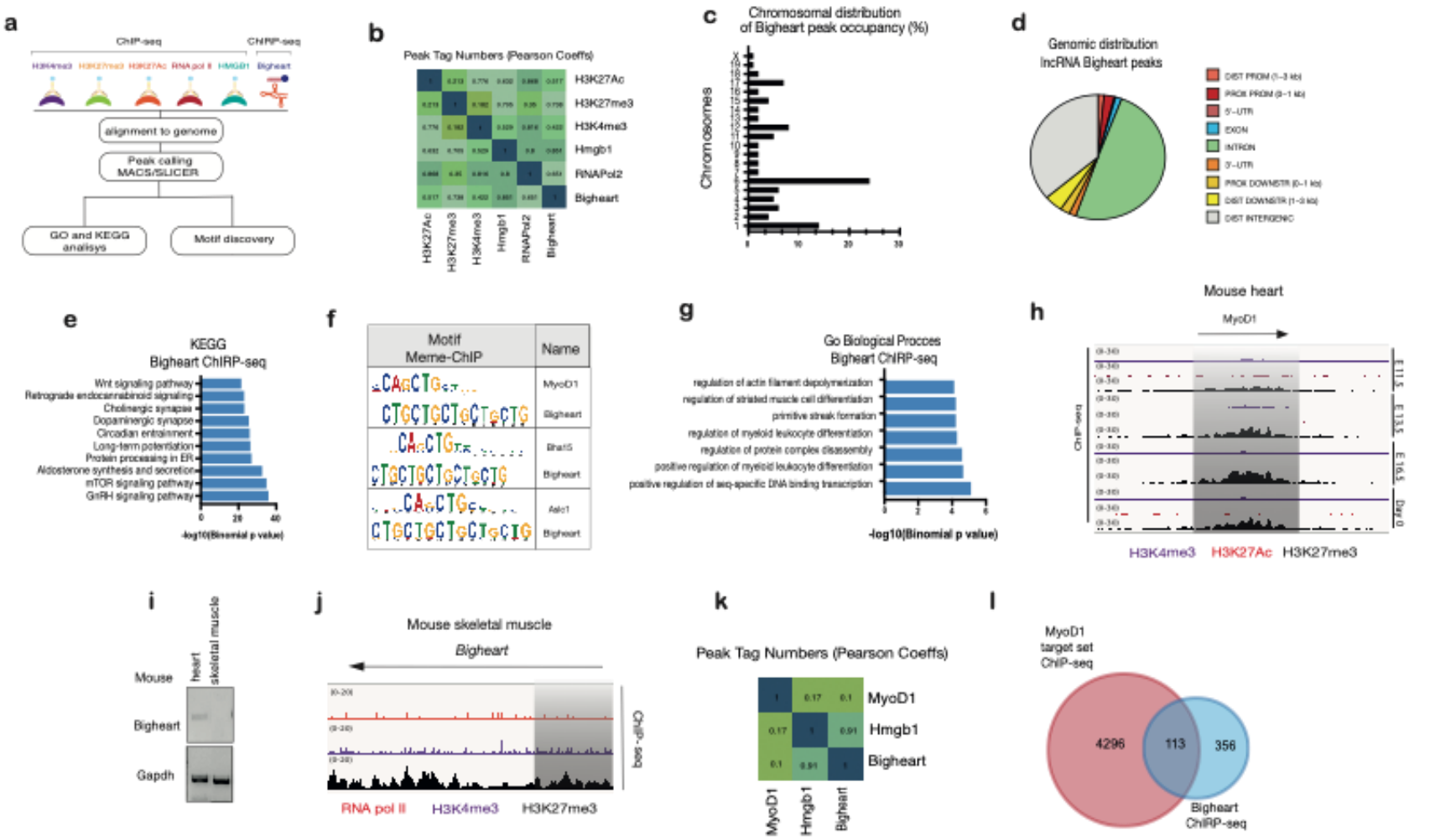
Supplementary table 6

List of all the genes used to performed ORA or GO analysis and result from the Venn diagrams

Supplementary table 7

KEGG original files with p-value and FDR. MeME-ChIP original files with original E-value and q-value. CMs merged peak calling from Easeq.

Figure 1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ChIP-seq
ChIRP-seq
alignment to genome
Peak calling MACS/SLICER
GO and KEGG analisys
Motif discovery
b
Peak Tag Numbers (Pearson Coeffs)
H3K27Ac
H3K27me3
H3K4me3
Hmgb1
RNAPol2
Bigheart
c
Chromosomal distribution of Bigheart peak occupancy (%)
Chromosomes
d
Genomic distribution lncRNA Bigheart peaks
e
KEGG
Bigheart ChIRP-seq
Wnt signaling pathway
Retrograde endocannabinoid signaling
Cholinergic synapse
Dopaminergic synapse
Circadian entrainment
Long-term potentiation
Protein processing in ER
Aldosterone synthesis and secretion
mTOR signaling pathway
GnRH signaling pathway
-log10(Binomial p value)
f
Motif Meme-ChIP
Name
MyoD1
Bigheart
Bhlh5
Bigheart
Ascl1
Bigheart
g
Go Biological Proces
Bigheart ChIRP-seq
regulation of actin filament depolymerization
regulation of striated muscle cell differentiation
primitive streak formation
regulation of myeloid leukocyte differentiation
regulation of protein complex disassembly
positive regulation of myeloid leukocyte differentiation
positive regulation of seq-specific DNA binding transcription
-log10(Binomial p value)
h
Mouse heart
MyoD1
ChIP-seq
E11.5
E13.5
E16.5
Day 0
H3K4me3
H3K27Ac
H3K27me3
i
heart
skeletal muscle
Mouse
Bigheart
Gapdh
j
Mouse skeletal muscle
Bigheart
ChIRP-seq
RNA pol II
H3K4me3
H3K27me3
k
Peak Tag Numbers (Pearson Coeffs)
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Hmgb1
Bigheart
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4296
113
356
Bigheart ChIRP-seq

Figure 2

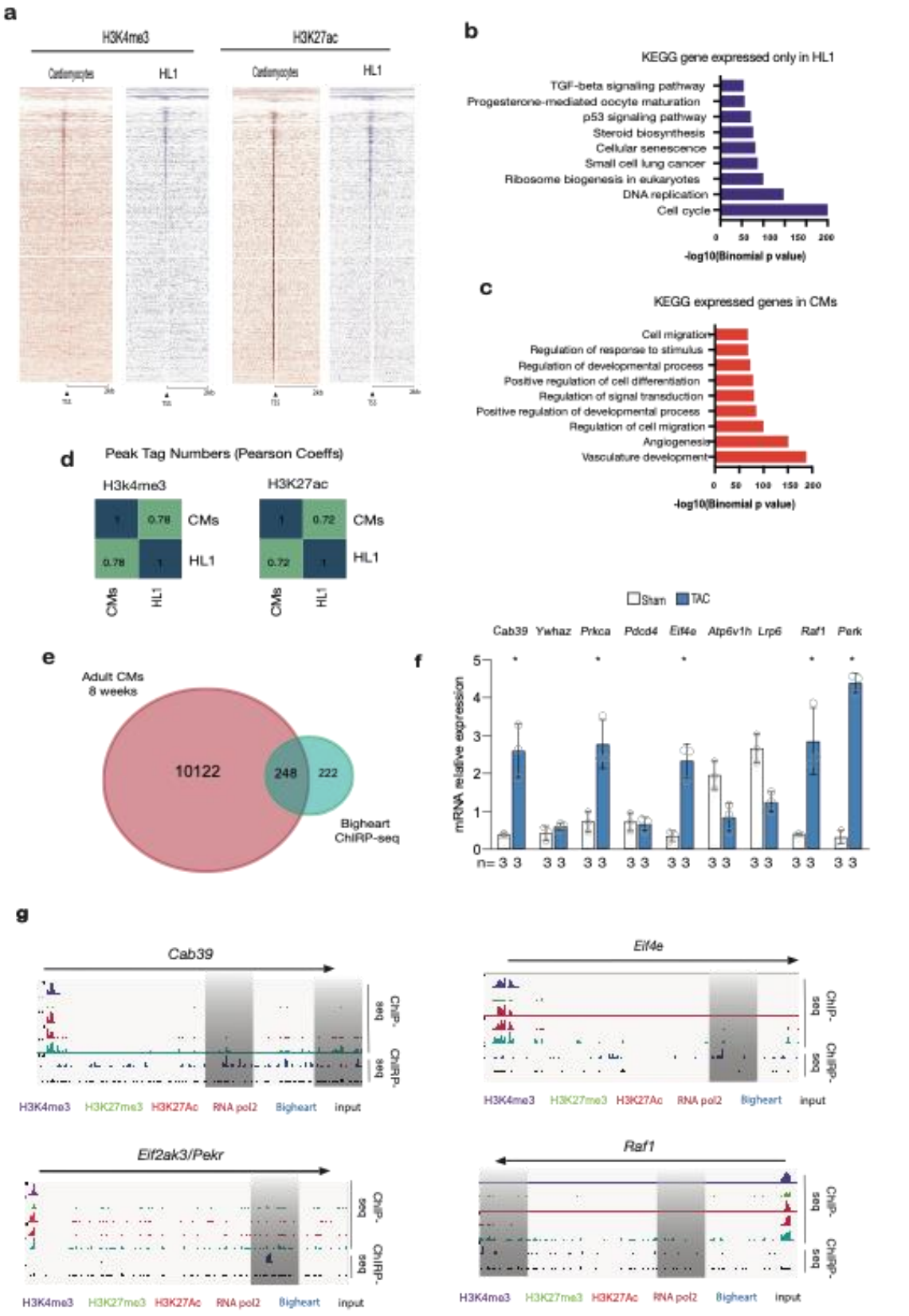
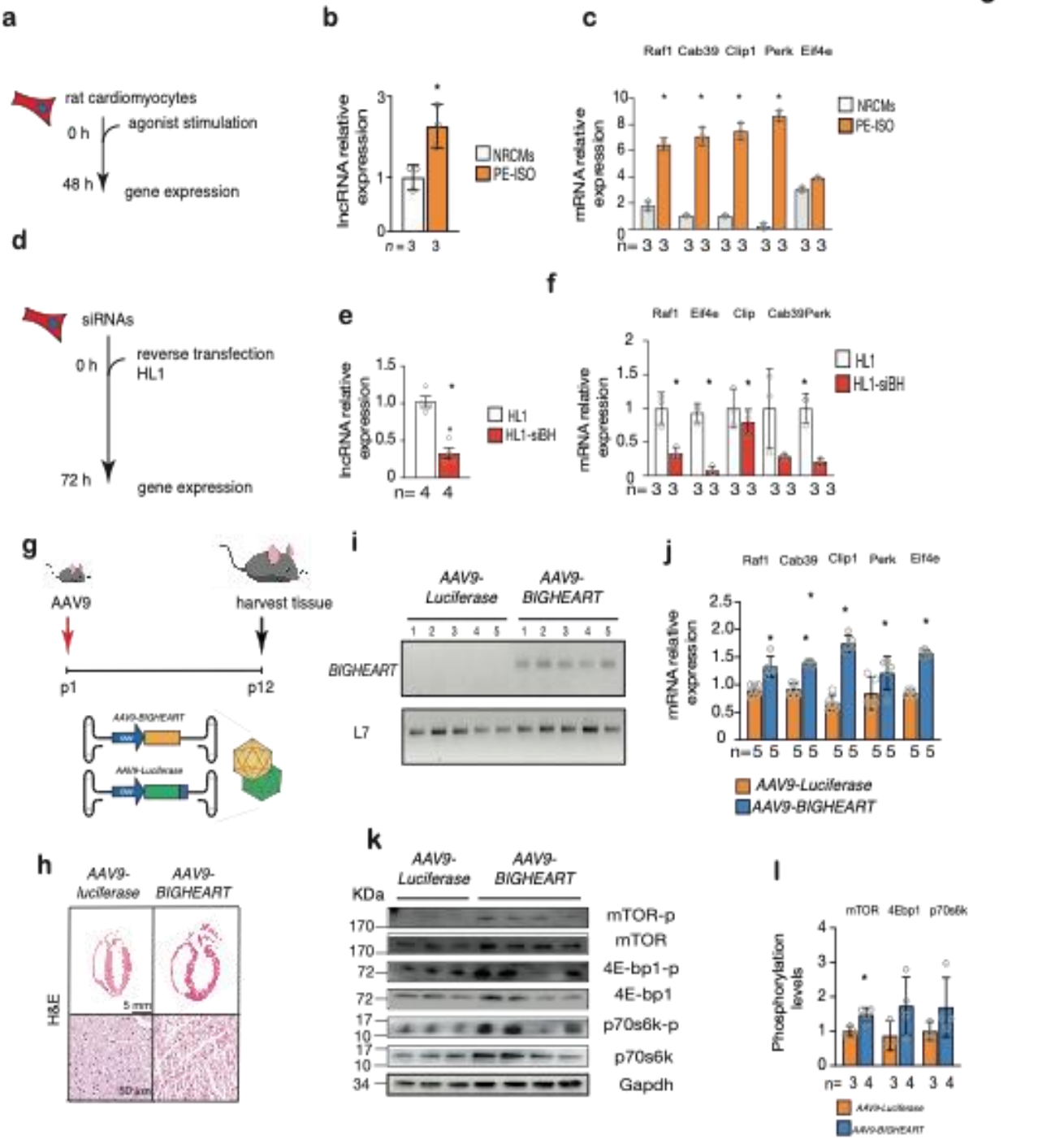

a
H3K4me3
H3K27ac
Cardiomyocytes
HL1
b
KEGG gene expressed only in HL1
TGF-beta signaling pathway
Progesterone-mediated oocyte maturation
p53 signaling pathway
Steroid biosynthesis
Cellular senescence
Small cell lung cancer
Ribosome biogenesis in eukaryotes
DNA replication
Cell cycle
0 50 100 150 200
-log10(Binomial p value)
c
KEGG expressed genes in CMs
Cell migration
Regulation of response to stimulus
Regulation of developmental process
Positive regulation of cell differentiation
Regulation of signal transduction
Positive regulation of developmental process
Regulation of cell migration
Angiogenesis
Vasculature development
0 50 100 150 200
-log10(Binomial p value)
d
Peak Tag Numbers (Pearson Coeffs)
H3k4me3
H3K27ac
1
0.78
0.72
CMs
HL1
e
Adult CMs
8 weeks
10122
248
222
Bigheart
ChIRP-seq
f
Sham
TAC
Cab39 Ywhaz Prkca Pdcd4 Eif4e Atp6v1h Lrp6 Raf1 Perk
mRNA relative expression
n= 3 3 3 3 3 3 3 3 3
g
Cab39
Eif4e
Eif2ak3/Pekr
Raf1
ChIP-seq
ChIRP-seq
H3K4me3 H3K27me3 H3K27Ac RNA pol2 Bigheart input

Figure 3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rat cardiomyocytes
0 h
agonist stimulation
48 h
gene expression
b
lncRNA relative expression
NRCMs
PE-ISO
n = 3 3
c
Raf1 Cab39 Clip1 Perk Eif4e
mRNA relative expression
NRCMs
PE-ISO
n= 3 3 3 3 3 3 3 3 3 3
d
siRNAs
0 h
reverse transfection HL1
72 h
gene expression
e
lncRNA relative expression
HL1
HL1-siBH
n= 4 4
f
Raf1 Eif4e Clip Cab39/Perk
mRNA relative expression
HL1
HL1-siBH
n= 3 3 3 3 3 3 3 3 3 3
g
AAV9
harvest tissue
p1
p12
AAV9-BIGHEART
AAV9-Luciferase
i
AAV9-Luciferase
AAV9-BIGHEART
1 2 3 4 5 1 2 3 4 5
BIGHEART
L7
j
Raf1 Cab39 Clip1 Perk Eif4e
mRNA relative expression
n=5 5 5 5 5 5 5 5 5 5
AAV9-Luciferase
AAV9-BIGHEART
h
AAV9-luciferase
AAV9-BIGHEART
H&E
5 mm
50 µm
k
AAV9-Luciferase
AAV9-BIGHEART
KDa
170 mTOR-p
170 mTOR
72 4E-bp1-p
72 4E-bp1
17 p70s6k-p
10
17 p70s6k
10
34 Gapdh
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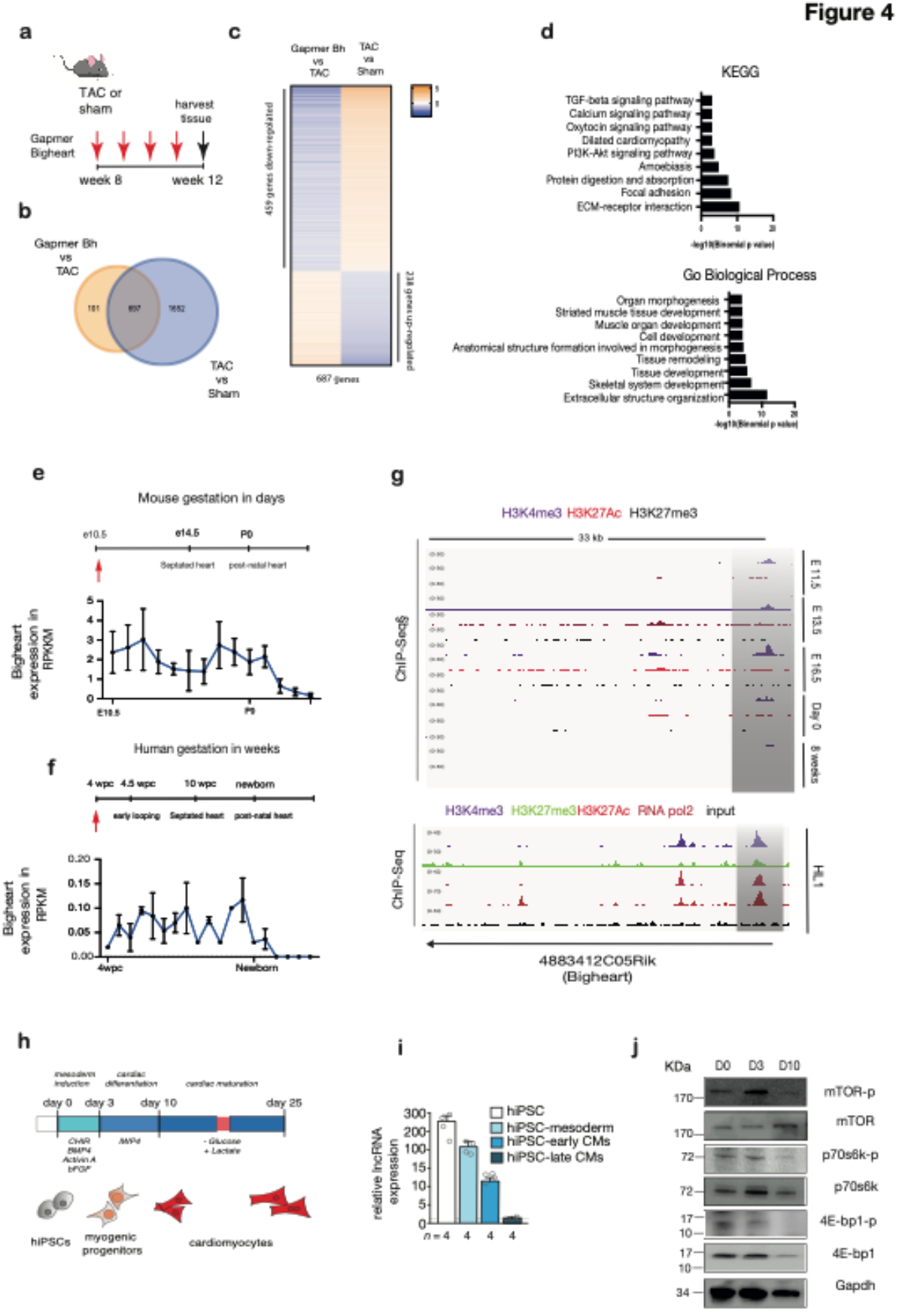
mTOR 4Ebp1 p70s6k
Phosphorylation levels
n= 3 4 3 4 3 4
AAV9-Luciferase
AAV9-BIGHEART

Figure 4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TAC or sham
harvest tissue
Gapmer Bigheart
week 8
week 12
b
Gapmer Bh vs TAC
181
697
1652
TAC vs Sham
c
Gapmer Bh vs TAC
TAC vs Sham
459 genes down-regulated
238 genes up-regulated
687 genes
d
KEGG
TGF-beta signaling pathway
Calcium signaling pathway
Oxytocin signaling pathway
Dilated cardiomyopathy
PI3K-Akt signaling pathway
Amoebiasis
Protein digestion and absorption
Focal adhesion
ECM-receptor interaction
Go Biological Process
Organ morphogenesis
Striated muscle tissue development
Muscle organ development
Cell development
Anatomical structure formation involved in morphogenesis
Tissue remodeling
Tissue development
Skeletal system development
Extracellular structure organization
e
Mouse gestation in days
e10.5
e14.5
P0
Septated heart
post-natal heart
Bigheart expression in RPKM
E10.5
P0
f
Human gestation in weeks
4 wpc
4.5 wpc
10 wpc
newborn
early looping
Septated heart
post-natal heart
Bigheart expression in RPKM
4wpc
Newborn
g
H3K4me3 H3K27Ac H3K27me3
33 kb
ChIP-Seq§
E11.5
E13.5
E16.5
Day 0
8 weeks
H3K4me3 H3K27me3 H3K27Ac RNA pol2 input
ChIP-Seq
HL1
4883412C05Rik
(Bigheart)
h
mesoderm induction
cardiac differentiation
cardiac maturation
day 0
day 3
day 10
day 25
CHIR
BMP4
Activin A
bFGF
IWP4
- Glucose
+ Lactate
hiPSCs
myogenic progenitors
cardiomyocytes
i
relative lncRNA expression
hiPSC
hiPSC-mesoderm
hiPSC-early CMs
hiPSC-late CMs
n = 4
4
4
4
j
KDa
D0
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170
mTOR
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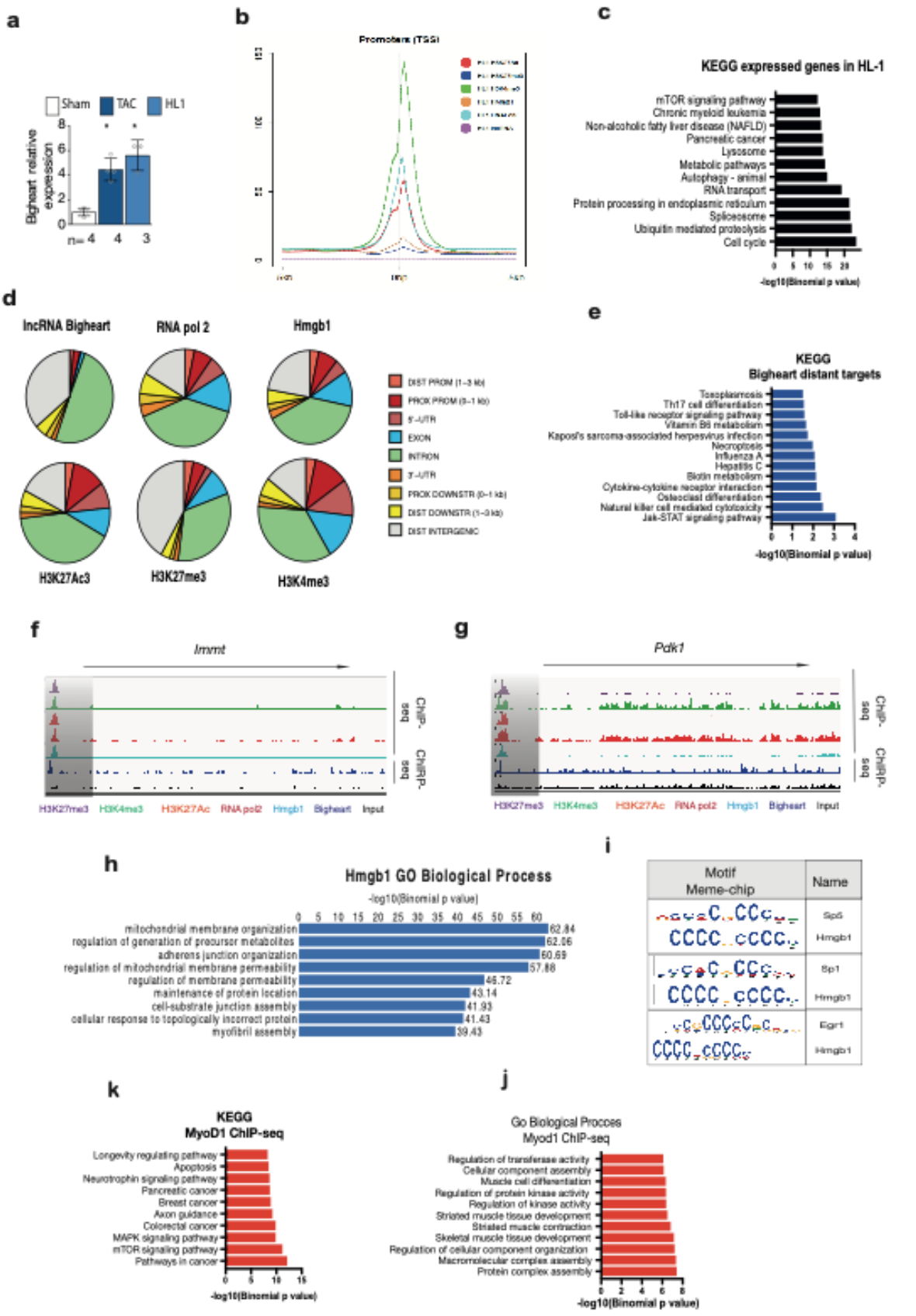
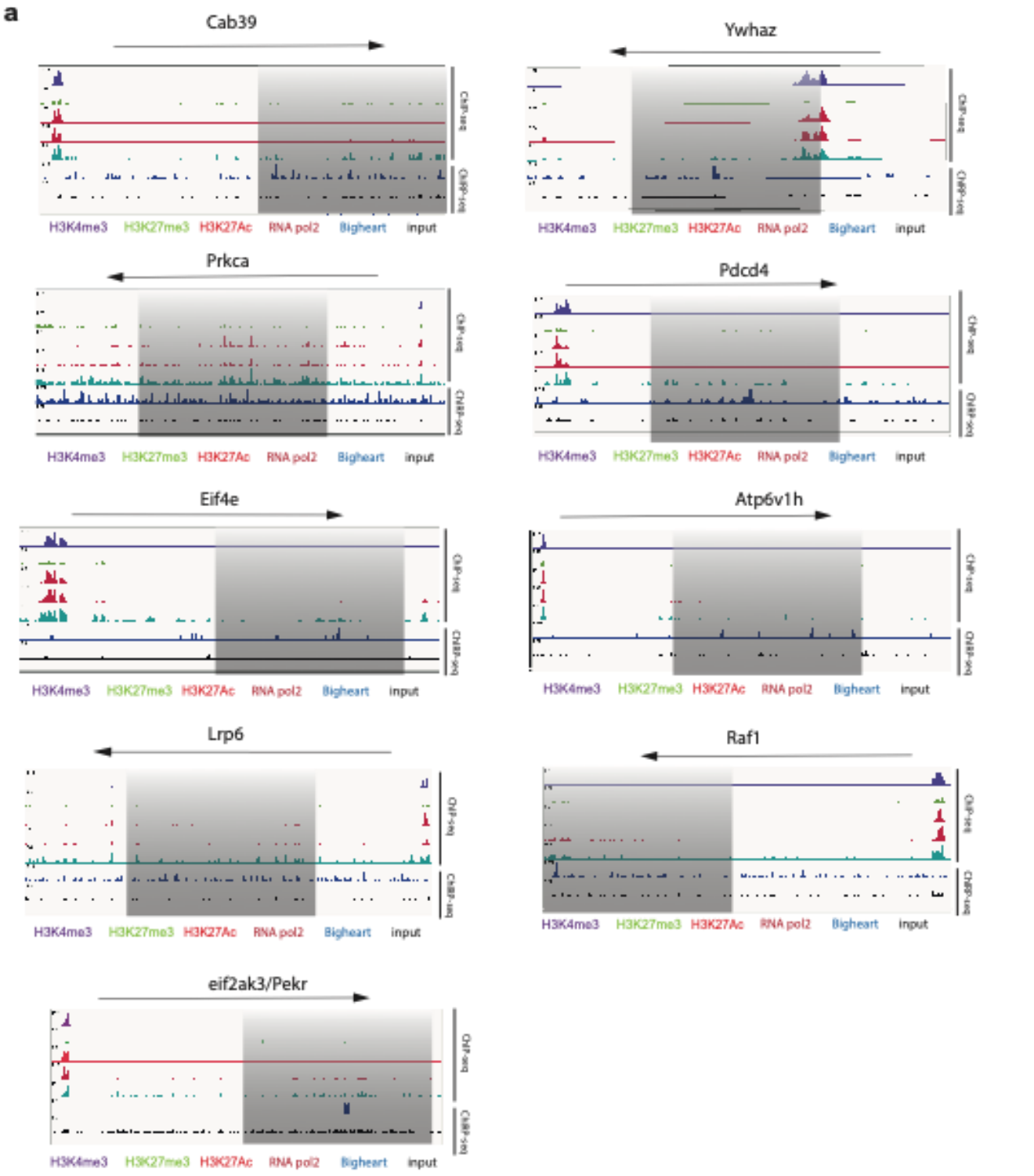
Gapdh

Supplementary Figure 1



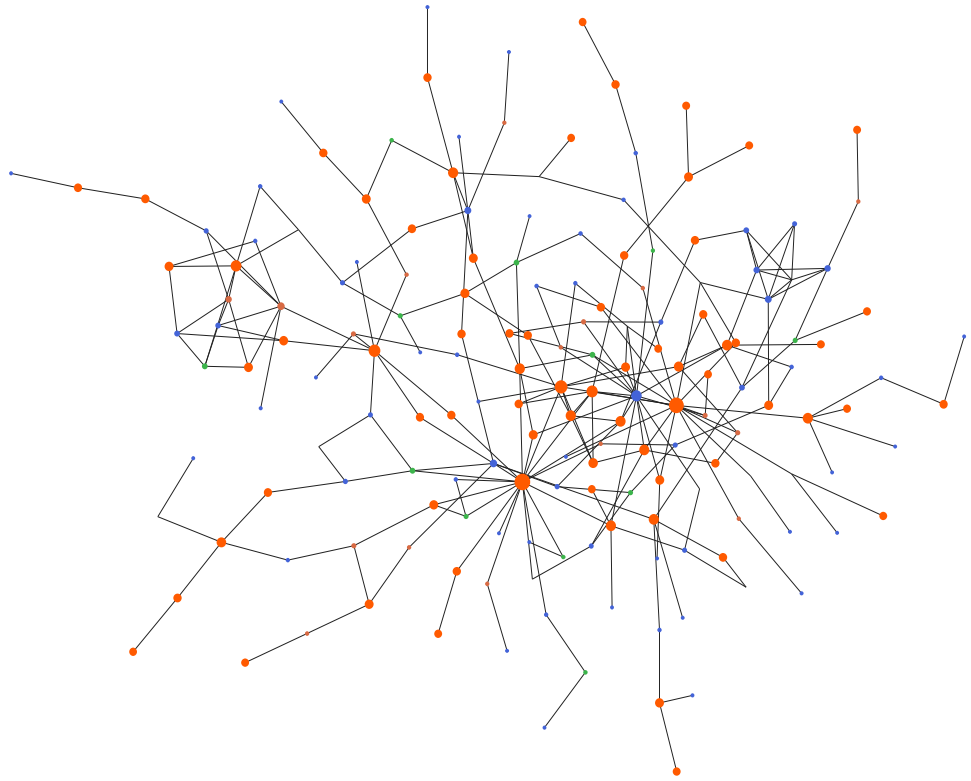
Supplementary figure 2



Supplementary figure 3

Network analisys pathway (based on Signor 2.0)

a



(GapMer BH vs TAC)

b

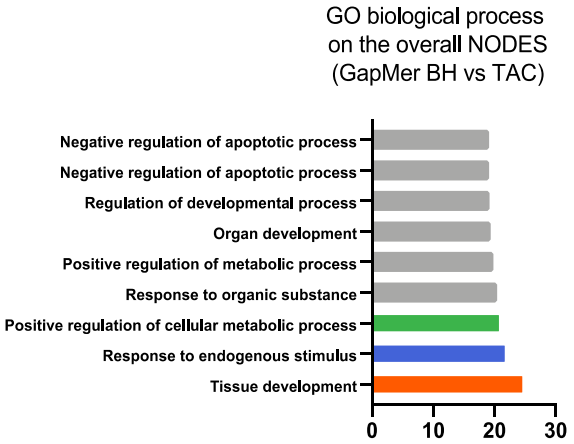


TABLE 1

Primers list used for PCR and qPCR

Name	**Primer Forward**	**Primer Reverse**
L7 (mouse)	GAAGCTCATCTATGAGAAGGC	AAGACGAAGGAGCTGCAGAAC
Bigheart (rat/mouse)	GCATGAAGAGCAAGGTGTATGG	ATCTCGAGTGGATCAGCCCT
Bigheart **(human)**	GGTGGTGTCGTTTCCAGTGA	ATCACCCGTTCATCCATCCG
Gapdh (human)	GGGTCCCAGCTTAGGTTCAT	CCCAATACGGCCAAATCCGT
Clip1 (rat)	GCTCAAGATCGGAGACAGGG	ACTGTAGCCACCTCAAGATCA
Cab39 (rat)	CCCGTCAAGCAGGTAGAGTG	TGGGTGCAGATGTATTCAACAGT
Raf1 (rat)	CAGAGGCAGTGAGTCCGC	GACAGCGGTGGTTGAGAGAA
Perk (rat)	TCTGTACAAGGCTGTCACTCA	AGATCCCACGTCCAAATCCC
Eif4e (rat)	GAAGGAAGGTGGACTGGCTC	CCATGTTTTAGCCCGGCAGA
Lrp6 (rat)	ACTAACCTTGGACGAAGGGC	GGAGGAGGCAGCAAGGTAAA
Clip1 (mouse)	GGAAACAGGGAGAAGCCTGA	GAGCCCCTAAATCCTCCACG
Cab39 (mouse)	GACAACCTCAGAGGCGAAGG	CTTCCACGGCACTCTACCTG
Raf1 (mouse)	GGATAGCCTGAGAGCGTCTTC	AAGAATCCGTGAGCTTGCCA
Perk (mouse)	TTCCCTACAAGCCCAAAGGC	TCAGACTCCTTCCGCTGCC
Eif4e (mouse)	ACAGTCCTTACCACAGCACAC	ATCGAGGTCACTCCGTCTCT
Lrp6 (mouse)	TGCAAACAGACGGGACTTGA	GTCAGTCCGTTTGGCCAGTA

TABLE 2

Antibody list used for Western blot and ChIP-seq

Name	Catalog Number	brand
mTOR	2983	Cell signaling
Phospho-mTOR	2971s	Cell signaling
4E-BP1	9452	Cell signaling
Phospho-4E-BP1	9451	Cell signaling
P70s6k	2708	Cell signaling
Phospho-P70s6k	9206	Cell signaling
Gapdh	MAB374	millipore

TABLE 3

Probe used for ChIRP-seq

Probe #	Probe (5'-> 3')	Probe Position on Bigheart
1	CACTAGTCTGCTGCTGCATG	25
2	AGCTGAAGAAAGCACACAGT	129
3	AGCTGAAGAAAGCACACAGT	229
4	AAATCTCGAGTGGATCAGCC	334
5	CAGGAAAGATGGCATTTCCA	435
6	TCTTTGTAGACTAGGCTGGC	545
7	CAGGAGGCCAAGACGTATAA	646
8	CGAGCAGCATTTTATCTCTG	770
9	TCAGCGATTTCCAAAGGGAT	888
10	GGTTCTTCGAGAAATCCATG	997

Chapter 5

Summary and General Discussion

The full extension of the processes that govern the development of cardiac hypertrophy are still a matter of debate, and, for the most part, remain unknown. Researchers throughout the years focused their attention on identifying key players that drive this process, successfully charting proteins, mRNA, miRNA, and cirRNA responsible for sustaining cardiac hypertrophy. In this thesis, we attempted to contribute to this collective effort by studying potential therapeutic targets belonging to the class of Long noncoding RNAs (LncRNAs). We started in **Chapter 1** by exploring the characteristics

of heart failure and the molecular basis of cardiac hypertrophy. Moreover, we introduced the latest developments in the field of heart disease, focusing on the study of non-coding RNA (ncRNA). Next, in **Chapter 2** we reviewed the recent advancements in the study of a specific and promising class of ncRNA suitable for therapeutic uses, called Long non-coding RNAs (*LncRNAs).* We then described in detail their characteristics, functions, and possible applications for medical use. Lastly, we listed LncRNAs known to be involved in cardiac diseases and particularly in cardiac hypertrophy. Next, In **Chapter 3** we utilized two established mouse models with early-onset cardiac hypertrophy and heart failure to identify previously undescribed LncRNAs differentially expressed in the hypertrophic mammalian heart. Specifically, we used mice subjected to transverse aortic constriction (TAC)[1] to induce sustained cardiac pressure overload, and mice with sustained calcineurin activity in the heart (Myh6-CnA)[2]. An important aspect we immediately noticed by employing a combined lncRNA/mRNA array on total RNA isolated from these models, was that while on the histological level both TAC and Myh6-CnA mice showed substantial signs of cardiac remodeling, as well as the characteristic reactivation of fetal genes, they exbibit a quite diverse transcriptional profile. We noticed a significant number of differentially expressed LncRNAs (respectively 6,107 and 7,025). However, only a fraction of ~750 LncRNAs were common to both models and followed a similar trend of down- or upregulation upon cardiac hypertrophy. Such a difference could be partially explained by the different methods by which cardiac remodeling is induced in TAC and Myh6-CnA models. In TAC, adult mice are exposed through surgery to severe cardiac pressure overload[3], which eventually degenerates into myocardial hypertrophy. Instead, Myh6-CnA are transgenic mice with constitutional activation of CnA[4], which leads to a profound hypertrophy response in juvenile mice, and over time can develop into heart failure. The difference in age, as well as the different types of stimulation, are the probable cause of these transcriptional discrepancies.

Cardiac hypertrophy is the attempt of the heart to adapt to specific stress[5]. Therefore, different stimulations such as pressure overload or constitutional expression of CnA could trigger a similar but not identical response of the heart that eventually would show a similar phenotype, in this case, cardiac hypertrophy. Nevertheless, several LncRNAs were differentially expressed in both models, suggesting that these common LncRNAs could be directly or indirectly involved in the response of hypertrophic growth. Among this fraction of commonly dysregulated LncRNAs, we identified 4833412C05RIK-201, which we termed *Bigheart*, which was previously described in a conditional knockout mouse in which the heterogeneous nuclear ribonucleoprotein U *Hnrnpu* gene was deleted in the heart. The authors noticed

Bigheart was expressed at relatively high levels in newborn mice but decreases dramatically after 1 week. However, deletion of hnRNP U leads to the persistent expression of *Bigheart*, and the accumulation of an alternatively spliced *Bigheart* isoform. Furthermore, it was also observed that the induction of *Bigheart* occurs in several heart failure models. Regardless of this promising evidence, the molecular functions of *Bigheart* were overlooked and their involvement in cardiac development and cardiac remodeling not explored[6]. Therefore, in **Chapter 3** and **Chapter 4**, we focused our efforts on characterizing *Bigheart* in the context of cardiac remodeling and heart failure. Since LncRNAs are poorly conserved among different species, compared with other classes of RNAs like mRNAs, miRNAs, and tRNA,[7] However, we could identify a human homolog for *Bigheart* based on synteny[8]. Furthermore, in **Chapter 4**, we established that similarly, in mice and humans, *Bigheart* is strongly expressed in earlier stages of cardiac differentiation, and results decreased in expression in later stages of maturation. We were able to replicate these findings in an hiPSC-derived cardiomyocytes (hiPSC-CMs) based model. Therefore, it was interesting observe a significant re-expression of *Bigheart* in conditions of pathological hypertrophy. It has been established that most genes observed during fetal cardiac development are rapidly re-expressed during cardiac remodeling. Those genes are collectively named as a part of a "fetal gene program"[9], and we established that *Bigheart follows* the same course. Next, to further investigate this LncRNA, we used RNA interference to silence endogenous *Bi*gheart in cultured primary cardiomyocytes*,* and we demonstrated its requirement in agonist-induced cardiomyocyte hypertrophy. Conversely, we employed a serotype 9 adeno-associated viral (AAV9) vector[10] to evoke overexpression of this LncRNA, resulting in a significant enlargement of the hearts infected with AVV9-BIGHEART compared to those from mice injected with the control AAV9.

Finally, we silenced endogenous *Bigheart in vivo* with a specific Gapmer, to evaluate its contribution to pressure overload-induced cardiac growth. Combined, our findings demonstrated that *Bigheart* is required for cardiomyocytes to induce a full hypertrophic response. The next step was establishing how this LncRNA was regulated upon cardiac remodeling. One of the main transcriptional effectors involved in early onset of cardiac hypertrophy response is the nuclear factor of activated t-cells (NFAT), and the calcineurin/NFAT axis is one of the major pathways involved in evoking a hypertrophic response[11]. In fact, the translocation of NFAT into the nucleus upon dephosphorylation by calcineurin allows this transcriptional factor to induce various genes related to cardiac remodeling[12]. Our findings in **Chapter 3** proved that NFAT could directly induce an active transcription of this LncRNA. Additionally, we noticed the existence

of an evolutionary conserved NFAT consensus binding site in the promoter area of the *Bigheart* gene. Notably, NFAT is known to regulate the expression of several "fetal genes," for instance the brain natriuretic peptide B (*Nppb*) and Natriuretic Peptide A (*Nppa*), again showing an interesting parallel between *Bigheart* and the fetal gene program[13]. Finally, we focused our attention on establishing the molecular function of *Bigheart.* We did not find evidence *of cis*-action. Therefore, we focused our attention on Trans-action. Silencing of *Bigheart* in mouse hearts subjected to pressure overload and following RNA-seq showed a strong impact on the expression of Regulator of calcineurin 1(*Rcan1),* an enhancer of calcineurin-NFAT signaling. *Rcan1* has two distinct promoters, encoding for two different isoforms, *Rcan 1.1* and *Rcan 1.4*[14]. We established that this LncRNA can bind both. This result was confirmed by the overexpression of *Bigheart* with AAV9 in postnatal hearts. Moreover, Chromatin Isolation by *RNA* Purification (ChIRP)[15] further proved a direct interaction between this LncRNA and *Rcan1*. Interestingly, *Rcan1.4* harbors several NFAT binding sites upstream of exon 4. Therefore, we speculated that the same transcriptional factor could also direct a LncRNA on the same gene. Although, the transcriptional regulation of *Rcan1.1* and *Rcan1.4* has not yet been well characterized, and several mechanisms could converge to ensure a rapid response to cardiac stress, including the integration of LncRNAs. Lastly, we established the protein partners of *Bigheart*. A LncRNA, which shows Trans-action tends to collaborate mostly, but not exclusively, with chromatin-modifying enzymes and nucleosome-remodeling factors[16]. In line with this, and by employing mass spectrometry, we re-evaluated the physical interaction of *Bigheart* with a chromatin-remodeling protein heterogeneous nuclear ribonucleoprotein F (hnRNP F) [17]and High-mobility group box 1 (Hmgb1)[18]. Based on this result and data of ChiRP-seq, we also hypothesized that *Bigheart* caused an active and open chromatin structure in *Rcan1* promoters. Despite **Chapter 3** revealing sophisticated positive feedback in the Rcan1/calcineurin/NFAT signaling regulated by the LncRNA *Bigheart,* there are some points that require additional investigations. Firstly, although both silencing and overexpression experiments demonstrated the significant impact of this LncRNA on R*can1*, and ChiRP-seq showed a direct bind of Hmgb1 and *Bigheart* on this gene, our experiments did not prove unequivocally that *Bigheart* changes the state of chromatin in the *Rcan1* promoter, and additional evidence is necessary to conclude this contention. For instance, an interesting approach would be to use Assay for Transposase-Accessible Chromatin using sequencing (ATAC-seq)[19] in cardiomyocytes that overexpress *Bigheart* against normal cardiomyocytes, to properly evaluate the chromatin conformation as well as accessibility upon *Bigheart* expression. Secondly, although we have proved the direct

interaction between this LncRNA and chromatin remodeler Hmgb1, we did not perform any functional experiments that validate this assumption, therefore, silencing of Hmgb1 and hnRNP F would give further indications on their role as a partners of *Bigheart.* Collectively, our studies in **Chapter 3** demonstrated how *Bigheart* is an important regulator of the hypertrophic process, able to establish powerful positive feedback for the calcineurin/NFAT signaling. Comprehensively, in **Chapter 3**, we have focused our attention on studying a single regulatory mechanism. However, trans LncRNAs can usually regulate a large number of genes simultaneously. Consequently, in **Chapter 4,** we examined the role of *Bigheart* by using high-throughput ChIRP-Seq for large-scale chromatin analysis. With this system we were able to establish the positions on the chromatin of *Bigheart* on a genome-wide scale. Therefore, we performed ChiRP-seq on HL-1 cells, which provided a list of 469 genes as targets of this LncRNA. Interestingly, most of these genes are related to developmental processes, rather than pathological growth. Genes identified to participate in embryonic development are also important in postnatal control of cell growth and differentiation. However, these genes are also involved in driving cancer progression. The model we employed, HL-1 is an immortalized mouse cardiomyocyte cell line, derived from an atrial tumour, and commonly used to study normal cardiomyocyte function[20]. Interestingly in HL-1 this *Bigheart* is abundantly expressed. On the other hand, in normal cardiomyocytes, this LncRNA is highly expressed in earlier stages of differentiation and upon pathological hypertrophy[21]. Instead, it is reduced in mature cells and cardiomyocytes in physiological condition[22]. On the account of this discrepancy, we first established the reliability of this model in our study against cardiomyocytes. Comparison of HL-1 versus cardiomyocytes with ChiP-seq data for marker of positive transcription H3K4me3 and H3K27ac, showed a similar but not identical transcriptional profile, with a Pearson correlation coefficient of 0.78 for 0.72 for H3K4me3 and H3K27ac. Furthermore, only 248 genes from our list of *Bigheart* targets are expressed in adult cardiomyocyte. Therefore, we focused on these 248 genes. Remarkably, among the different targets of our LncRNA in this list, we have identified many genes linked to the mTOR pathway[23]. This pathway is well-established as participating in cardiac hypertrophy onset and progression[24], as well as facilitating *cancer* cell growth and proliferation [25]. mTOR is an atypical serine/threonine kinase pivotal to the heart[26]. Several studies employing cardiac-specific mTOR knockout mice showed a dramatic mortality, and the survivors were affected by severe cardiac dilation and signs of terminal cardiac failure[26]. Accordingly, further studies showed that mTOR is involved during the adulthood in maintenance of cardiac structure and stress response. Increased mTOR activation is present during the

cardiomyocyte hypertrophic response to β-adrenergic stimulation, angiotensin-II and IGF-1[27]. Additionally, inhibition of mTOR signaling, prevented cardiac hypertrophy in established TAC-induced hypertrophy mice[28,29]. Consequently, it has been established that mTOR signaling participates in the regulation of embryonic cardiovascular development and controls normal postnatal growth.[25] Hence, accordingly with the result collected from our ChIRP-seq, we hypothesized a central role of *Bigheart* in influencing this pathway. In line with this hypothesis, in **Chapter 4**, we observed that *Bigheart* and Hmgb1 physically bind several genes associated with the mTOR pathway. Furthermore, upon overexpression of *Bigheart* with an AAV9 viral vector, we detected a strong phosphorylation of S6 kinase 1 (S6K1) and 4E (eIF4E)-binding protein 1 (4E-BP1), downstream effectors of the mTOR pathway[30], suggesting the activation of this pathway is influenced by *Bigheart* . Next, we noticed that *Bigheart* could participate in the regulation of many genes involved in muscle development. In *vivo* silencing of *Bigheart,* and the subsequent RNA-seq revealed that many genes involved in muscle development are altered upon silencing, suggesting a correlation between this LncRNA and development. Furthermore, we noticed that *Bigheart* binds the DNA with a motif similar to the muscle-specific protein Myogenic Differentiation 1 (MyoD1). MyoD1 is a member of the family of the helix-loop-helix(HLH) [31]*proteins* dimeric transcription factors, usually involved in orchestrating lineage specification and cell commitment[32]. Accordingly, MyoD1 promotes the transcription of muscle-specific genes and plays a role in muscle differentiation and myocyte commitment. Interestingly, MyoD1 is not expressed in the heart at any stage of the embryogenesis or later. Cardiac differentiation relies on a series of transcriptional factors, which orchestrate the cell commitment. The core of this network includes members of the basic helix-loop-helix (bHLH) family HAND1 and HAND2, the T-box protein family (TBX5), the MADS-box family (MEF2, SRF), and GATA zinc-finger family (GATA4, GATA6, etc.).[33] However, many muscles-specific skeletal genes are expressed in early stage of cardiac differentiation, but the full network that regulates this process[34] is still unclear. The mechanisms and factors responsible for transcriptional regulation of mTOR in cardiac cells are still partially unknown[35]. Nevertheless, a recent study showed that MyoD1 have conserved recognition sites in mTOR promoters as well as several other pivotal genes of this signaling[36]. This is confirmed by MyoD1 ChiP-seq data collected from C1C12 cells[37]. Therefore**,** we conjecture that Bigheart could replace MyoD1 during cardiac differentiation by interacting with similar targets of the mTOR signaling pathway. In **Chapter 4**, we cross-referred data of ChiRP-seq and ChIP-seq to show that *Bigheart* and MyoD1 share a restricted number of targets, most of which are related to the mTOR pathway

regulation. mTOR signaling in skeletal muscle growth and hypertrophy is relatively well understood.[38,39] However, the mTOR dynamic in cardiogenesis is still under investigation. The expression pattern shown throughout the cardiac differentiation of *Bigheart* in humans and mice, along with the same results collected in hiPSC-derived cardiomyocytes (hiPSC-CMs), provide some insight into the molecular mechanism that intertwined this LncRNA and the mTOR signaling. We noticed that *Bigheart* is strongly expressed in earlier stages of cardiac differentiation, and results decreased in later stages of maturation. Surprisingly, in hiPSC-CMs, mTOR signaling followed precisely the same trend, suggesting that *Bigheart* could influence the activation of this pathway. LncRNA, which shows Trans-action often can regulate several genes and therefore influence an array of pathways simultaneously. According with the data already collected, we speculated that mTOR could be among them. This theory is further validated in HL-1 cells, where ChiRP-seq revealed that *Bigheart* binds to several mTOR-related genes. Moreover, the Silencing of this LncRNA negatively affects the expression of many genes of the mTOR pathway. Similarly, Silencing of *Bigheart* in induced pressure overload mouse hearts and following RNA-seq showed a strong impact on the PI3K-Akt pathway, upstream Regulator of the mTOR, and part of the PI3K/AKT/mTOR axis. [40] On the other hand, overexpression of *Bigheart* in postnatal mice hearts with AAV9 strongly induced mTOR signaling and was sufficient to induce hypertrophic growth. Collectively, our data suggest a contribution of *Bigheart* to cardiac development through the regulation of the mTOR pathway. The contribution of this pathway to embryogenesis has already been studied. Cardiac deletion of mTOR is associated with embryonic lethality[41]. Furthermore, disruption in mTOR-related genes such Raf1[42,43], and Eif4e[44] (both targets of *Bigheart*) also leads to severe cardiac hypertrophy. [45] The limitations of our experimental models prevented us from precisely evaluating its role in cardiogenensis and whether these same mechanisms are also regulated in the pathological development of cardiac hypertrophy. One of the limitations of our experiments was the impossibility of verifying the magnitude of the contribution of *Bigheart* in embryonic development in *vivo* and *vitro*. A possible experiment to overcome this barrier would be to develop a *Bigheart* knockout (KO) iPSCs*.* The differentiation in cardiomyocytes of this transgenic line (hIPSC-CMs) could provide several insights into this LncRNAs functions. A similar approach is already used to study the LncRNA *Cyrano,* an essential lncRNA for the maintenance of pluripotency and self-renewal in ESCs, in this case the strategy used to generate *Cyrano* KO cells was a dual guide RNA (gRNA) CRISPR/Cas9 followed by the selection of one heterozygous and one homozygous KO clone for further analysis[46], a similar strategy could undoubtedly be used to further explore *Bigheart*

functions. Alternatively, Knock-out mice for *Bigheart*, (BH^{null}/BH^{null}) or conditional Knock-out (*Bigheart* cKO) could be employed to estimate its impact on embryogenesis, as previously applied with several other lncRNA, like NEAT1[47], Maltat1[48] and Gomafu [49]. Furthermore, the improvement of the CRISPR)/Cas9 platform provides a powerful genome-editing tool that can safely generate lncRNA loss-of-function mouse models[50]. A further limitation of these results comes from the model used for the sequencing, the cardiomyocyte-like cell line HL-1. Although as already mentioned, HL-1 are partially suitable for studying cardiac models of adult cells, *Bigheart* seems to be mainly involved in embryonic development in immature cells, and hypertrophic response in adult cardiomyocyte. These two cell types have a different accessibility of the chromatin[51,52] as already noticed previously[53,54] and possibly a much larger number of targets for this LncRNA. Therefore, more accurate models to study *Bigheart* would be hiPSC-CMs harvested in early stages of cardiac differentiation [55] *versus* primary neonatal cardiomyocytes that undergo cardiomyocyte hypertrophy, both subjected to ChiRP-seq against this LncRNA. In a final instance, another important and overlooked aspect to further investigate is the role of Bigheart in HL-1, a cancer-like cell of tumor origin, and in broader terms the possibility that *Bigheart* could be involved in some pathway relevant to both cancer and cardiac hypertrophy. As suggested from the literature, the molecular signals involved in cellular survival and cell growth are as important in the onset of cardiac hypertrophic as in tumor progression[56]. Furthermore, cell proliferation and cardiac hypertrophy share similar risk factors, and in line with some recent studies, early stages of cardiac remodeling promote cancer progression and metastasis[57]. Hyperactivation in the mTOR signaling is commonly observed in various types of cancers[58], and several of our experiments suggest a link between Bigheart expression and mTOR activation. Nevertheless, we did not collect sufficient evidence to confirm this theory. Although our knowledge of the functions of *Bigheart* in the heart are still limited, in this thesis we have identified and characterized the contribution of this LncRNA to the regulation of two important axes, the calcineurin/NFAT signaling and the mTOR pathway. Finally with our work in **Chapter 3** and **Chapter 4** we provide evidence that this LncRNA could be a promising therapeutic target for cardiac treatment. In fact from our experiments, we noticed that the reduction of *Bigheart* by Gapmer blunts the calcineurin / NFAT signaling, by regulating Rcan1(**Chapter 3**). Similarly, the silencing with siRNA reduce the expression of several genes involved in the mTOR signaling(**Chapter 4**). these pathways are well-known for their pivotal role in cardiac hypertrophy and our experiments showed that *Bigheart* modulation reduces cardiac hypertrophy in *vivo* and

vitro. Furthermore, we set strong bases for further study which will clarify and expand our understanding of the molecular mechanisms of this LncRNA.

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Chapter 6

Impact Paragraph

In the last years, the world came to face incredible challenges concerning health and welfare through the COVID-19 pandemic. However, we are also experiencing an equally concerning and less acknowledged threat, regarding the incessant rise in cardiac diseases. Cardiac hypertrophy and consequential heart failure represent one of the most deadly and widespread diseases of society today, with tremendous economic and sociological consequences. Almost 550,000 new cases of heart failure are diagnosed each year, and currently, there are about 26 million cases worldwide [1]. This represents the largest patient base after cancer patients, for an annual cost of about $ 15,000 per patient in therapies and around 40 billion dollars for the healthcare system[2]. Additionally, the number of patients suffering from this type of disorder is constantly increasing, and at least 50 million cases are estimated to be registered in 2030[3]. As mentioned previously throughout this thesis, the current therapies and treatments lack efficiency, especially in patients with severe heart failure[4]. Therefore, the development of new approaches and methods to deal with those conditions is a fundamental task for healthcare. To improve the management of patients with severe HF, we need to improve our knowledge of the biological pathways that govern this disease. Already, the expanding understanding of the molecular mechanisms of cardiac disease helped to reduce mortality drastically[5]. However as mentioned, these efforts are far from sufficient[6]. In the last 20 years, basic research has revealed that different microRNAs and LncRNAs have fundamental roles during cardiac pathologies[7]. Modulation of such molecules can be used to reverse the pathology outcomes. At present, the relevance of ncRNAs in hypertrophic heart disease is well acknowledged. The next goal of the scientific community is the implementation of conventional therapies with new ncRNAs-based treatments with increased precision, that will further decrease mortality. In this context, basic research plays a critical role to direct future strategies. In this thesis, we investigated the role of the long non-coding RNA (LncRNA) *"Bigheart"* in cardiac hypertrophy. Our evidence suggests that this LncRNA has a crucial role in leading to maladaptive hypertrophy. We also provide the evidence that the modulation of this LncRNA can bring beneficial effects, such as the reduction of the hypertrophic remodeling in mice after TAC surgery in *vivo*. (**Chapter** 3 and **Chapter** 4). Therefore, we propose *Bigheart* as a potential target in patients suffering from cardiac hypertrophy. Although the application of ncRNAs-based therapies is still in the design phase, new cardiac hypertrophy hallmark LncRNAs such as *Bigheart* will provide the basis for the development of new clinical methodologies, and insights into the underlying mechanisms of HF and hypertrophy. Furthermore, the scientific evidence we reported in this thesis can prove to be particularly interesting for

researchers in the cardiovascular field, since there is still a dire need for pharmaceutical treatment approaches, and there is a strong competition to identify novel targets. Further investigation of the list of *Bigheart* targets identified by ChiRP-seq described in **Chapter 4** can lead to the identification of specific new genes involved in cardiac hypertrophy onset, and therefore help to develop a better-tailored therapy for patients. Similarly, the aforementioned list could help to identify novel genes involved in cardiogenesis and prove useful in identifying new targets for the study of regenerative therapies. This type of basic research aside, academic value has a significant impact on several other groups. Pharmaceutical companies, clinicians, and researchers, as well as patients with cardiac hypertrophy, will profit the most from the result of this thesis, which highlights a new and very important candidate for the treatment of this condition. Additionally, this evidence confirms the value of investing in molecules or drugs directed against LncRNA such as *Bigheart* to improve the outcome for patients with cardiac conditions. Currently, a business of over 1 billion euros per project has been estimated for the development of a new drug for cardiac hypertrophy [8]. The use of *Bigheart* inhibitors could generate economic success for pharmaceutical companies and generate jobs in different sectors over a period of almost 20 years. The development of a new drug passes through different stages, but it is possible to divide then into two main classes, pre-clinical and clinical trials. The first part of the pre-clinical trial concerns the development of the drug itself. This step is mostly focused on the hypothesis generation and the conceptual mechanism of action of the drug, immediately followed by the proof of concept and its efficacy, usually archived using in *vitro* models (cells lines or primary cells) and animal models (frequently rats or mice). Furthermore, in this phase, possible structural modifications are studied to increase the absorption efficiency or the effectiveness of the drug. The second stage concerns the non-clinical study on at least 3 different species to ensure product safety, effect, pharmacokinetics, and pharmacodynamics. In the third phase referred to as clinical trials the drug is tested on humans, where its effectiveness, side effects, and therapeutic dose are evaluated for a period of 3-7 years. Finally, after a total review of all results, the drug is authorized for marketing, which will, however, be followed by a further period of supervision for a period of 5-10 years[9]. Many of these steps require the registration of patents and specialized personnel. Additionally, the involvement of various companies, start-ups, and marketing agencies generates a business of several million. Although there are still no inhibitors of LncRNAs on the market, Antisense RNA against protein targets are marketed since 1998 (e.g., Vitravene or EXONDYS 51). More recently miR-92 is entered in phase I of the clinical trial for the treatment of ischemic heart (NCT03603431 and NCT03494712) and

overall, 16 microRNAs-bases trials are currently ongoing [10]. Considering the enormous success of Vitravene or EXONDYS 51 and the rapid development of vaccines with mRNAs bases it is likely that several researchers will start to look into lncRNA therapy soon. Unfortunately, as described in **Chapter 2,** the tools for a correct and controllable modulation of LncRNA in humans are still under development. GapMERs and siRNAs have proven to be effective in several animal models in pre-clinical trials[11], but there is still no experimental evidence of their use in humans, and no clinical trial for such a molecule has been announced yet. Additionally, new gene therapy strategies such as CRISPR Interference, capable of modulating gene expression without DNA modifications are also considered options today[12]. Therefore, the development of reliable LncRNAs inhibitors represents not only a great economic opportunity but also a significant advancement in medical technology. Considering the data presented in our thesis, we can conclude that we have collected the evidence necessary for the development of the pre-clinical phase, at which, however, it is necessary to implement the experimentation on pigs and no-humane primate models before the clinical application. The main problems to address before setting a clinical project are a reliable delivery system and the controllable and selective modulation of *Bigheart*. In our thesis, we have proven that GapMers are an efficient and reliable silencing system in neonatal rat cardiomyocytes, and several studies prove similar results also in human cells[13]. The next step will be to test its effectiveness in a complex system such as humans, and what chemical modification adds to increase or modulate its efficiency[14] This further information will still require many studies about it, with the development of further academic positions (Ph.Ds. or post-docs), new grants and funding, guaranteeing an economic gain for universities and biotechnological companies.

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Biography

Nicoló Mangraviti was born in Messina, Italy the 30th of July 1992. In 2014 he graduated in biotechnology from the University of Sannio, Italy with a thesis about alternative splicing. Then he moved to Rome to attend the master's degree where he graduated *cum laude* in Genetic and Molecular Biology applied to research, at the University La Sapienza. He spent the last year of his master's degree working in the Paediatric hospital Bambino Gesu of Rome under the supervision of Dr. Angela Gallo and Prof. Dr. Alessandro Fatica. Focusing his research on "ADAR2 activities in Glioblastoma", a project supported by AIRC (Italian association of Cancer Research), after the master's degree, he spent an additional year in Dr. Gallo's lab as a researcher. During this period, he developed a great interest in Molecular Mechanism and RNA biology, focused especially on LncRNA biology. Nicoló then moved to the Netherlands to work

as a Ph.D. student in the department of Molecular Genetics at Maastricht university under the supervision of Prof. Dr. Leon J. De Windt. His work concentrated on the characterization of the LncRNA *Bigheart* in cardiac hypertrophy. During this period, he has been a candidate for the Young investigation award (YIH) from the Dutch-German meeting 2020 and winning the YIH at the meeting for international society for Heart Research in Turin 2021. At the present time, Nicoló is employed as a postdoctoral fellow at Yale University in Connecticut under the supervision of Dr. Nadya Dimitrova, continuing his research on LncRNA biology.