Mouse Models to Study Inherited Cardiomyopathy

8

Mohammad Bakhtiar Hossain, Mohammad Bohlooly-Y, and Ralph Knöll

Contents

8.1 Introduction 291
8.2 Inherited Cardiomyopathies 291
8.3 Overview of Genetic Interventions 293
8.3.1 Random Transgenesis 293
8.3.2 Knockout (KO) 294
8.3.3 Conditional Knockout 295
8.3.4 Knock In (KI) 295
8.3.5 Precision Genetic Engineering (PGE) 296
8.3.6 Tet-Off/Tet-On 297
8.3.7 Humanized Mouse Model 297
8.3.8 Reporter Mouse Model 298
8.4 Examples: Mouse Models of Cardiomyopathies 298
8.4.1 Phospholamban (Pln) 298
8.4.2 Calcineurin (CaN) 300
8.4.3 Titin (TTN) 300
8.4.4 Myosin Binding Protein C 3 (Mybpc3) 301
8.4.5 Cysteine-Rich Protein 3 (Csrp3/Mlp) 304
8.5 Conclusion 306
References 306

M. B. Hossain · R. Knöll (✉)
Integrated Cardio Metabolic Center (ICMC), Myocardial Genetics, Karolinska Institutet, University Hospital, Heart and Vascular Theme, Huddinge, Sweden

Bioscience Research & Early Development, Cardiovascular, Renal and Metabolic Diseases (CVRM), Biopharmaceuticals R&D, AstraZeneca, Mölndal, Sweden
e-mail: Ralph.Knoell@KI.se

M. Bohlooly-Y
Discovery Sciences, BioPharmaceuticals R&D, AstraZeneca, Gothenburg, Sweden

J. Erdmann, A. Moretti (eds.), *Genetic Causes of Cardiac Disease*, Cardiac and Vascular Biology 7, https://doi.org/10.1007/978-3-030-27371-2_8

Abstract

Cardiovascular diseases, including cardiomyopathy and associated heart failure, are the number one cause of death worldwide, but our ability to interfere with these devastating diseases is limited. Cardiomyopathies, which are mainly due to genetic causes, make a significant portion of heart failure. Current pharmacological treatment of cardiovascular diseases focuses on symptoms rather than the underlying cellular mechanisms, and the gaps in our understanding of cellular mechanisms of the disease are profound. Elucidation of these mechanisms is a central issue in cardiovascular biology and important for designing new treatment for cardiovascular diseases. While significant progress has been made in using in vitro systems in deciphering the mechanisms and finding innovative solutions for cardiovascular disease treatment, including the use of induced pluripotent stem cell (iPSC) derivatives, suitable in vivo models are more difficult to develop. Among several different species, mouse models are rather inexpensive, easily manipulatable, reproducible, physiologically representative of human disease, and ethically acceptable. This chapter will provide a brief overview of genetics of heart failure, largely focusing on genetically altered mouse models and experimental approaches applicable to cardiovascular research.

List of Abbreviations

AD	Autosomal dominant
AR	Autosomal recessive
ARVC	Arrhythmogenic right ventricular cardiomyopathy
BNP	Brain natriuretic peptide
cKO	Conditional knockout
CPVT	Catecholaminergic polymorphic ventricular tachycardia
CRISPR	Clustered regularly interspaced short palindromic repeats
CSRP3/Csrp3 (or MLP/Mlp)	Cysteine-rich protein 3
CVD	Cardiovascular disease
DCM	Dilated cardiomyopathy
ECM	Extracellular matrix
HCM	Hypertrophic cardiomyopathy
KI	Knock in
KO	Knockout
LV	Left ventricle
LVNC	Left ventricular noncompaction
PGE	Precision genetic engineering
PKA	Protein kinase A
PLN/Pln	Phospholamban
RCM	Restrictive cardiomyopathy

SR	Sarcoplasmic reticulum
SRF	Serum response factor
TALEN	Transcription activator-like effector nucleases
ZFN	Zinc finger nuclease

8.1 Introduction

According to the World Health Organization (WHO), cardiovascular disease (CVD) is considered as the major cause of morbidity and mortality [1]. During the course of the last decades, impressive achievements have been made in diagnosis and treatment of cardiovascular disease, relying to a large extent on the use of experimental animal models. Despite this, no permanent cures exist for the overwhelming majority of cardiovascular diseases. As such, heart failure can only be treated by heart transplantation, but only a very limited number of donor hearts are available and its therapeutic potential is limited by the complications of long-term allograft vasculopathy. Innovative therapies can be developed by tissue engineering, autologous and allogenic cell therapies, gene therapy and genome editing—but their development depends to a large extent on extensive animal experimentation.

Human heart samples, either non-transplantable or end-stage failing, usually obtained at the time of transplantation, are valuable for identifying underlying disease causing molecular pathways, but their number is limited and they exhibit significant variability due to differences in genetics, epigenetics, environment, and different therapeutic regimens. Therefore, we need to develop preclinical testing for relevant CVD models, including heart failure. The closer the heart or body weight of the animal is in comparison to humans, the more similar are the hearts. Therefore, large animal models, like nonhuman primates and dogs, are much more relevant, but experimentations with these animals require extensive ethical considerations and are expensive to perform, which limits their widespread use.

Rodent models, especially mice, are often used in CVD research since they are easy to handle, have a short gestation time, are genetically manipulatable, and have relatively low maintenance costs—making them much more suitable for high-throughput screening. Despite phylogenetically quite distant from humans and some pathophysiological features of disease, including electrophysiology, and their response to pharmacological interventions being significantly different, mouse models have become the method of choice in biomedical research during the last decades.

8.2 Inherited Cardiomyopathies

Cardiomyopathies are a heterogenous group of myocardial diseases associated with mechanical or electrical dysfunction that usually exhibit inappropriate ventricular hypertrophy or dilation [2]. Primary cardiomyopathies are predominantly confined

to the myocardium, while secondary cardiomyopathies result from systemic diseases associated with heart failure. Major types of cardiomyopathy includes hypertrophic cardiomyopathy (HCM), dilated cardiomyopathy (DCM), arrhythmogenic right ventricular cardiomyopathy (ARVC), and restrictive cardiomyopathy (RCM). Genetic mutations have been reported to account for a significant percentage of all cardiomyopathies, and it is likely that a genetic component exists for all (figure).

- Hypertrophic cardiomyopathy (HCM) is characterized morphologically and defined by a hypertrophied, nondilated left ventricle (LV) in the absence of other systemic or cardiac diseases that are capable of producing the magnitude of wall thickening evident (e.g., systemic hypertension, aortic valve stenosis). About 60% of all HCM cases are due to mutations in genes encoding sarcomeric proteins. The MYH7:p.R403Q mutation in the cardiac β-myosin heavy chain was the first mutation shown to cause cardiomyopathy [3, 4].
- Dilated forms of cardiomyopathy (DCM) are characterized by ventricular chamber enlargement and systolic dysfunction with normal LV wall thickness. About 35% of all DCM patients carry mutations in genes encoding sarcomeric or cytoskeletal components.
- Arrhythmogenic right ventricular cardiomyopathy (ARVC) involves the right ventricle predominantly with progressive loss of myocytes and fatty or fibrofatty tissue replacement, resulting in regional (segmental) or global abnormalities. About 50% of all ARVC cases can be traced back to mutations, especially in genes encoding desmosomal components.
- Primary restrictive cardiomyopathy (RCM) is a rare form of heart disease and a cause of heart failure that is characterized by normal or decreased volume of both ventricles associated with biatrial enlargement, normal LV wall thickness and atrioventricular (AV) valves, impaired ventricular filling with restrictive physiology, and normal (or near normal) systolic function.
- Left ventricular noncompaction (LVNC) is a recently recognized congenital cardiomyopathy characterized by adistinctive ("spongy") morphological appearance of the LV myocardium. Noncompaction involves predominantly the distal (apical) portion of the LV chamber with deep intertrabecular recesses (sinusoids) in communication with the ventricular cavity, resulting from an arrest in the normal embryogenesis.

However, while the overwhelming majority of cardiomyopathies are autosomal dominant, more complex modes of inheritance, such as homozygous, double heterozygous, and compound heterozygous mutations, are existent which underlie recessive, X-linked, or mitochondrial types of inheritance (Table 8.1). While a lot of valuable information were gathered from careful clinical and genetic examination of human subjects, significant molecular insights were revealed from the generation and analysis of genetically altered mouse models.

Table 8.1 Most important genetic cardiomyopathies, prevalences, mode of inheritance, and related genes

Disease	Prevalence	Inheritance	Genes/structure
HCM	1:500	Mainly AD	Sarcomere
DCM	1:500–1:2000	Mainly AD, AR, mitochondrial, X-linked	Sarcomere, cytoskeleton, signal transduction ECM
ARVC	1:2000–1:5000		Desmosome
RCM	Rare	AD, AR, mitochondrial, X-linked	Sarcomere (troponin)
LVNC	Rare	AD, AR, mitochondrial, X-linked	DCM related

AD autosomal dominant, *AR* autosomal recessive, *HCM* hypertrophic cardiomyopathy, *DCM* dilated cardiomyopathy, *ECM* extracellular matrix, *ARVC* arrhythmogenic right ventricular cardiomyopathy, *RCM* restrictive cardiomyopathy, *LVNC* left ventricular noncompaction

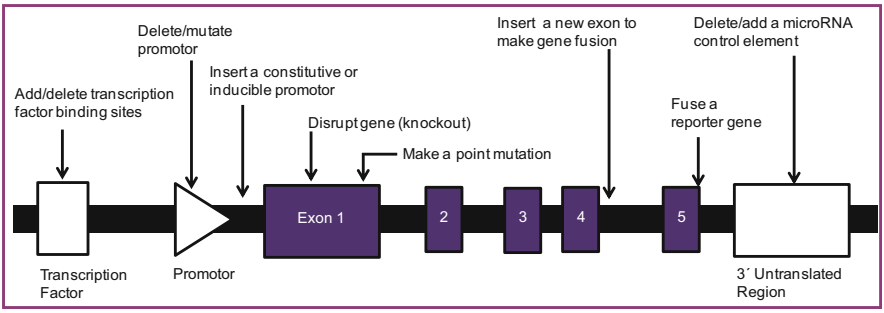

Fig. 8.1 Examples of various genetic changes that can be introduced into mice

8.3 Overview of Genetic Interventions

Genetic engineering can be applied to create model animals that mimic human conditions and gene therapy. Genetically modified mice are the most common genetically engineered animal model and used to study heart disease, including cardiomyopathies. Homologous recombination using a targeting vector that consists of a modified version of the endogenous gene can change the gene or interest. Furthermore, we can introduce different functional changes of a specific gene into a mouse locus through gene targeting (Fig. 8.1).

8.3.1 Random Transgenesis

A transgenic mouse model can be generated by random insertion of a foreign DNA sequence/fragment into the genome by random transgenesis. The insertion of the foreign DNA usually results in a gain of function (expression of a new gene) or

overexpression of endogenous genes. The classical method used for the generation of transgenic mice is via pronuclear injection where a transgene is injected into a fertilized mouse egg with subsequent random integration of the gene within the mouse genome. These models are the simplest with very little molecular biology work and require a relatively short development time. However, the biggest disadvantages of this model come from the unpredictability of the location of genomic integration and the number of inserted gene copies. Random insertion of genetic elements into the mouse genome can lead to the misregulated expression of essential genes. The inserted transgene may also be subjected to variegation and positional effects, such as gene silencing, which may lead to altered phenotypic outcomes.

8.3.2 Knockout (KO)

We can determine the function of a specific gene in an organism by creating a KO rendering that specific gene nonfunctional and by inferring the differences between the knockout organism and normal or wild-type littermate controls. The original gene knockout technique was developed by Martin Evans, Oliver Smithies, and Mario Capecchi [5–7]. It is necessary that we know the sequence of the gene to create a KO organism. The traditional process of creating a KO organism starts with constructing an appropriate plasmid where the mutant version of the gene of interest is incorporated. The plasmid construct is engineered with an aim that the faulty version of the gene will recombine with the target gene. Embryonic stem cells are transfected with the plasmid, along with a "transgene" whose overexpression indicates plasmid transfection, and screened by antibiotic selection (Fig. 8.2). Recombination occurs in the region of that sequence within the gene, resulting in the insertion of a faulty sequence to disrupt the gene. The targeted embryonic stem

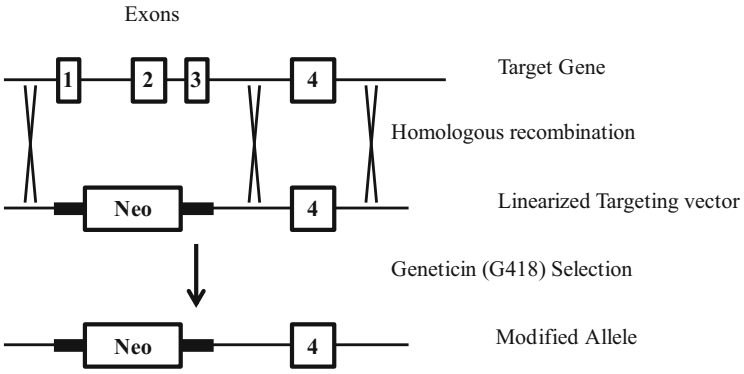

Fig. 8.2 The process of homologous recombination employed in mouse model development. A gene KO model is generated where the neomycin selection cassette replaces part of the gene coding sequence, rendering the gene nonfunctional (Adapted from Gama Sosa et al. [8])

cells are inserted into early embryos, and chimeric organism is generated. Chimeric animals are selectively bred to produce heterozygous animals which are needed to obtain homozygous mutation carriers.

8.3.3 Conditional Knockout

In contrast, a conditional KO allows gene deletion in a tissue (e.g., liver, heart)- or time-specific (a specific stage in development) manner. In tissue-specific conditional KO, a gene is inactivated in target tissue(s) only; in all other tissues, the gene is fully functional. A target gene can also be inactivated at particular time(s) of interest, e.g., mimicking adult-onset condition with more physiological responses and disease relevance. Conditional KO animals are useful to study the role of individual genes in living organisms; as in traditional gene knockout, embryonic death can occur from a gene mutation. Conditional KO organism is most commonly created by introducing short sequences called lox (locus of recombination) sites around the gene of interest and introduced into the germline via the same mechanism as KO (Fig. 8.3). This germline can then be crossed to another line containing Cre recombinase, which is a viral enzyme that can recognize these sequences. This system is inducible by tetracycline or by other means that activate transcription of the Cre recombinase gene or by tamoxifen that activates transport of the Cre recombinase protein to the nucleus. Activation of the system leads to either deletion or inversion of the genes between the two lox sites, depending on their orientation.

8.3.4 Knock In (KI)

Gene KI is a targeted insertion involving one-for-one substitution of DNA sequence information in a genetic locus. This technique usually relies on homologous recombination for gene replacement. Embryonic stem cells with specific gene modification are then implanted into a viable blastocyst, which will grow into a mature chimeric mouse with some cells having the modifications introduced to the embryonic stem cells. Subsequent offspring of the chimeric mouse will then have the gene KI [9]. The first KI mouse model in heart failure research was described by Geisterfer-Lowrance et al., where HCM was generated by introducing $Arg^{403} \rightarrow Gln$ mutation into the α cardiac myosin heavy chain (MHC) mouse locus [10]. This elegant study was a "proof-of-concept" showing that a specific mutation is the underlying cause of a devastating human disease. Later on, this mouse model was used to study the role of sarcomere mutations in the development of HCM and successfully used in drug discovery where the beneficial effects of MYK-461 is discovered [11]. We can design KI models where the transgene is introduced into the permissive ROSA26 gene locus, which is well suited for gene overexpression and speedy KI model development [12]. Reporter genes or any other genes of interest can be introduced into ROSA26 gene locus.

A point mutation can be introduced in a single defined base pair location of a gene of interest. The resulting model expresses a mutant protein instead of the wild type if

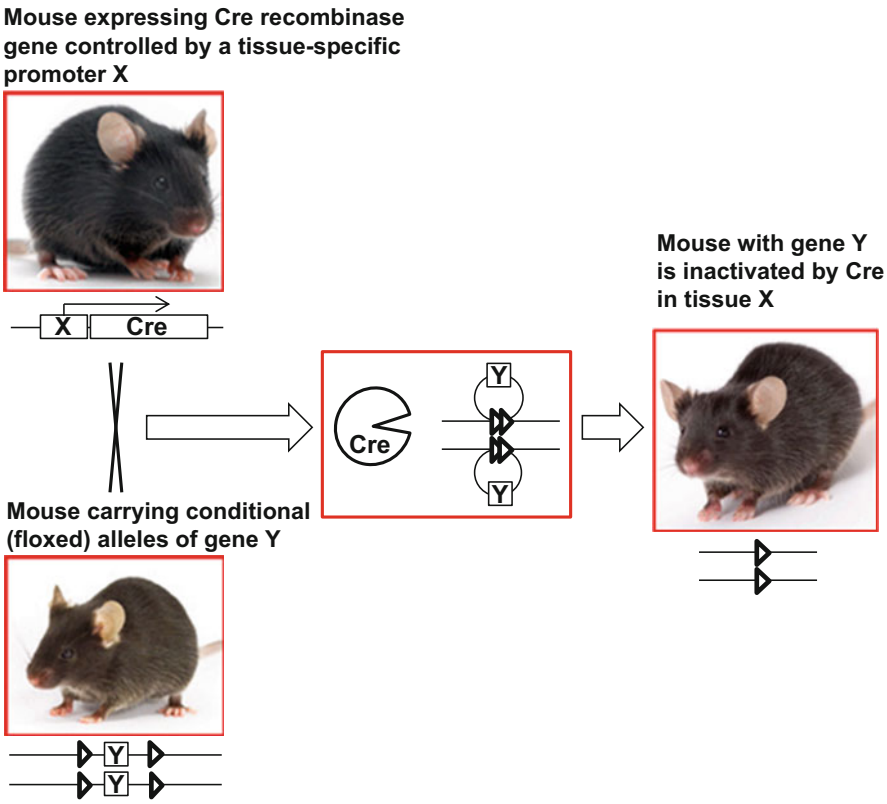

Fig. 8.3 The Cre-loxP system is used extensively in mouse models for cell type- and tissue-specific as well as temporally regulated genetic alteration. Mice that express Cre recombinase under the control of a tissue-specific promoter are crossed with mice that constitutively express a "floxed" genetic region, meaning that the region is flanked by loxP sites. Cre-mediated recombination excises any region of DNA in between, leaving behind a single loxP site

the point mutation is in the coding sequence, but the expression pattern is maintained. Point-mutant models have several potential applications including the study of the causal role of mutations in human pathological conditions, in vivo functionality of an enzyme active site, or protein binding site. Point mutations may also be used as a complementary strategy or an alternative strategy to the more common KO mouse model.

8.3.5 Precision Genetic Engineering (PGE)

PGE focuses on the development of site-directed modification methods in specific DNA sequences to introduce new traits in the models. Zinc finger domains, a zinc ion containing protein domain that can recognize specific DNA sequence, can be

engineered to fuse with DNA cleavage domain to form zinc finger nucleases (ZFNs). These engineered DNA-binding proteins that facilitate targeted editing of the genome by creating double-strand breaks in DNA at specific sites [13]. Transcription activator-like effector nucleases (TALEN) are other sets of chimeric nucleases composed of programmable, sequence-specific DNA-binding modules fused with nonspecific DNA cleavage domain, which can cut DNA at specific locations [14]. ZFNs and TALENs enable homology-directed repair at specific genomic locations after inducing DNA double-strand breaks. Recent development of CRISPR/Cas9 system also allows us accurate and successful gene insertions with ease [15]. The CRISPR/Cas system is a prokaryotic immune system that confers resistance to foreign genetic elements [16]. We can manipulate cellular genome specifically at a desired location—thus allowing existing genes to be removed—by delivering the Cas9 nuclease complexed with a synthetic guide RNA (gRNA) into a cell [17], and we can generate complete gene KO mice in a single step [18].

8.3.6 Tet-Off/Tet-On

Different antibiotics, e.g., tetracycline or its derivatives, can induce gene expression. This method was first described by Professors Hermann Bujard and Manfred Gossen at the University of Heidelberg in 1992 [19]. In this system, transcription is turned on/off in the presence of an antibiotic in a reversible way, providing an advantage over KO and KI where the gene inactivation is irreversible. A tetracycline response element (TRE) is 7 repeats of a 19-nucleotide sequence, and this sequence is recognized by tetracycline repressor (tetR). A tetracycline-controlled transactivator (tTA) was developed by Gossen and Bujard by fusing tetR with the C-terminal domain of VP16, an essential transcriptional activation domain from herpes simplex virus. In this system, tet-inducible promoter is repressed by the presence of tetracycline and known as Tet-Off. In a subsequent report, Gossen et al. [20] used random mutagenesis to identify which amino acids of tetR are important for tetracycline-dependent repression. Mutation of these residues created a reverse Tet repressor (rTetR) which relies on the presence of tetracycline for transcription induction (Tet-On).

8.3.7 Humanized Mouse Model

A humanized mouse is a biological model in which a mouse gene is replaced by either a part of or the entire equivalent human gene. The human protein is expressed in mouse in the same cells and tissues instead of the mouse protein. Humanized mouse models are important tools for studying pathological conditions, preclinical research, and compound efficacy in vivo to ensure translatability between various human- and species-specific in vitro and in vivo models. The human protein is expressed typically using the mouse promoter and regulatory regions to ensure natural expression and maintain functionality of the protein. Humanized models

provide a far better predictive tool to model human disease and efficacy testing of therapeutic compounds over wild-type mice [21]. By replacing key murine genes with their human counterparts, these models offer greater predictive ability that enables us to increase the impact of research on human diseases, speed up drug discovery, and reduce the time-to-market of new therapeutics.

8.3.8 Reporter Mouse Model

A reporter mouse model is a model in which a target gene is modified for the purpose of monitoring its promoter activity. This is done through the replacement of coding sequence of the gene with the coding sequence of a marker, such as green fluorescent protein (GFP). Reporter mouse models can be used to monitor a target protein's expression, localization, and trafficking and are powerful tools to monitor transcriptional activity of a promoter in vivo. We can also generate reporter models where we avoid knocking out the endogenous gene and use polycistronic technology to couple the expression of the reporter gene with the endogenous promoter, thus allowing co-expression of both target and reporter genes [22].

8.4 Examples: Mouse Models of Cardiomyopathies

To describe a complete overview, listing the myriads of genetically altered mouse models used in heart failure research, is beyond the scope of this review. Therefore, we chose to highlight some of the models we think are probably most important in identifying the roles of key proteins in cardiomyopathies (Fig. 8.4) (we apologize to many authors who contributed to this exciting field but not being mentioned here).

8.4.1 Phospholamban (Pln)

One of the first genetically altered mouse models to study the importance of individual proteins in myocardial contractility was the phospholamban knockout model [23]. Phospholamban is an important regulator of Ca^{2+}-ATPase in the cardiac sarcoplasmic reticulum. This protein was first discovered by Arnold Martin Katz and coworkers in 1974 [24] and is an integral membrane protein of 52 amino acids encoded by *PLN* gene. Phospholamban is a substrate for the cAMP-dependent protein kinase (PKA) in cardiac muscle. Although it functions as an inhibitor of Ca^{2+}-ATPase, phosphorylation by PKA relieves the inhibition. The mouse phospholamban gene was cloned from 129/SvJ mouse. An internal *Hin*dIII fragment was replaced by *Xho* I-*Sal* I fragment of the polyadenylation signal-deficient *neo* gene from pMCl*neo*. Embryonic stem cells were electroporated with this targeting fragment. Phospholamban-targeted ES cells were injected into C57Bl/6 blastocysts and transferred to pseudopregnant females. Heterozygous mutant mice were mated to generate homozygous mutant. Phospholamban ablation in mice showed no gross

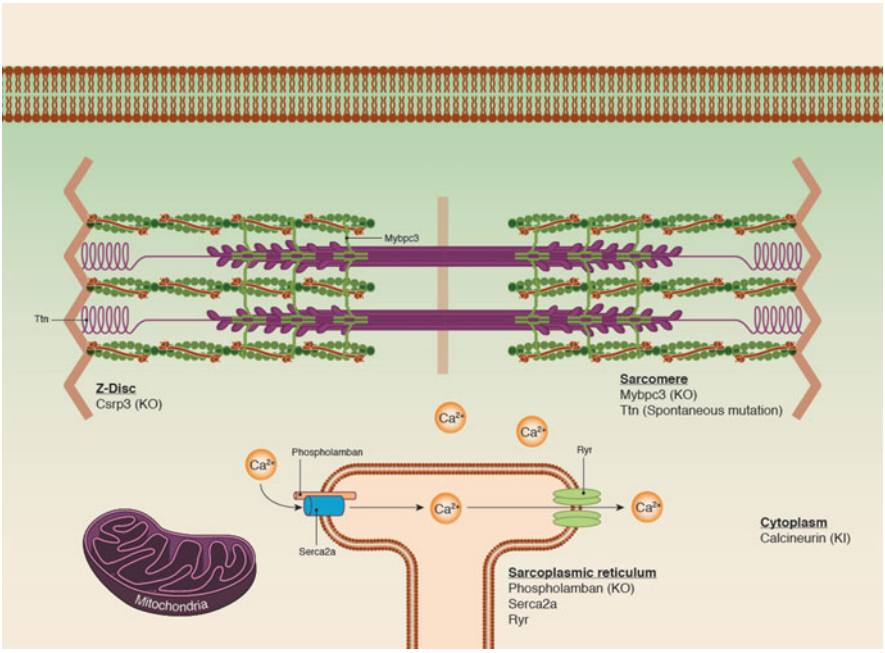

Fig. 8.4 Different cardiomyocyte proteins that were genetically altered in mouse models described in this report. The corresponding mouse models generated to decipher the function and significance are indicated

developmental abnormalities, but mice exhibited enhanced myocardial performance. The time to peak pressure and the time to half-relaxation were significantly shorter in absence of phospholamban. These mice provided an attractive system for elucidation of the regulatory effects of phospholamban on sarcoplasmic reticulum Ca^{2+}-ATPase.

Phospholamban KO mice were crossbred with a well-characterized mouse model of DCM, which harbors a deficiency in muscle-specific LIM protein (MLP; please see also CSRP3 section). Phospholamban ablation led to a dramatic rescue of the cardiomyopathy phenotype of MLP KO mice [25]. Later on, phospholamban KO mice were also crossbred with TNF1.6 mice, which develop heart failure as a consequence of cardiac-specific overexpression of tumor necrosis factor alpha (TNF-α). The resulting offsprings with phospholamban ablation showed improved calcium transients but not cardiac functions [26]. Phospholamban KO mice were also crossbred with tropomyosin-mutant (α-TmE180G) mice, which mimic mutation and features from human hypertrophic cardiomyopathy. The resulting progeny (PLN KO/Tm180) displayed a rescued hypertrophic phenotype with improved morphology and cardiac function [27]. More recent work showed that phospholamban ablation in ryanodine receptor (RyR2)-mutant mice breaks spontaneous Ca^{2+} waves, which is a major cause of Ca^{2+}-mediated arrhythmias [28]. A marked increase in sarcoplasmic reticulum Ca^{2+} leaking was also observed. Despite

increased Ca^{2+} leakage, phospholamban ablation in these mice protected against catecholaminergic polymorphic ventricular tachycardia (CPVT) [28].

The PLN:p.R14del, highly prevalent in the Netherlands, is associated with severe DCM and ARVC. Recently, iPS-derived cardiomyocytes from patients have been successfully used to model this disease in the "petri dish" [29, 30]; however, suitable in vivo models are also needed to develop novel cures for this otherwise lethal disease.

8.4.2 Calcineurin (CaN)

Calcineurin is a heterodimer of a 58 to 64 kDa catalytic subunit, calcineurin A (CnA), and a 19 kDa regulatory subunit, calcineurin B (CnB), well known as one of the major four Ser/Thr phosphatases found in eukaryotic cells and implicated in cardiac hypertrophy and associated heart failure. The role of calcineurin (CaN) in cardiac hypertrophy was first described by Molkentin et al. in 1998 [31]. Transgenic mice that express activated forms of calcineurin were generated by cloning a constitutively active form of calcineurin A catalytic subunit with 5′*Sal* I and 3′*Hin* DIII linkers into an expression vector containing α-*MHC* promoter. Similarly, mice expressing human NF-AT3 were generated by cloning a DNA sequence encoding amino acids 317–902 of human NF-AT3 into α-*MHC* expression vector. These sequences were injected into fertilized oocytes, and resulting oocytes were transferred into the oviducts of pseudopregnant mice. Cardiac hypertrophy and heart failure mimicking human heart disease were observed in the transgenic mice expressing calcineurin and NF-AT3. More importantly, the authors showed that pharmacologic agents that inhibit calcineurin activity block in vivo and in vitro hypertrophy. Calcineurin A β-deficient animals (CnAβ−/−) have been generated; the only phenotype reported is smaller hearts in comparison to their wild-type littermate controls [32]. However, calcineurin also appears to be cardioprotective, at least under conditions of ischemia reperfusion [33].

8.4.3 Titin (TTN)

Titin is important in the contraction of striated muscle tissue and connects Z line to M line in sarcomere (for a brief review, please see Tabish et al. [34]). It functions as a molecular spring and responsible for the passive elasticity of muscle. It is the largest known protein with 244 individually folded domains [35]—and these domains unfold when the protein is stretched and refold when the tension is removed [36]. Gerull et al. first reported that titin mutations cause familial DCM in autosomal-dominant fashion in 2002 [37]. Later on, it was shown in a large cohort of subjects that truncating mutations in titin account up to 25% of all causes of dilated cardiomyopathy [38]. These data have largely been confirmed by other authors (for review Tabish et al. [34] and Marston et al. [39]). The first titin mutation reported in mice arose spontaneously on C57BL/6 J background, causing muscular

dystrophy with myositis [40]. A KI mouse model was generated with a previously identified 2-bp insertion mutation. Homozygous mutation caused sarcomere formation defects in embryonic heart and was shown to be embryonically lethal [41]. Heterozygous mice were viable and showed normal cardiac morphology and function. However, when these heterozygous mice were chronically exposed to angiotensin II, they developed marked left ventricular dilatation with impaired fractional shortening and diffused myocardial fibrosis—mimicking human DCM [41].

Titin mutation was recently (2014) added to human familial clinical genetic testing of cardiomyopathies, which resulted in an additional 10% genetic diagnosis of DCM [42]. Frameshift mutations in the titin gene are a major cause of DCM. A proof-of-concept was established recently showing that disruption in the *titin* reading frame due to truncating mutations can be restored by exon skipping. This RNA-based strategy was shown to work in both patient cardiomyocytes and in mouse heart with DCM, providing us with a potential treatment option for DCM [43].

8.4.4 Myosin Binding Protein C 3 (Mybpc3)

Myosin binding protein C (MYBPC) is a crucial component of the sarcomere and an important regulator of muscle function. While mutations in the cardiac isoform of MYBPC (MYBPC3) are well-known causes of cardiomyopathies, such as HCM and DCM, the underlying molecular mechanisms are not well understood. A variety of MYBPC3 mutations have been studied in great detail and several corresponding mouse models have been generated. Most MYBPC3 mutations may cause haploinsufficiency, and with it, they may cause a primary increase in calcium sensitivity that could explain major clinical features of HCM patients such as the hypercontractile phenotype and the well-known secondary effects such as myofibrillar disarray, fibrosis, myocardial hypertrophy, and remodeling including arrhythmogenesis. However, the presence of poison peptides, mutant sarcomeric proteins that incorporate into myofibrils and act as dominant negative proteins, in some cases cannot be fully excluded, and most probably other mechanisms are also at play. Here we discuss various MYBPC3 mouse models and their implications in cardiomyopathy.

MYBPC3 mutations causing human HCM were first reported in 1995 [44, 45]. Indeed HCM is a frequent disease, affecting 1:500 individuals [2], and depending on the population analyzed, MYBPC3 mutations are found in up to 40–50% of the genotyped HCM patients [46]. To date more than 350 different MYBPC3 mutations have been reported in patients with HCM[47]. In general, MYBPC3 mutations are associated with a slightly lower penetrance (those with disease-causing mutations that have clinical manifestations), with later onset of disease, and with milder forms of disease progression in comparison to other HCM-causing mutations located, for example, in the beta myosin heavy chain (MYH7) gene [48, 49]. However this general statement may not necessarily be

true for all MYBPC3 mutations, and indeed some MYBPC3 mutations are associated with a poor prognosis.

Other MYBPC3 mutations primarily found in DCM patients have also been reported, for example, the Asn948Thr missense mutation [50]. In addition, the MYBPC3 Arg502Trp mutation with a frequency of about 2.4% is the most common HCM-causing mutation among individuals of European descent in the USA [51]. Other mutations may be prevalent in different European populations, such as in the Netherlands, where three different founder mutations are present: (1) the c.2373_2374insG MYBPC3 which is present in the great majority of HCM patients (up to 8 to 25%) and where (2) the c.2864_2865delCT and (3) the c.2827C>T mutations occur in about 5% of HCM patients each [52, 53]. Although MYBPC3 mutations are established as well-known causes of cardiomyopathy, the underlying molecular mechanisms are not well defined. Genetically altered mouse models provided significant new insights, which will be discussed in the next paragraphs.

To gain more insight into the underlying molecular mechanisms, Mybpc3 has been deleted in genetically altered mouse models by two independent groups. Loss of this protein is not associated with any embryonic lethality, and MYBPC3 also is not essential for sarcomere formation, but its absence results in profound eccentric hypertrophy in the homozygous animals [54, 55]. Hemodynamic analysis of the mice where exons 1 and 2 have been deleted was performed by the Carrier group, revealing the presence of normal contractility but severe diastolic defects. In addition, heterozygous animals develop septal hypertrophy, a hallmark of HCM [54]. However, these animals were engineered such that they harbor a complete ablation of the gene and therefore are useful to identify basic mechanisms, but the overwhelming majority of human mutation carriers express mutant mRNAs and probably proteins, which makes it difficult to relate these data directly to humans.

Therefore, in addition to the pure knockout models, wild-type and truncated MYBPC3, which mimic a certain type of human mutations leading to the loss of carboxyterminal domain including titin and myosin binding sites, were overexpressed in mouse models. Overexpression of the truncated form, but not the wild-type protein, led to the appearance of HCM features, including hypertrophy, and an increase in calcium sensitivity [56]. Whereas abovementioned transgenes were well expressed at the mRNA and protein levels, overexpression of a Mybpc3 mutant lacking only the myosin binding domain resulted in the expression of only very modest levels of mutant protein (i.e., about 5%) which led to a mild hypertrophy and heart failure phenotype [57].

Two additional KI mouse models have been engineered to carry Mybpc3 mutations found in patients which affect titin and myosin binding. Interestingly, animals homozygous for these mutations develop a DCM-like phenotype with depressed contractility and hypertrophy [58]. Another KI mouse model was generated such that the mutant protein did not contain the amino terminal myosin binding domain. The mutant protein was readily integrated into the sarcomeres of heterozygous and homozygous animals and was protein kinase A (PKA) phosphorylatable, and no major structural defects of sarcomere were detected. However, this mutation was associated with a significant increase in calcium

sensitivity [59]. An additional mouse KI model, based on a G>A transition located on the last nucleotide of exon 6 and which was found in a patient with HCM, has been generated by Lucie Carrier's group. Interestingly this mutation gives rise to three different mRNAs: (1) missense mutation; (2) nonsense due to exon skipping, frameshift, and premature stop codon; and (3) deletion/insertion as nonsense but with additional partial retention of a downstream intron which restores the reading frame and leads to an almost full-length protein. Homozygous animals develop hypertrophy, interstitial fibrosis, and decreased myocardial function, whereas heterozygous animals do not have any obvious phenotype [60]. Additional genetically altered mouse models have been generated to study MYBPC3 phosphorylation, which will be discussed in the next part.

8.4.4.1 MYBPC3 Phosphorylation

Human MYBPC3 contains at least four phosphorylation sites that are localized inside the myosin binding motif (serines 275, 284, 304, with an additional phosphorylation site not unequivocally identified) [61]); the mouse myosin binding motif contains three to four sites (serines 273, 282, 302, (305)) [62–64]. These phosphorylation sites have been studied in various transgenic mouse models; for example, lines have been established where the phosphorylation sites (Ser273, Ser282, and Ser302), along with two adjacent sites that could be potentially phosphorylated (Thr272, Thr281), were converted to alanines. While overexpression of wild-type MYBPC3 was able to rescue the MYBPC3 null phenotype, the non-phosphorylatable MYBPC3 was not [65]. Transgenic mice with complete deletion of phosphorylation motif "LAGAGRRTS" show an increase in cardiac contractility and relaxation and an increase in the phosphorylation of the remaining MYBPC3, troponin I, and phospholamban [66]. On the other hand, overexpression of a phosphomimetic MYBPC3 in a Mybpc3 null background showed subtle changes in sarcomeric ultrastructure characterized by increased distances between the thick filaments, indicating that phosphomimetic MYBPC3 affects thick-thin filament relationship. The loss of MYBPC3 phosphorylation has been observed in failing human hearts, and strategies to increase its phosphorylation may have cardioprotective effects [61, 67].

Although compelling evidences suggest that MYBPC3 phosphorylation modulates contractility by controlling the proximity of the myosin heads to actin, the precise molecular mechanism remains unclear (reviewed in [68]). MYBPC3 phosphorylation can abolish its ability to interact with the S2 region of the myosin heavy chain in vitro, but it may enhance MYBPC3 interactions with the thin filament. Alternatively, dephosphorylation results in strong binding of Mybpc3 to the myosin head, probably preventing its force-generating interaction with actin [67, 69]. However, recent data indicate that MYBPC3 may act synergistically with the myosin regulatory light chain to enhance cross-bridge formation by altering the interaction of the myosin head with actin. It may well be that this interaction depends on phosphorylation of either Mybpc3 or regulatory light chain and may well be able to provide another mechanism how phosphorylation of sarcomeric proteins may affect protein-protein interactions and kinetics of force development [70]. Although

it is well known that the distance between thick and thin filaments is a major determinant of calcium sensitivity and the loss of MYBPC3 is associated with increased calcium sensitivity, the precise molecular mechanism of how MYBPC3 phosphorylation affects this system is unknown. Part of this problem is that PKA phosphorylation decreases calcium sensitivity via troponin I phosphorylation, which leads to a decrease in troponin C calcium sensitivity. However Mybpc3 phosphorylation, which appears to decrease calcium sensitivity as well, has no effect on interfilament spacing [70]. Further studies, based on various transgenic animals, will certainly help to answer this question.

8.4.5 Cysteine-Rich Protein 3 (Csrp3/Mlp)

Cysteine-rich protein 3 (CSRP3) or muscle LIM protein (MLP) is a myogenic factor and was first described in 1994 [71]. MLP is a LIM-only protein consisting of 194 amino acids that constitute two LIM domains, each followed by a glycine-rich domain. Soon after its initial description, the protein became important in cardiovascular research when it was demonstrated in 1997 [72] that Csrp3-deficient mice developed hypertrophy followed by DCM later in life—at that time the first genetically altered organism reproducing this devastating disease. Perhaps even more important was that human CSRP3 mutations were able to cause human forms of cardiomyopathy [73–77]. The precise mechanisms by which CSRP3 mutations cause these diseases are not fully elucidated yet. As mentioned before, additional ablation of phospholamban (Pln) in Csrp3$^{-/-}$ mice exhibiting normal or enhanced SR-calcium ATPase function [78] rescues the heart failure phenotype (i.e., no heart failure in Pln and Csrp3 double KO mice; please see PLN section) [25]. It was also shown that Csrp3$^{-/-}$cardiomyocytes are defective in producing brain natriuretic peptide (BNP), a sensitive cardiac mechanical load marker, when exposed to mechanical stress but not to endothelin (Gq-coupling receptor agonist) or phenylephrine (α-adrenergic agonist), suggesting that the primary defect is not located in the downstream pathway but in the initial sensing of the stretch stimulus [76]. Thus, CSRP3 was suggested to be part of a macromolecular, cardiac mechanical stretch sensor.

CSRP3 was found to be present in different cellular compartments, including the sarcomeric Z-disc/I-band of different species such as drosophila [79], mouse [72, 80–82], and humans [76]. It was also reported to be present in the costamere (where β1-spectrin interacts with MLP) [83, 84], in the intercalated disc (where N-RAP interacts with MLP) [85], and in the cytoplasm (actin) [71], as well as in the nucleus [80]. However, Geier and coworkers found MLP mostly cytoplasmic and not at the Z-disc [86]. Boateng and colleagues demonstrated quite elegantly that CSRP3 in cardiac myocytes is present in two different molecular forms: the oligomeric form, which is present at the sarcolemma and the cytoskeleton, and the monomeric form, which is exclusively located in the nucleus [80]. Interestingly, after myocardial infarction and pressure overload, in both animal model and failing human hearts, nuclear CSRP3 levels in the myocardium increased at the expense of

nonnuclear form. In failing human hearts, almost no CSRP3 was detectable outside the nucleus. Additional work by Boateng and coworkers provided stronger evidence for nucleocytoplasmic shuttling of CSRP3 based on putative nuclear localization signal (NLS) [87]. The possible role of NLS was first suggested by Fung et al. when a clone with sequence homology of rat muscle LIM protein was isolated and characterized from a human fetal heart cDNA library [88]. Significant insights on the functional properties of nuclear CSRP3 were provided by using cell-penetrating synthetic peptide containing the putative nuclear localization signal (RKYGPK) of CSRP3—which inhibited any shuttling of endogenously synthesized CSRP3 to the nucleus, thus documenting functionality of this NLS [87]. Inhibition of nuclear translocation prevented the increased protein accumulation, usually seen with phenylephrine treatment, in isolated cardiomyocytes, thus identifying an important role of CSRP3 in controlling agonist-stimulated hypertrophy. Interestingly, cyclic strain of myocytes with prior NLS treatment resulted in disarrayed sarcomeres—an observation that has also been made in cardiomyocytes of Csrp3 KO mice [71]. Inhibition of nuclear shuttling during cyclic-mechanical strain prevents increased protein synthesis and BNP expression, suggesting that CSRP3 is required for remodeling of myofilaments and altered gene expression. A direct link between BNP gene expression and Csrp3 has been shown earlier [76], and research from other groups with heterozygous Csrp3 KO mice with myocardial infarction suggest similar association [82]. Although the functional significance of nuclear Csrp3, like myocyte hypertrophy following biomechanical stress, gene expression, and sarcomere assembly, has been demonstrated in this study, more questions are arising. One of the most important questions is how a small protein-adaptor molecule like MLP can exert its actions in different cellular compartments including the nucleus. The prohypertrophic phosphatase calcineurin has been previously identified as a cytoskeletal target/interacting partner of Csrp3 [82, 89, 90], and it was demonstrated that CSRP3 at the Z-disc is necessary for calcineurin activation in cardiac myocytes [82, 89, 90].

What are the *nuclear* targets of CSRP3 in cardiac myocytes? As CSRP3 does not bind DNA directly, one could speculate that it interacts with DNA by stress-responsive cardiac transcription factors like GATA4, GATA6, AP1, or serum response factor (SRF). In fact, it was previously shown that CSRP2, which is closely related to CSRP3, can bind GATAs and SRF and thereby enhance gene expression in smooth muscle cells [91]. By linking different transcription factors, as shown by the complex formation via protein-protein interaction between two LIM-domain containing proteins [92], CSRP3 may integrate the activities of multiple nuclear regulatory proteins in order to coordinate gene expression. It is also possible that CSRP3 facilitates nucleocytoplasmic shuttling of its nonnuclear target calcineurin, which has been shown to translocate to the nucleus in hypertrophied cardiac myocytes [93]. Research on CSRP3 was predominantly focused on its cytoskeletal function. Although the interaction of CSRP3 with different transcription factors, such as MyoD, MRF4, and myogenin, was shown in skeletal myocytes [94], and CSRP3 nuclear localization in right ventricular murine hypertrophy following increased biomechanical stress [95], the functional significance of this NLS

remained elusive until the report by Boateng and coworkers [87]. In this context, we have previously shown that CSRP3 also interacts with the transcription factor ZBTB17, which is important for survival signaling [96].

It should be noted that CSRP3 becomes acetylated within its NLS on position K69 [81], a modification thought to affect calcium sensitivity, which may affect CSRP3's nucleocytoplasmic shuttling. It will be interesting to analyze the effects of human CSRP3 mutations on its nuclear localization and analyze their ability to affect specific genes, particularly of K69R mutation, found in an individual affected by DCM and endocardial fibroelastosis [77]. Besides acetylation, other posttranslational modifications such as phosphorylation, sumoylation, and/or polyubiquitinylation may also affect CSRP3 function [97].

8.5 Conclusion

Due to many advantages over large animal models, mouse models are commonly used in CV research, both in academia and big pharma. They a have a short life span, which allows scientists to follow the natural course of disease at an accelerated pace. More importantly, mice can be genetically modified, allowing for rapid establishment of proof-of-principle that can later be extended to larger animal models. Nevertheless, mice have disadvantages, such as being phylogenetically distant from humans, and some physiological features and their response to pharmacological treatments may not be comparable to humans. Therefore, translational aspects and the value of genetically altered mice must be interpreted with caution. However, genetically altered mouse models provided us with fundamental insights in cardiac Ca^{2+} homeostasis/signaling (Pln), hypertrophism and survival signaling (calcineurin), patho-mechanisms of general heart failure (Csrp3, Mlp), phosphorylation (Mybpc3), and potential routes to therapy (Ttn). Although we made significant progress, more efforts are necessary to better understand the underlying molecular mechanisms of heart failure and to exploit the knowledge for innovative therapies to benefit the mankind.

Acknowledgments Ralph Knöll is supported by funds provided by the Integrated Cardio Metabolic Centre at the Karolinska Institute (ICMC/KI), Leducq Foundation, German Research Foundation (Kn 448/10-2), and Hjärt och Lungfonden in Sweden.

We apologize to the many authors having contributed to this exiting field but have not been mentioned here.

References

1. Stevens G, Mascarenhas M, Mathers C. Global health risks: progress and challenges. Bull World Health Organ. 2009;87(9):646.
2. Maron BJ, Towbin JA, Thiene G, Antzelevitch C, Corrado D, Arnett D, Moss AJ, Seidman CE, Young JB. Contemporary definitions and classification of the cardiomyopathies: an American Heart Association Scientific Statement from the Council on Clinical Cardiology, Heart Failure

and Transplantation Committee; Quality of Care and Outcomes Research and Functional Genomics and Translational Biology Interdisciplinary Working Groups; and Council on Epidemiology and Prevention. Circulation. 2006;113(14):1807–16.
3. Geisterfer-Lowrance AA, Kass S, Tanigawa G, Vosberg HP, McKenna W, Seidman CE, Seidman JG. A molecular basis for familial hypertrophic cardiomyopathy: a beta cardiac myosin heavy chain gene missense mutation. Cell. 1990;62(5):999–1006.
4. Watkins H, Rosenzweig A, Hwang DS, Levi T, McKenna W, Seidman CE, Seidman JG. Characteristics and prognostic implications of myosin missense mutations in familial hypertrophic cardiomyopathy. N Engl J Med. 1992;326(17):1108–14.
5. Capecchi MR. Generating mice with targeted mutations. Nat Med. 2001;7(10):1086–90.
6. Evans MJ. The cultural mouse. Nat Med. 2001;7(10):1081–3.
7. Smithies O. Forty years with homologous recombination. Nat Med. 2001;7(10):1083–6.
8. Gama Sosa MA, De Gasperi R, Elder GA. Animal transgenesis: an overview. Brain Struct Funct. 2010;214(2–3):91–109.
9. Manis JP. Knock out, knock in, knock down--genetically manipulated mice and the Nobel Prize. N Engl J Med. 2007;357(24):2426–9.
10. Geisterfer-Lowrance AA, Christe M, Conner DA, Ingwall JS, Schoen FJ, Seidman CE, Seidman JG. A mouse model of familial hypertrophic cardiomyopathy. Science. 1996;272 (5262):731–4.
11. Green EM, Wakimoto H, Anderson RL, Evanchik MJ, Gorham JM, Harrison BC, Henze M, Kawas R, Oslob JD, Rodriguez HM, Song Y, Wan W, Leinwand LA, Spudich JA, McDowell RS, Seidman JG, Seidman CE. A small-molecule inhibitor of sarcomere contractility suppresses hypertrophic cardiomyopathy in mice. Science. 2016;351(6273):617–21.
12. Soriano P. Generalized lacZ expression with the ROSA26 Cre reporter strain. Nat Genet. 1999;21(1):70–1.
13. Geurts AM, Cost GJ, Freyvert Y, Zeitler B, Miller JC, Choi VM, Jenkins SS, Wood A, Cui X, Meng X, Vincent A, Lam S, Michalkiewicz M, Schilling R, Foeckler J, Kalloway S, Weiler H, Menoret S, Anegon I, Davis GD, Zhang L, Rebar EJ, Gregory PD, Urnov FD, Jacob HJ, Buelow R. Knockout rats via embryo microinjection of zinc-finger nucleases. Science. 2009;325(5939):433.
14. Boch J. TALEs of genome targeting. Nat Biotechnol. 2011;29(2):135–6.
15. Ruan J, Li H, Xu K, Wu T, Wei J, Zhou R, Liu Z, Mu Y, Yang S, Ouyang H, Chen-Tsai RY, Li K. Highly efficient CRISPR/Cas9-mediated transgene knockin at the H11 locus in pigs. Sci Rep. 2015;5:14253.
16. Joung J, Konermann S, Gootenberg JS, Abudayyeh OO, Platt RJ, Brigham MD, Sanjana NE, Zhang F. Genome-scale CRISPR-Cas9 knockout and transcriptional activation screening. Nat Protoc. 2017;12(4):828–63.
17. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science. 2012;337 (6096):816–21.
18. Zuo E, Cai YJ, Li K, Wei Y, Wang BA, Sun Y, Liu Z, Liu J, Hu X, Wei W, Huo X, Shi L, Tang C, Liang D, Wang Y, Nie YH, Zhang CC, Yao X, Wang X, Zhou C, Ying W, Wang Q, Chen RC, Shen Q, Xu GL, Li J, Sun Q, Xiong ZQ, Yang H. One-step generation of complete gene knockout mice and monkeys by CRISPR/Cas9-mediated gene editing with multiple sgRNAs. Cell Res. 2017;27(7):933–45.
19. Gossen M, Bujard H. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc Natl Acad Sci U S A. 1992;89(12):5547–51.
20. Gossen M, Freundlieb S, Bender G, Muller G, Hillen W, Bujard H. Transcriptional activation by tetracyclines in mammalian cells. Science. 1995;268(5218):1766–9.
21. Garcia S, Freitas AA. Humanized mice: current states and perspectives. Immunol Lett. 2012;146(1–2):1–7.

22. Tsukiyama T, Kato-Itoh M, Nakauchi H, Ohinata Y. A comprehensive system for generation and evaluation of induced pluripotent stem cells using piggyBac transposition. PLoS One. 2014;9(3):e92973.
23. Luo W, Grupp IL, Harrer J, Ponniah S, Grupp G, Duffy JJ, Doetschman T, Kranias EG. Targeted ablation of the phospholamban gene is associated with markedly enhanced myocardial contractility and loss of beta-agonist stimulation. Circ Res. 1994;75(3):401–9.
24. Tada M, Kirchberger MA, Repke DI, Katz AM. The stimulation of calcium transport in cardiac sarcoplasmic reticulum by adenosine 3′:5′-monophosphate-dependent protein kinase. J Biol Chem. 1974;249(19):6174–80.
25. Minamisawa S, Hoshijima M, Chu G, Ward CA, Frank K, Gu Y, Martone ME, Wang Y, Ross J Jr, Kranias EG, Giles WR, Chien KR. Chronic phospholamban-sarcoplasmic reticulum calcium ATPase interaction is the critical calcium cycling defect in dilated cardiomyopathy. Cell. 1999;99(3):313–22.
26. Janczewski AM, Zahid M, Lemster BH, Frye CS, Gibson G, Higuchi Y, Kranias EG, Feldman AM, McTiernan CF. Phospholamban gene ablation improves calcium transients but not cardiac function in a heart failure model. Cardiovasc Res. 2004;62(3):468–80.
27. Gaffin RD, Pena JR, Alves MS, Dias FA, Chowdhury SA, Heinrich LS, Goldspink PH, Kranias EG, Wieczorek DF, Wolska BM. Long-term rescue of a familial hypertrophic cardiomyopathy caused by a mutation in the thin filament protein, tropomyosin, via modulation of a calcium cycling protein. J Mol Cell Cardiol. 2011;51(5):812–20.
28. Bai Y, Jones PP, Guo J, Zhong X, Clark RB, Zhou Q, Wang R, Vallmitjana A, Benitez R, Hove-Madsen L, Semeniuk L, Guo A, Song LS, Duff HJ, Chen SR. Phospholamban knockout breaks arrhythmogenic Ca(2)(+) waves and suppresses catecholaminergic polymorphic ventricular tachycardia in mice. Circ Res. 2013;113(5):517–26.
29. Karakikes I, Stillitano F, Nonnenmacher M, Tzimas C, Sanoudou D, Termglinchan V, Kong CW, Rushing S, Hansen J, Ceholski D, Kolokathis F, Kremastinos D, Katoulis A, Ren L, Cohen N, Gho JM, Tsiapras D, Vink A, Wu JC, Asselbergs FW, Li RA, Hulot JS, Kranias EG, Hajjar RJ. Correction of human phospholamban R14del mutation associated with cardiomyopathy using targeted nucleases and combination therapy. Nat Commun. 2015;6:6955.
30. Stillitano F, Turnbull IC, Karakikes I, Nonnenmacher M, Backeris P, Hulot JS, Kranias EG, Hajjar RJ, Costa KD. Genomic correction of familial cardiomyopathy in human engineered cardiac tissues. Eur Heart J. 2016;37(43):3282–4.
31. Molkentin JD, Lu JR, Antos CL, Markham B, Richardson J, Robbins J, Grant SR, Olson EN. A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. Cell. 1998;93(2):215–28.
32. Bueno OF, Wilkins BJ, Tymitz KM, Glascock BJ, Kimball TF, Lorenz JN, Molkentin JD. Impaired cardiac hypertrophic response in Calcineurin Abeta -deficient mice. Proc Natl Acad Sci U S A. 2002;99(7):4586–91.
33. Bueno OF, Lips DJ, Kaiser RA, Wilkins BJ, Dai YS, Glascock BJ, Klevitsky R, Hewett TE, Kimball TR, Aronow BJ, Doevendans PA, Molkentin JD. Calcineurin Abeta gene targeting predisposes the myocardium to acute ischemia-induced apoptosis and dysfunction. Circ Res. 2004;94(1):91–9.
34. Tabish AM, Azzimato V, Alexiadis A, Buyandelger B, Knoll R. Genetic epidemiology of titin-truncating variants in the etiology of dilated cardiomyopathy. Biophys Rev. 2017;9:207–23.
35. Labeit S, Kolmerer B. Titins: giant proteins in charge of muscle ultrastructure and elasticity. Science. 1995;270(5234):293–6.
36. Minajeva A, Kulke M, Fernandez JM, Linke WA. Unfolding of titin domains explains the viscoelastic behavior of skeletal myofibrils. Biophys J. 2001;80(3):1442–51.
37. Gerull B, Gramlich M, Atherton J, McNabb M, Trombitas K, Sasse-Klaassen S, Seidman JG, Seidman C, Granzier H, Labeit S, Frenneaux M, Thierfelder L. Mutations of TTN, encoding the giant muscle filament titin, cause familial dilated cardiomyopathy. Nat Genet. 2002;30(2):201–4.

38. Herman DS, Lam L, Taylor MR, Wang L, Teekakirikul P, Christodoulou D, Conner L, DePalma SR, McDonough B, Sparks E, Teodorescu DL, Cirino AL, Banner NR, Pennell DJ, Graw S, Merlo M, Di Lenarda A, Sinagra G, Bos JM, Ackerman MJ, Mitchell RN, Murry CE, Lakdawala NK, Ho CY, Barton PJ, Cook SA, Mestroni L, Seidman JG, Seidman CE. Truncations of titin causing dilated cardiomyopathy. N Engl J Med. 2012;366(7):619–28.
39. Marston S, Montgiraud C, Munster AB, Copeland O, Choi O, Dos Remedios C, Messer AE, Ehler E, Knoll R. OBSCN Mutations Associated with Dilated Cardiomyopathy and Haploinsufficiency. PLoS One. 2015;10(9):e0138568.
40. Garvey SM, Rajan C, Lerner AP, Frankel WN, Cox GA. The muscular dystrophy with myositis (mdm) mouse mutation disrupts a skeletal muscle-specific domain of titin. Genomics. 2002;79 (2):146–9.
41. Gramlich M, Michely B, Krohne C, Heuser A, Erdmann B, Klaassen S, Hudson B, Magarin M, Kirchner F, Todiras M, Granzier H, Labeit S, Thierfelder L, Gerull B. Stress-induced dilated cardiomyopathy in a knock-in mouse model mimicking human titin-based disease. J Mol Cell Cardiol. 2009;47(3):352–8.
42. Weintraub RG, Semsarian C, Macdonald P. Dilated cardiomyopathy. Lancet. 2017;390 (10092):400–14.
43. Gramlich M, Pane LS, Zhou Q, Chen Z, Murgia M, Schotterl S, Goedel A, Metzger K, Brade T, Parrotta E, Schaller M, Gerull B, Thierfelder L, Aartsma-Rus A, Labeit S, Atherton JJ, McGaughran J, Harvey RP, Sinnecker D, Mann M, Laugwitz KL, Gawaz MP, Moretti A. Antisense-mediated exon skipping: a therapeutic strategy for titin-based dilated cardiomyopathy. EMBO Mol Med. 2015;7(5):562–76.
44. Bonne G, Carrier L, Bercovici J, Cruaud C, Richard P, Hainque B, Gautel M, Labeit S, James M, Beckmann J, Weissenbach J, Vosberg HP, Fiszman M, Komajda M, Schwartz K. Cardiac myosin binding protein-C gene splice acceptor site mutation is associated with familial hypertrophic cardiomyopathy. Nat Genet. 1995;11(4):438–40.
45. Watkins H, Conner D, Thierfelder L, Jarcho JA, MacRae C, McKenna WJ, Maron BJ, Seidman JG, Seidman CE. Mutations in the cardiac myosin binding protein-C gene on chromosome 11 cause familial hypertrophic cardiomyopathy. Nat Genet. 1995;11(4):434–7.
46. Richard P, Charron P, Carrier L, Ledeuil C, Cheav T, Pichereau C, Benaiche A, Isnard R, Dubourg O, Burban M, Gueffet JP, Millaire A, Desnos M, Schwartz K, Hainque B, Komajda M. Hypertrophic cardiomyopathy: distribution of disease genes, spectrum of mutations, and implications for a molecular diagnosis strategy. Circulation. 2003;107(17):2227–32.
47. Carrier L, Mearini G, Stathopoulou K, Cuello F. Cardiac myosin-binding protein C (MYBPC3) in cardiac pathophysiology. Gene. 2015;573(2):188–97.
48. Niimura H, Bachinski LL, Sangwatanaroj S, Watkins H, Chudley AE, McKenna W, Kristinsson A, Roberts R, Sole M, Maron BJ, Seidman JG, Seidman CE. Mutations in the gene for cardiac myosin-binding protein C and late-onset familial hypertrophic cardiomyopathy. N Engl J Med. 1998;338(18):1248–57.
49. Watkins H, Ashrafian H, Redwood C. Inherited cardiomyopathies. N Engl J Med. 2011;364 (17):1643–56.
50. Daehmlow S, Erdmann J, Knueppel T, Gille C, Froemmel C, Hummel M, Hetzer R, Regitz-Zagrosek V. Novel mutations in sarcomeric protein genes in dilated cardiomyopathy. Biochem Biophys Res Commun. 2002;298(1):116–20.
51. Saltzman AJ, Mancini-DiNardo D, Li C, Chung WK, Ho CY, Hurst S, Wynn J, Care M, Hamilton RM, Seidman GW, Gorham J, McDonough B, Sparks E, Seidman JG, Seidman CE, Rehm HL. Short communication: the cardiac myosin binding protein C Arg502Trp mutation: a common cause of hypertrophic cardiomyopathy. Circ Res. 2010;106(9):1549–52.
52. Alders M, Jongbloed R, Deelen W, van den Wijngaard A, Doevendans P, Ten Cate F, Regitz-Zagrosek V, Vosberg HP, van Langen I, Wilde A, Dooijes D, Mannens M. The 2373insG mutation in the MYBPC3 gene is a founder mutation, which accounts for nearly one-fourth of the HCM cases in the Netherlands. Eur Heart J. 2003;24(20):1848–53.

53. Christiaans I, Nannenberg EA, Dooijes D, Jongbloed RJ, Michels M, Postema PG, Majoor-Krakauer D, van den Wijngaard A, Mannens MM, van Tintelen JP, van Langen IM, Wilde AA. Founder mutations in hypertrophic cardiomyopathy patients in the Netherlands. Netherlands heart journal: monthly journal of the Netherlands Society of Cardiology and the Netherlands Heart Foundation. 2010;18(5):248–54.
54. Carrier L, Knoll R, Vignier N, Keller DI, Bausero P, Prudhon B, Isnard R, Ambroisine ML, Fiszman M, Ross J Jr, Schwartz K, Chien KR. Asymmetric septal hypertrophy in heterozygous cMyBP-C null mice. Cardiovasc Res. 2004;63(2):293–304.
55. Harris SP, Bartley CR, Hacker TA, McDonald KS, Douglas PS, Greaser ML, Powers PA, Moss RL. Hypertrophic cardiomyopathy in cardiac myosin binding protein-C knockout mice. Circ Res. 2002;90(5):594–601.
56. Yang Q, Sanbe A, Osinska H, Hewett TE, Klevitsky R, Robbins J. A mouse model of myosin binding protein C human familial hypertrophic cardiomyopathy. J Clin Invest. 1998;102 (7):1292–300.
57. Yang Q, Sanbe A, Osinska H, Hewett TE, Klevitsky R, Robbins J. In vivo modeling of myosin binding protein C familial hypertrophic cardiomyopathy. Circ Res. 1999;85(9):841–7.
58. McConnell BK, Jones KA, Fatkin D, Arroyo LH, Lee RT, Aristizabal O, Turnbull DH, Georgakopoulos D, Kass D, Bond M, Niimura H, Schoen FJ, Conner D, Fischman DA, Seidman CE, Seidman JG. Dilated cardiomyopathy in homozygous myosin-binding protein-C mutant mice. J Clin Invest. 1999;104(12):1771.
59. Witt CC, Gerull B, Davies MJ, Centner T, Linke WA, Thierfelder L. Hypercontractile properties of cardiac muscle fibers in a knock-in mouse model of cardiac myosin-binding protein-C. J Biol Chem. 2001;276(7):5353–9.
60. Vignier N, Schlossarek S, Fraysse B, Mearini G, Kramer E, Pointu H, Mougenot N, Guiard J, Reimer R, Hohenberg H, Schwartz K, Vernet M, Eschenhagen T, Carrier L. Nonsense-mediated mRNA decay and ubiquitin-proteasome system regulate cardiac myosin-binding protein C mutant levels in cardiomyopathic mice. Circ Res. 2009;105(3):239–48.
61. Copeland O, Sadayappan S, Messer AE, Steinen GJ, van der Velden J, Marston SB. Analysis of cardiac myosin binding protein-C phosphorylation in human heart muscle. J Mol Cell Cardiol. 2010;49(6):1003–11.
62. Jia W, Shaffer JF, Harris SP, Leary JA. Identification of novel protein kinase A phosphorylation sites in the M-domain of human and murine cardiac myosin binding protein-C using mass spectrometry analysis. J Proteome Res. 2010;9(4):1843–53.
63. Yuan C, Guo Y, Ravi R, Przyklenk K, Shilkofski N, Diez R, Cole RN, Murphy AM. Myosin binding protein C is differentially phosphorylated upon myocardial stunning in canine and rat hearts-- evidence for novel phosphorylation sites. Proteomics. 2006;6(14):4176–86.
64. Yuan C, Sheng Q, Tang H, Li Y, Zeng R, Solaro RJ. Quantitative comparison of sarcomeric phosphoproteomes of neonatal and adult rat hearts. Am J Physiol Heart Circ Physiol. 2008;295 (2):H647–56.
65. Sadayappan S, Gulick J, Osinska H, Martin LA, Hahn HS, Dorn GW 2nd, Klevitsky R, Seidman CE, Seidman JG, Robbins J. Cardiac myosin-binding protein-C phosphorylation and cardiac function. Circ Res. 2005;97(11):1156–63.
66. Yang Q, Hewett TE, Klevitsky R, Sanbe A, Wang X, Robbins J. PKA-dependent phosphorylation of cardiac myosin binding protein C in transgenic mice. Cardiovasc Res. 2001;51(1):80–8.
67. Sadayappan S, Osinska H, Klevitsky R, Lorenz JN, Sargent M, Molkentin JD, Seidman CE, Seidman JG, Robbins J. Cardiac myosin binding protein C phosphorylation is cardioprotective. Proc Natl Acad Sci U S A. 2006;103(45):16918–23.
68. Schlossarek S, Mearini G, Carrier L. Cardiac myosin-binding protein C in hypertrophic cardiomyopathy: mechanisms and therapeutic opportunities. J Mol Cell Cardiol. 2011;50 (4):613–20.
69. Kulikovskaya I, McClellan G, Flavigny J, Carrier L, Winegrad S. Effect of MyBP-C binding to actin on contractility in heart muscle. J Gen Physiol. 2003;122(6):761–74.

70. Colson BA, Locher MR, Bekyarova T, Patel JR, Fitzsimons DP, Irving TC, Moss RL. Differential roles of regulatory light chain and myosin binding protein-C phosphorylations in the modulation of cardiac force development. J Physiol. 2010;588(Pt 6):981–93.
71. Arber S, Halder G, Caroni P. Muscle LIM protein, a novel essential regulator of myogenesis, promotes myogenic differentiation. Cell. 1994;79(2):221–31.
72. Arber S, Hunter JJ, Ross J Jr, Hongo M, Sansig G, Borg J, Perriard JC, Chien KR, Caroni P. MLP-deficient mice exhibit a disruption of cardiac cytoarchitectural organization, dilated cardiomyopathy, and heart failure. Cell. 1997;88(3):393–403.
73. Bos JM, Poley RN, Ny M, Tester DJ, Xu X, Vatta M, Towbin JA, Gersh BJ, Ommen SR, Ackerman MJ. Genotype-phenotype relationships involving hypertrophic cardiomyopathy-associated mutations in titin, muscle LIM protein, and telethonin. Mol Genet Metab. 2006;88 (1):78–85.
74. Geier C, Perrot A, Ozcelik C, Binner P, Counsell D, Hoffmann K, Pilz B, Martiniak Y, Gehmlich K, van der Ven PF, Furst DO, Vornwald A, von Hodenberg E, Nurnberg P, Scheffold T, Dietz R, Osterziel KJ. Mutations in the human muscle LIM protein gene in families with hypertrophic cardiomyopathy. Circulation. 2003;107(10):1390–5.
75. Hershberger RE, Parks SB, Kushner JD, Li D, Ludwigsen S, Jakobs P, Nauman D, Burgess D, Partain J, Litt M. Coding sequence mutations identified in MYH7, TNNT2, SCN5A, CSRP3, LBD3, and TCAP from 313 patients with familial or idiopathic dilated cardiomyopathy. Clin Transl Sci. 2008;1(1):21–6.
76. Knöll R, Hoshijima M, Hoffman HM, Person V, Lorenzen-Schmidt I, Bang ML, Hayashi T, Shiga N, Yasukawa H, Schaper W, McKenna W, Yokoyama M, Schork NJ, Omens JH, McCulloch AD, Kimura A, Gregorio CC, Poller W, Schaper J, Schultheiss HP, Chien KR. The cardiac mechanical stretch sensor machinery involves a Z disc complex that is defective in a subset of human dilated cardiomyopathy. Cell. 2002;111(7):943–55.
77. Mohapatra B, Jimenez S, Lin JH, Bowles KR, Coveler KJ, Marx JG, Chrisco MA, Murphy RT, Lurie PR, Schwartz RJ, Elliott PM, Vatta M, McKenna W, Towbin JA, Bowles NE. Mutations in the muscle LIM protein and alpha-actinin-2 genes in dilated cardiomyopathy and endocardial fibroelastosis. Mol Genet Metab. 2003;80(1–2):207–15.
78. Su Z, Yao A, Zubair I, Sugishita K, Ritter M, Li F, Hunter JJ, Chien KR, Barry WH. Effects of deletion of muscle LIM protein on myocyte function. Am J Physiol Heart Circ Physiol. 2001;280(6):H2665–73.
79. Mery A, Taghli-Lamallem O, Clark KA, Beckerle MC, Wu X, Ocorr K, Bodmer R. The Drosophila muscle LIM protein, Mlp84B, is essential for cardiac function. J Exp Biol. 2008;211(Pt 1):15–23.
80. Boateng SY, Belin RJ, Geenen DL, Margulies KB, Martin JL, Hoshijima M, de Tombe PP, Russell B. Cardiac dysfunction and heart failure are associated with abnormalities in the subcellular distribution and amounts of oligomeric muscle LIM protein. Am J Physiol Heart Circ Physiol. 2007;292(1):H259–69.
81. Gupta MP, Samant SA, Smith SH, Shroff SG. HDAC4 and PCAF bind to cardiac sarcomeres and play a role in regulating myofilament contractile activity. J Biol Chem. 2008;283 (15):10135–46.
82. Heineke J, Ruetten H, Willenbockel C, Gross SC, Naguib M, Schaefer A, Kempf T, Hilfiker-Kleiner D, Caroni P, Kraft T, Kaiser RA, Molkentin JD, Drexler H, Wollert KC. Attenuation of cardiac remodeling after myocardial infarction by muscle LIM protein-calcineurin signaling at the sarcomeric Z-disc. Proc Natl Acad Sci U S A. 2005;102(5):1655–60.
83. Flick MJ, Konieczny SF. The muscle regulatory and structural protein MLP is a cytoskeletal binding partner of betaI-spectrin. J Cell Sci. 2000;113.(Pt 9:1553–64.
84. Lange S, Gehmlich K, Lun AS, Blondelle J, Hooper C, Dalton ND, Alvarez EA, Zhang X, Bang ML, Abassi YA, Dos Remedios CG, Peterson KL, Chen J, Ehler E. MLP and CARP are linked to chronic PKCalpha signalling in dilated cardiomyopathy. Nat Commun. 2016;7:12120.

85. Ehler E, Horowits R, Zuppinger C, Price RL, Perriard E, Leu M, Caroni P, Sussman M, Eppenberger HM, Perriard JC. Alterations at the intercalated disk associated with the absence of muscle lim protein. J Cell Biol. 2001;153(4):763–72.
86. Geier C, Gehmlich K, Ehler E, Hassfeld S, Perrot A, Hayess K, Cardim N, Wenzel K, Erdmann B, Krackhardt F, Posch MG, Osterziel KJ, Bublak A, Nagele H, Scheffold T, Dietz R, Chien KR, Spuler S, Furst DO, Nurnberg P, Ozcelik C. Beyond the sarcomere: CSRP3 mutations cause hypertrophic cardiomyopathy. Hum Mol Genet. 2008;17(18):2753–65.
87. Boateng SY, Senyo SE, Qi L, Goldspink PH, Russell B. Myocyte remodelling in response to hypertrophic stimuli requires nucleocytoplasmic shuttling of muscle LIM protein. J Mol Cell Cardiol. 2009;47(4):426–35.
88. Fung YW, Wang R, Liew CC. Characterization of a human cardiac gene which encodes for a LIM domain protein and is developmentally expressed in myocardial development. J Mol Cell Cardiol. 1996;28(6):1203–10.
89. Heineke J, Molkentin JD. Regulation of cardiac hypertrophy by intracellular signalling pathways. Nat Rev Mol Cell Biol. 2006;7(8):589–600.
90. Jeong D, Kim JM, Cha H, Oh JG, Park J, Yun SH, Ju ES, Jeon ES, Hajjar RJ, Park WJ. PICOT attenuates cardiac hypertrophy by disrupting calcineurin-NFAT signaling. Circ Res. 2008;102 (6):711–9.
91. Chang DF, Belaguli NS, Iyer D, Roberts WB, Wu SP, Dong XR, Marx JG, Moore MS, Beckerle MC, Majesky MW, Schwartz RJ. Cysteine-rich LIM-only proteins CRP1 and CRP2 are potent smooth muscle differentiation cofactors. Dev Cell. 2003;4(1):107–18.
92. Schmeichel KL, Beckerle MC. The LIM domain is a modular protein-binding interface. Cell. 1994;79(2):211–9.
93. Hallhuber M, Burkard N, Wu R, Buch MH, Engelhardt S, Hein L, Neyses L, Schuh K, Ritter O. Inhibition of nuclear import of calcineurin prevents myocardial hypertrophy. Circ Res. 2006;99(6):626–35.
94. Kong Y, Flick MJ, Kudla AJ, Konieczny SF. Muscle LIM protein promotes myogenesis by enhancing the activity of MyoD. Mol Cell Biol. 1997;17(8):4750–60.
95. Ecarnot-Laubriet A, De Luca K, Vandroux D, Moisant M, Bernard C, Assem M, Rochette L, Teyssier JR. Downregulation and nuclear relocation of MLP during the progression of right ventricular hypertrophy induced by chronic pressure overload. J Mol Cell Cardiol. 2000;32 (12):2385–95.
96. Buyandelger B, Mansfield C, Kostin S, Choi O, Roberts AM, Ware JS, Mazzarotto F, Pesce F, Buchan R, Isaacson RL, Vouffo J, Gunkel S, Knoll G, McSweeney SJ, Wei H, Perrot A, Pfeiffer C, Toliat MR, Ilieva K, Krysztofinska E, Lopez-Olaneta MM, Gomez-Salinero JM, Schmidt A, Ng KE, Teucher N, Chen J, Teichmann M, Eilers M, Haverkamp W, Regitz-Zagrosek V, Hasenfuss G, Braun T, Pennell DJ, Gould I, Barton PJ, Lara-Pezzi E, Schafer S, Hubner N, Felkin LE, O'Regan DP, Brand T, Milting H, Nurnberg P, Schneider MD, Prasad S, Petretto E, Knoll R. ZBTB17 (MIZ1) Is Important for the Cardiac Stress Response and a Novel Candidate Gene for Cardiomyopathy and Heart Failure. Circ Cardiovasc Genet. 2015;8 (5):643–52.
97. Wende AR. Post-translational modifications of the cardiac proteome in diabetes and heart failure. Proteomics Clin Appl. 2016;10(1):25–38.