(12) STANDARD PATENT (11) Application No. **AU 2018297274 B2**
(19) AUSTRALIAN PATENT OFFICE

(54) Title
Treatment of heart disease by inhibition of the action of muscle A-kinase anchoring protein (mAKAP)

(51) International Patent Classification(s)
A61K 31/713 (2006.01) ***A61P 9/00*** (2006.01)
A61K 38/45 (2006.01) ***A61P 9/10*** (2006.01)
A61K 48/00 (2006.01) ***C07K 14/075*** (2006.01)

(21) Application No: **2018297274** (22) Date of Filing: **2018.07.05**

(87) WIPO No: **WO19/010301**

(30) Priority Data

(31) Number	(32) Date	(33) Country
62/529,224	**2017.07.06**	**US**

(43) Publication Date: **2019.01.10**
(44) Accepted Journal Date: **2023.05.18**

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(56) Related Art
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LEE ET AL.: "AKAP6 inhibition impairs myoblast differentiation and muscle regeneration: Positive loop between AKAP6 and myogenin", SCIENTIFIC REPORTS, vol. 5, no. 16523, 13 November 2015 (2015-11-13), pages 1 - 14, XP055565478
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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
10 January 2019 (10.01.2019)

WIPO | PCT

(10) International Publication Number
WO 2019/010301 A1

(51) International Patent Classification:
A61K 31/713 (2006.01) *A61P 9/00* (2006.01)
A61K 38/45 (2006.01) *A61P 9/10* (2006.01)
A61K 48/00 (2006.01) *C07K 14/075* (2006.01)

(21) International Application Number:
PCT/US2018/040913

(22) International Filing Date:
05 July 2018 (05.07.2018)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/259,224 06 July 2017 (06.07.2017) US

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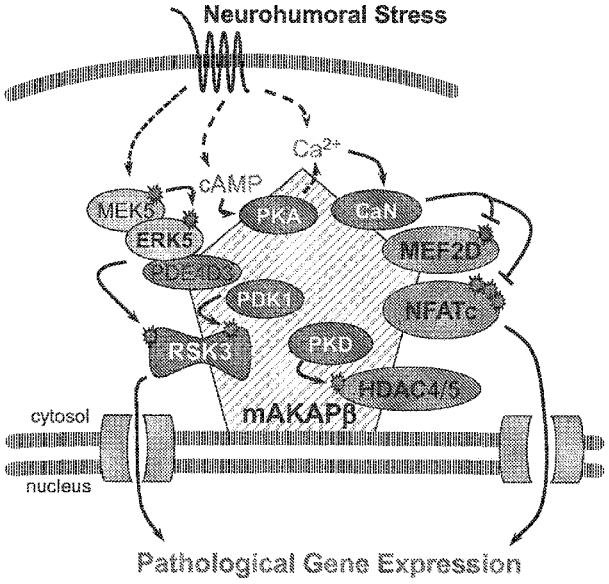
(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

WO 2019/010301 A1

(54) **Title:** TREATMENT OF HEART DISEASE BY INHIBITION OF THE ACTION OF MUSCLE A-KINASE ANCHORING PROTEIN (MAKAP)

FIGURE 7



(57) **Abstract:** The present invention provides a method of protecting the heart from damage, by administering to a patient at risk of such damage, a pharmaceutically effective amount of a composition which inhibits the interaction of RSK3 and mAKAPβ, or the expression or activity of one or both of those molecules. This composition is preferably in the form of an siRNA construct, more preferably an shRNA construct, which inhibits the expression of mAKAPβ.

[Continued on next page]

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

TREATMENT OF HEART DISEASE BY INHIBITION OF THE ACTION OF MUSCLE A-KINASE ANCHORING PROTEIN (mAKAP)

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 62/529,224, filed July 6, 2017, which is hereby incorporated by reference in its entirety into the present application.

STATEMENT OF GOVERNMENTAL SUPPORT

[0002] This invention was made with Government support under contract RO1 HL 075398 awarded by the National Institutes of Health. The Government has certain rights in this invention.

BACKGROUND OF THE INVENTION

[0003] The heart is capable of undergoing hypertrophic growth in response to a variety of stimuli. Hypertrophic growth may occur as the result of physical training such as running or swimming. However, it also occurs as the result of injury or in many forms of heart disease. Hypertrophy is the primary mechanism by which the heart reduces stress on the ventricular wall. When the growth is not accompanied by a concomitant increase in chamber size, this is called concentric hypertrophy. Hypertrophy occurs as the result of an increase in protein synthesis and in the size and organization of sarcomeres within individual myocytes. For a more thorough review of cardiac remodeling and hypertrophy, see Kehat (2010) and Hill (2008), each herein incorporated by reference in their entirety. The prevailing view is that cardiac hypertrophy plays a major role in heart failure. Traditional routes of treating heart failure include afterload reduction, blockage of beta-adrenergic receptors (β-ARs) and use of mechanical support devices in afflicted patients. However, the art is in need of additional mechanisms of preventing or treating cardiac hypertrophy.

[0004] *AKAPs and Cardiac Remodeling*

[0005] Ventricular myocyte hypertrophy is the primary compensatory mechanism whereby the myocardium reduces ventricular wall tension when submitted to stress because of myocardial infarction, hypertension, and congenital heart disease or neurohumoral activation. It is associated with a nonmitotic growth of cardiomyocytes, increased myofibrillar organization, and upregulation of specific subsets of "fetal" genes that are normally expressed during embryonic life (Frey 2004, Hill 2008). The concomitant aberrant cardiac contractility, Ca^{2+} handling, and myocardial energetics are associated with maladaptive changes that include interstitial fibrosis and cardiomyocyte death and increase the risk of developing heart failure and malignant arrhythmia (Cappola 2008, Hill 2008). Increased in prevalence by risk factors such as smoking and obesity, heart failure is a syndrome that affects about six million Americans and has an annual incidence of 1% of senior citizens (Roger 2011). Since the five-year survival rate after diagnosis is still very poor (lower than 50%), many efforts have been made during the last years to define the molecular mechanisms involved in this pathological process.

[0006] Cardiac hypertrophy can be induced by a variety of neuro-humoral, paracrine, and autocrine stimuli, which activate several receptor families including G protein-coupled receptors, cytokine receptors, and growth factor tyrosine kinase receptors (Brown 2006, Frey 2004). In this context, it is becoming increasingly clear that A-kinase anchoring proteins (AKAPs) can assemble multiprotein complexes that integrate hypertrophic pathways emanating from these receptors. In particular, recent studies have now identified anchoring proteins including mAKAP and AKAP-Lbc and D-AKAP1 that serve as scaffold proteins and play a central role in organizing and modulating hypertrophic pathways activated by stress signals.

[0007] As the organizers of "nodes" in the intracellular signaling network, scaffold proteins are of interest as potential therapeutic targets (Negro 2008). In cells, scaffold proteins can organize multimolecular complexes called "signalosomes," constituting an important mechanism responsible for

specificity and efficacy in intracellular signal transduction (Scott and Pawson 2009). Firstly, many signaling enzymes have broad substrate specificity. Scaffold proteins can co-localize these pleiotropic enzymes with individual substrates, selectively enhancing the catalysis of substrates and providing a degree of specificity not intrinsic to the enzyme's active site (Scott and Pawson 2009). Secondly, some signaling enzymes are low in abundance. Scaffold proteins can co-localize a rare enzyme with its substrate, making signaling kinetically favorable. Thirdly, since many scaffolds are multivalent, scaffold binding can orchestrate the co-regulation by multiple enzymes of individual substrate effectors. Muscle A-kinase anchoring protein (mAKAP, a.k.a. AKAP6) is a large scaffold expressed in cardiac and skeletal myocytes and neurons that binds both signaling enzymes such as protein kinase A (PKA) and the Ca^{2+}/calmodulin-dependent phosphatase Calcineurin (CaN) that have broad substrate specificity and signaling enzymes such as p90 ribosomal S6 kinase 3 (RSK3) that is remarkably low in abundance (Figs. 1 and 7) (Kapiloff 1999, Michel 2005, Pare 2005, Wang 2015). mAKAPβ is the alternatively-spliced isoform expressed in myocytes, in which cells it is localized to the outer nuclear membrane by binding the integral membrane protein nesprin-1α (Pare 2005).

[0008] mAKAPβ "signalosomes" regulate cardiac remodeling by multiple mechanisms (Figs. 1 and 7) (Passariello 2015). By binding a diverse set of signaling molecules, some constitutively and some in response to stress-related stimuli, mAKAPβ dynamically organizes multiple signaling modules that transduce cAMP, mitogen-activated protein kinase (MAPK), calcium, phosphoinositide, and hypoxic stress signals (Marx 2000, Kapiloff 2001, Dodge-Kafka 2005, Wong 2008, Kapiloff 2009, Li 2010, Vargas 2012, Kritzer 2014). mAKAP was originally identified by its binding to PKA, and mAKAPβ signalosomes contain all of the required machinery for cAMP synthesis, degradation and function, including adenylyl cyclase 5, type 4D3 phosphodiesterase (PDE4D3), and the cAMP targets type IIα PKA and exchange protein activated by cAMP-1 (Epac1) (Kapiloff 1999, Dodge 2001,

Dodge-Kafka 2005, Kapiloff 2009). Notably, all of these signaling enzymes have been implicated in myocyte remodeling, and binding of PKA to mAKAPβ has been shown to be important for myocyte hypertrophy (Okumura 2003, Dodge-Kafka 2005, Lehnart 2005, Pare 2005). In addition, mAKAPβ binds a wide variety of other proteins important for myocyte stress responses: MEK5 and ERK5 MAP-kinases, 3-phosphoinositide-dependent protein kinase-1 (PDK1), RSK3, phospholipase Cε (PLCε), protein kinase Cε (PKCε), protein kinase D (PKD, PKCμ), the protein phosphatases CaN (Aβ isoform) and PP2A, the type 2 ryanodine receptor (RyR2), the sodium/calcium exchanger NCX1, ubiquitin E3-ligases involved in HIF1α regulation, and myopodin (Marx 2000, Kapiloff 2001, Schulze 2003, Dodge-Kafka 2005, Michel 2005, Pare 2005, Pare 2005, Dodge-Kafka and Kapiloff 2006, Faul 2007, Wong 2008, Kapiloff 2009, Zhang 2011, Vargas 2012, Li , Zhang 2013). Bound to mAKAPβ, these signaling molecules co-regulate the transcription factors hypoxia-inducible factor 1α (HIF1α), myocyte enhancer factor-2 (MEF2), and nuclear factor of activated T-cell (NFATc), as well as type IIa histone deacetylases (HDAC4/5) (Wong 2008, Li 2010, Li 2013, Kritzer 2014).

[0009] Consistent with its role as a scaffold protein for stress-related signaling molecules in the cardiac myocyte, depletion of mAKAPβ in rat neonatal ventricular myocytes in vitro inhibited hypertrophy induced by α-adrenergic, β-adrenergic, endothelin-1, angiotensin II, and leucine inhibitor factor/gp130 receptor signaling (Dodge-Kafka 2005, Pare 2005, Zhang 2011, Guo 2015). *In vivo*, along with attenuating hypertrophy induced by short-term pressure overload and chronic β-adrenergic stimulation, mAKAP gene targeting in the mouse inhibited the development of heart failure following long-term pressure overload, conferring a survival benefit (Kritzer 2014). Specifically, mAKAP gene deletion in the $mAKAP^{fl/fl}$;Tg(Myh6-cre/Esr1*), tamoxifen-inducible, conditional knock-out mouse reduced left ventricular hypertrophy, while greatly inhibiting myocyte apoptosis, and interstitial fibrosis, left atrial hypertrophy, and pulmonary edema (wet lung weight) due to transverse aortic

constriction for 16 weeks (Kritzer 2014).

[0010] mAKAP gene targeting is also beneficial following myocardial infarction. Permanent ligation of the left anterior descending coronary artery (LAD) in the mouse results in myocardial infarction, including extensive myocyte death, scar formation, and subsequent left ventricular (LV) remodeling. Four weeks following LAD ligation, mAKAP conditional knock-out mouse had preserved LV dimensions and function when to compared to infarcted control cohorts. mAKAP conditional knock-out mice had preserved LV ejection fraction and indexed atrial weight compared to controls, while displaying a remarkable decrease in infarct size.

[0011] In cardiomyocytes, mAKAPβ is localized to the nuclear envelope through an interaction with nesprin-1α (Pare 2007). mAKAPβ assembles a large signaling complex that integrates hypertrophic signals initiated by α1-adrenergic receptors (α1- ARs) and β-ARs, endothelin-1 receptors, and gp130/leukemia inhibitor factor receptors (Figure 46A of of U.S. Patent No. 9,132,174) (Dodge-Kafka 2005, Pare 2005). Over the last few years, the molecular mechanisms as well as the signaling pathways whereby mAKAPβ mediates cardiomyocyte hypertrophy have been extensively investigated. It is now demonstrated that mAKAPβ can recruit the phosphatase calcineurin Aβ (CaNAβ) as well as the hypertrophic transcription factor nuclear factor of activated T cells c3 (NFATc3) (Li 2010). In response to adrenergic receptor activation, anchored CaNAβ dephosphorylates and activates NFATc3, which promotes the transcription of hypertrophic genes (Figure 46A of U.S. Patent No. 9,132,174) (Li 2010). The molecular mechanisms controlling the activation of the pool of CaNAβ bound to the mAKAPβ complex are currently not completely understood but seem to require mobilization of local Ca^{2+} stores. In this context, it has been shown that mAKAP favors PKA-induced phosphorylation of RyR2 (Kapiloff 2001), which, through the modulation of perinuclear Ca^{2+} release, could activate CaNAβ (Figure 46A of U.S. Patent No. 9,132,174). In line with this hypothesis, the deletion of the

PKA anchoring domain from mAKAPβ has been shown to suppress the mAKAP-mediated hypertrophic response (Pare 2005). On the other hand, recent findings indicate that mAKAPβ also binds phospholipase Cε(PLCε) and that disruption of endogenous mAKAPβ-PLCεcomplexes in rat neonatal ventricular myocytes inhibits endothelin 1-induced hypertrophy (Zhang 2011). This suggests that the anchoring of PLCε to the nuclear envelope by mAKAPβ controls hypertrophic remodeling. Therefore, it is also plausible that at the nuclear envelope, PLCε might promote the generation of inositol 1,4,5-trisphosphate, which through the mobilization of local Ca^{2+} stores, might promote the activation of CaNAβ and NFATc3 bound to mAKAPβ (Figure 46 of U.S. Patent No. 9,132,174).

[0012] In cardiomyocytes, the dynamics of PKA activation within the mAKAP complex are tightly regulated by AC5 (Kapiloff 2009) and the PDE4D3 (Dodge-Kafka 2005, Dodge 2001) that are directly bound to the anchoring protein. The mAKAP-bound AC5 and upstream β-AR may be localized within transverse tubules adjacent to the nuclear envelope (Escobar 2011). In response to elevated cAMP levels, mAKAP-bound PKA phosphorylates both AC5 and PDE4D3 (Dodge-Kafka 2005, Dodge 2001, Kapiloff 2009). This induces AC5 deactivation and PDE4D3 activation, which locally decreases cAMP concentration and induces deactivation of anchored PKA (Figure 46A of U.S. Patent No. 9,132,174). Dephosphorylation of PDE4D3 is mediated by the phosphatase PP2A that is also associated with mAKAPβ (Figure 46A of U.S. Patent No. 9,132,174) (Dodge-Kafka 2010). Collectively, these findings suggest that the mAKAP complex generates cyclic pulses of PKA activity, a hypothesis that was supported experimentally by live cell imaging studies (Dodge-Kafka 2005).

[0013] *AKAPs and Hypoxia*

[0014] Myocardial oxygen levels need to be maintained within narrow levels to sustain cardiac function. During ischemic insult, in response to conditions of reduced oxygen supply (termed hypoxia), cardiomyocytes mobilize hypoxia-inducible factor 1α (HIF-1α), a transcription factor that promotes a

wide range of cellular responses necessary to adapt to reduced oxygen (Semenza 2007). Transcriptional responses activated by HIF-1α control cell survival, oxygen transport, energy metabolism, and angiogenesis (Semenza 2007). Under normoxic conditions, HIF-1α is hydroxylated on two specific proline residues by the prolyl hydroxylase domain proteins (PHDs) and subsequently recognized and ubiquitinated by the von Hippel-Lindau protein (Jaakkola 2001, Maxwell 1999). Ubiquitinated HIF-1α is targeted to the proteasome for degradation. On the other hand, when oxygen concentration falls, the enzymatic activity of PHD proteins is inhibited. Moreover, PHD proteins are ubiquitinated by an E3 ligase named "seven in absentia homolog 2 (Siah2)" and targeted for proteasomal degradation (Nakayama 2004). This inhibits HIF-1α degradation and allows the protein to accumulate in the nucleus where it promotes gene transcription required for the adaptive re- sponse to hypoxia. In line with this finding, the delivery of exogenous HIF-1α improves heart function after myocardial infarction (Shyu 2002), whereas cardiac overexpression of HIF-1α reduces infarct size and favors the formation of capillaries (Kido 2005).

[0015] Recent findings indicate that mAKAP assembles a signaling complex containing HIF-1α, PHD, von Hippel-Lindau protein, and Siah2 (Wong 2008). This positions HIF-1α in proximity of its upstream regulators as well as to its site of action inside the nucleus. In this configuration, under normoxic conditions, negative regulators associated with the mAKAP complex favor HIF-1α degradation (Wong 2008). On the other hand, during hypoxia, the activation of Siah2 within the mAKAP complex promotes HIF-1α stabilization, allowing the transcription factor to induce transcription (Wong 2008). Therefore, mAKAP assembles a macromolecular complex that can favor degradation or stabilization of HIF-1α in cardiomyocytes in response to variations of oxygen concentrations. In this context, mAKAP could play an important role in cardiomyocyte protection during cardiac ischemia, when coronary blood flow is reduced or interrupted. By coordinating the

molecular pathways that control HIF-1α stabilization in cardiomyocytes, mAKAP might favor HIF-1α-mediated transcriptional responses, controlling the induction of glycolysis (which maximizes ATP production under hypoxic conditions), the efficiency of mitochondrial respiration, and cell survival during ischemia (Semenza 2009).

[0016] *RSKs and Cardiac Remodeling*

[0017] Myofibrillar assembly driving nonmitotic growth of the cardiac myocyte is the major response of the heart to increased workload (Kehay 2010). Although myocyte hypertrophy per se may be compensatory, in diseases such as hypertension and myocardial infarction, activation of the hypertrophic signaling network also results in altered gene expression ("fetal") and increased cellular apoptosis and interstitial fibrosis, such that left ventricular hypertrophy is a major risk factor for heart failure. Current therapy for pathologic hypertrophy is generally limited to the broad downregulation of signaling pathways through the inhibition of upstream cell membrane receptors and ion channels (McKinsey 2007). Novel drug targets may be revealed through the identification of signaling enzymes that regulate distinct pathways within the hypertrophic signaling network because of isoform specificity or association with unique multimolecular signaling complexes.

[0018] p90 ribosomal S6 kinases (RSK) are pleiotropic extracellular signal–regulated kinase (ERK) effectors with activity that is increased in myocytes by most hypertrophic stimuli (Anjum 2008, Sadoshima 2005, Kodama 2000). In addition, increased RSK activity has been detected in explanted hearts from patients with end-stage dilated cardiomyopathy (Takeishi 2002). There are 4 mammalian RSK family members that are ubiquitously expressed and that overlap in substrate specificity (Anjum 2008). RSKs are unusual in that they contain 2 catalytic domains, N-terminal kinase domain and C-terminal kinase domain (Figure 4A of U.S. Patent No. 9,132,174, Anjum 2008). The N-terminal kinase domain phosphorylates RSK substrates and is activated by sequential phosphorylation of the C-

terminal kinase domain and N-terminal kinase domain by ERK (ERK1, ERK2, or ERK5) and 3'-phosphoinositide- dependent kinase 1 (PDK1), respectively (Anjum 2008).

[0019] By binding scaffold proteins, RSKs may be differentially localized within subcellular compartments, conferring isoform-specific signaling like when bound to the scaffold protein muscle A-kinase anchoring protein (mAKAP) (Michael 2005). PDK1 activation of RSK was enhanced by co-expression with the mAKAP scaffold in a recombinant system. In cardiac myocytes, mAKAPβ (the alternatively spliced form expressed in muscle cells) organizes signalosomes that transduce cAMP, mitogen- activated protein kinase, Ca^{2+}, and hypoxic signaling by binding a diverse set of enzymes, ion channels, and transcription factors (Kritzer 2012).

[0020] In the United States, heart failure affects 5.7 million people, and each year 915,000 new cases are diagnosed (Writing Group 2016). The prevalence and incidence of heart failure are increasing, mainly because of increasing life span, but also because of the increased prevalence of risk factors (hypertension, diabetes, dyslipidemia, and obesity) and improved survival rates from other types of cardiovascular disease (myocardial infarction [MI] and arrhythmias) (Heidenreich 2013). First-line therapy for patients with heart failure includes angiotensin-converting enzyme (ACE) inhibitors and β-adrenergic receptor blockers (β-blockers) that can improve the survival and quality of life of such patients, as well as reduce mortality for those with left ventricular dysfunction (Consensus 1987). Subsequent or alternative therapies include aldosterone and angiotensin II receptor blockers, neprilysin inhibitors, loop and thiazide diuretics, vasodilators, and I_f current blockers, as well as device-based therapies (Ponikowski 2016). Nevertheless, the 5-year mortality for symptomatic heart failure remains 50%, including >40% mortality for those post-MI (Heidenreich 2013, Gerber 2016). There is a clear need to develop new effective therapies to treat patients with heart failure, as well as to prevent its development in the context of other cardiovascular diseases such coronary artery disease,

hypertension, and valvular disease.

[0021]

SUMMARY OF THE INVENTION

[0022] The following brief summary is not intended to include all features and aspects of the present invention, nor does it imply that the invention must include all features and aspects discussed in this summary.

[0023] The present inventors have discovered methods of treating cardiac pathological processes by inhibiting the signaling properties of individual mAKAP signaling complexes using drugs that target unique protein-protein interactions. Such a therapeutic strategy offers an advantage over classical therapeutic approaches because it allows the selective inhibition of defined cellular responses.

[0024] In particular, the present inventors have found that disrupting mAKAP-mediated protein-protein interactions can be used to inhibit the ability of mAKAP to coordinate the activation of enzymes that play a central role in activating key transcription factors that initiate the remodeling process leading to cardiac hypertrophy.

[0025] Specifically, the inventors have discovered that inhibiting the binding interaction between type 3 ribosomal S6 kinase (RSK3) and mAKAPβ can protect the heart from damage caused by various physical stresses, for example pressure overload and prolonged exposure to high levels of catecholamines.

[0026] Thus, the present invention comprises, in certain aspects a method for protecting the heart from damage, by administering to a patient at risk of such damage, a pharmaceutically effective amount of a composition which inhibits the interaction of RSK3 and mAKAPβ.

[0027] The invention also relates to a method of treating heart disease, by administering to a patient a pharmaceutically effective amount of a composition which inhibits the interaction of RSK3

and mAKAPβ.

[0028] The invention also relates to compositions which inhibit the interaction of RSK3 and mAKAPβ.

[0029] In still other embodiments, the inhibitors include any molecule that inhibits the expression or activity of of RSK3 and mAKAPβ.

[0030] The foregoing and other objects, features and advantages of the invention will be apparent from the following more particular description of preferred embodiments of the invention, as illustrated in the accompanying drawings in which like reference characters refer to the same parts throughout the different views. The drawings are not necessarily to scale, emphasis instead being placed upon illustrating the principles of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0031] The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

[0032] Fig. 1. **Model for RSK3 signaling.** MAP-Kinase signaling induced by α1-adrenergic receptor (α1-AR) stimulation and potentially other upstream signals activates anchored RSK3 in conjunction with PDK1 at anchored sites, including at perinuclear mAKAPβ signalosomes. Targets for RSK3 may include cytosolic and nuclear proteins, especially those involved in the regulation of hypertrophic gene expression.

[0033] Fig. 2. **Shows the amino acid sequence of human RSK3 (SEQ ID NO: 1).**

[0034] Fig. 3. **Shows the amino acid sequence of rat mAKAP (SEQ ID NO: 2).** – Note that within this document, references to mAKAP sequences, whether labelled "mAKAPβ" or "mAKAP" are according to the numbering for the mAKAPα alternatively-spliced form which contains within the

entirety of mAKAPβ and is identical to the originally published mAKAP sequence as shown in this figure (Kapiloff 1999, Michel 2005). "mAKAP" is also referred to as "AKAP6" in reference databases and the literature.

[0035] **Fig. 4** shows cDNA cloning, *in vitro* translation, and detection of endogenous RSK3 by immunoblotting. Fig. 4(a) shows the complete nucleotide (SEQ ID NO:3) and deduced amino acid (SEQ ID NO:4) sequence of human RSK3. (Zhao (1995) The sequence was derived from a full-length cDNA clone. The deduced RSK3 protein sequence is indicated in the one-letter amino acid code beginning at the first methionine residue preceding the 733-codon open reading frame and terminating at the asterisk. Highly conserved amino acid residues among the known protein kinases are shown in boldface type. The unique N-terminal region of RSK3 (which bears no homology to RSK1 or RSK2) is underlined; the putative bipartite nuclear targeting motif is indicated by parentheses. An in-frame stop codon upstream of the first methionine is indicated (***). This nucleotide sequence was submitted to the EMBL-GenBank data library and assigned accession number X85106. Fig. 4(b) shows *in vitro* translation of RSK3. In vitro transcripts were generated with T7 polymerase from the vector alone (lane 1) or from the vector with an RSK3 insert by using T7 polymerase (lane 2, sense oriented) or Sp6 polymerase (lane 3, antisense oriented). Subsequent in vitro translation was performed with rabbit reticulocyte lysate in the presence of [^{35}S]methionine. Proteins were then resolved by SDSPAGE (10% polyacrylamide) followed by autoradiography. Fig. 4(c) shows immunoblotting with RSK3-specific antiserum. Antiserum N-67 was raised against a peptide (KFA VRRFFSVYLRR) derived from the unique N-terminal region of RSK3 (residues 7 to 20 of SEQ ID NO: 1). In this example, proteins derived from human skin fibroblasts were separated by SDS-PAGE followed by Western immunoblotting. Blots were probed with preimmune serum or N-67. A band of 83 kDa was detected when N-67 was used.(Zhao (1995)

[0036] **Fig. 5. Shows the nucleotide sequence of human RSK3 (SEQ ID NO: 5).**

[0037] **Fig. 6. Shows the nucleotide sequence of rat mAKAP (SEQ ID NO: 6).**

[0038] **Fig. 7. mAKAPβ Signalosomes Drive Pathological Remodeling.** ERK pathways are activated by stress signaling. Anchored RSK3 is activated by ERK and 3'-phosphoinositide-dependent protein kinase 1 (PDK1)-catalyzed phosphorylation (Martinez 2015). Calcineurin (CaN) bound to mAKAPβ is important for the activation of MEF2D and NFATc transcription factors (Li 2010, Li 2013). mAKAP-anchored protein kinase D (PKD) dephosphorylates type IIa histone deacetylases, de-repressing gene expression (Zhang 2013, Kritzer 2014). Together these pathways promote pathological gene expression.

[0039] **Fig. 8. Sequence of human mAKAP (AKAP6) mRNA (SEQ ID NO:11)** - ref seq XM_017021808.1 with shRNA sequences (#1-3) marked. Numbering is for nucleotide sequence. Encoded animo acids are indicated above.

[0040] **Fig 9. Map of pscA-TnT-mAKAP shrna (#3) plasmid.**

[0041] **Fig. 10. Nucleotide sequence of pscA-TnT-mAKAP shrna (#3) plasmid (SEQ ID NO:12)** with key features and some restriction enzymes sites indicated.

[0042] **Fig. 11. Design of a scAAV-mAKAP shRNA biologic drug.** A. mAKAP mRNA sequences form human (SEQ ID NO:13), swine (SEQ ID NO:14), mouse (SEQ ID NO:15) and rat (SEQ ID NO:16). The boxed sequence is shRNA target #3. B. AAV Design. A truncated right ITR confers self-complementarity. cTnT is the cardiac myocyte-specific promoter. C. Western blot of total heart extracts for mAKAPβ 3 weeks after injection into adult mice with $5x10^{11}$ vg IV scAAV-mAKAP shRNA #3 or scAAV-control shRNA (containing a random sequence). NI – non-injected control.

[0043] **Fig. 12. Inhibition of *mAKAPβ post-MI Ameliorates Left Ventricular (LV) Systolic Dysfunction in C57BL/6 mice. A.*** Experimental design. Myocardial infarction (MI) was induced by

permanent ligation of the left anterior coronary artery survival surgery. Sham-operated mice underwent all but coronary artery ligation. AAV IV refers to tail vein injection of AAV biologic. ***B.*** Administration of a single intravenous injection of scAAV-mAKAP-shRNA #3 (shR-mAKAP-MI) 3 days post-MI resulted in amelioration of systolic dysfunction as evidenced by normalization of ejection fraction (EF) from day 2 to weeks 2, 4 and 8 post-MI. In contrast, mice treated with scAAV-control-shRNA (shRNA-Ctrl-MI) displayed a progressive deterioration of EF after ischemic injury. At 8 weeks post MI, EF in shRNA-mAKAP-MI was not significantly different than either mAKAP- and control-shRNA- injected Sham-operated animals (44.3±3.3 vs. 54.6±1.7 & 54.5±2.0%), while significantly greater than that for control-shRNA-MI animals (18.0±4.3%, P<0.**0001).**

[0044] **Fig. 13. Post-mortem gravimetrics for mice studied in Figure 12.** scAAV-mAKAP-shRNA #3 treatment improved overall heart weight, biventricular weight, atrial weight, and wet lung weight.

DETAILED DESCRIPTION OF THE INVENTION

[0045] As discussed above, AKAP-based signaling complexes play a central role in regulating physiological and pathological cardiac events. As such, the present inventors have examined inhibiting the signaling properties of individual AKAP signaling complexes using drugs that target unique protein-protein interactions as an approach for limiting cardiac pathological processes. Such a therapeutic strategy offers an advantage over classical therapeutic approaches since it allows the selective inhibition of defined cellular responses.

[0046] Anchoring proteins including mAKAP are therapeutic targets for the treatment of cardiac hypertrophy and heart failure. In particular, the present inventors have found that disrupting AKAP-mediated protein-protein interactions can be used to inhibit the ability of mAKAP to coordinate the activation of enzymes that play a central role in activating key transcription factors that initiate the

remodeling process leading to cardiac hypertrophy.

[0047] In particular, the inventors have found that type 3 ribosomal S6 kinase (RSK3) binds mAKAPβ directly via the unique N-terminal domain of RSK3, defining a novel enzyme-scaffold interaction. The inventors have found that anchored RSK3 regulates concentric cardiac myocyte growth, revealing an isoform-specific target for therapeutic intervention in pathologic cardiac hypertrophy. Delivery of such peptides that might inhibit RSK3-mAKAPβ interaction can be enhanced by the use of cell-penetrating sequences such as the transactivator of transcription peptide and polyarginine tails, or conjugation with lipid-derived groups such as stearate. Stability may also be enhanced by the use of peptidomimetics [i.e., peptides with structural modifications in the original sequence giving protection against exo- and endoproteases without affecting the structural and functional properties of the peptide. Alternatively, as shown in Figure 41 of U.S. Patent No. 9,132,174, peptides can be delivered by intracellular expression via viral-based gene therapy vectors.

[0048] The inventors have also found that small molecule disruptors can be used to target specific interaction within AKAP-based complexes. Small molecule disruptors can be identified by combining rational design and screening approaches. Such compounds can be designed to target-specific binding surfaces on AKAPs, to disrupt the interaction between AKAPs and PKA in cardiomyocytes and to enhance the contractility of intact hearts for the treatment of chronic heart failure.

[0049] The present invention relates to methods of treating any cardiac condition, which is initiated through the interaction of RSK3 and mAKAPβ. Such cardiac dysfunction can result in signs and symptoms such as shortness of breath and fatigue, and can have various causes, including, but not limited to hypertension, coronary artery disease, myocardial infarction, valvular disease, primary cardiomyopathy, congenital heart disease, arrhythmia, pulmonary disease, diabetes, anemia,

hyperthyroidism and other systemic diseases.

[0050] In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook et al, "Molecular Cloning: A Laboratory Manual" (4th Ed., 2012); "Current Protocols in Molecular Biology" Volumes I-III [Ausubel, R. M., ed. (1994)]; "Cell Biology: A Laboratory Handbook" Volumes I-III [J. E. Celis, 3rd ed. (2005))]; "Current Protocols in Immunology" Volumes I-III [Coligan, J. E., ed. (2005)]; "Oligonucleotide Synthesis" (M.J. Gait ed. 1984); "Nucleic Acid Hybridization" [B.D. Hames & S.J. Higgins eds. (1985)]; "Transcription And Translation" [B.D. Hames & S.J. Higgins, eds. (1984)]; "Animal Cell Culture" [R.I. Freshney, ed. (1986)]; "Immobilized Cells And Enzymes" [IRL Press, (1986)]; B. Perbal, "A Practical Guide To Molecular Cloning" (1984); C. Machida, "Viral Vectors for Gene Therapy: Methods and Protocols" (2010); J. Reidhaar-Olson and C. Rondinone, "Therapeutic Applications of RNAi: Methods and Protocols" (2009).

[0051] The following definitions and acronyms are used herein:

[0052] **ANF** atrial natriuretic factor

[0053] **CTKD** C-terminal kinase domain

[0054] **ERK** extracellular signal-regulated kinase

[0055] **FBS** fetal bovine serum

[0056] **GFP** green fluorescent protein

[0057] **Iso** isoproterenol

[0058] **LIF** leukemia inhibitory factor

[0059] **mAKAP** muscle A-kinase anchoring protein

[0060] **MI** myocardial infarction

[0061] **NTKD** N-terminal kinase domain

[0062] **PDK1** 3'phosphoinositide-dependent kinase 1

[0063] **PE** phenylephrine

[0064] **RBD** RSK binding domain

[0065] **RSK** p90 ribosomal S6 kinase

[0066] **siRNA** small interfering RNA oligonucleotide

[0067] **shRNA** short hairpin RNA

[0068] **TAC** transverse aortic constriction

[0069] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by those of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described. Generally, nomenclatures utilized in connection with, and techniques of, cell and molecular biology and chemistry are those well known and commonly used in the art. Certain experimental techniques, not specifically defined, are generally performed according to conventional methods well known in the art and as described in various general and more specific references that are cited and discussed throughout the present specification. For purposes of the clarity, following terms are defined below.

[0070] The present invention recognizes that the interaction of RSK3 and mAKAPβ mediates various intracellular signals and pathways which lead to cardiac myocyte hypertrophy and/or dysfunction. As such, the present inventors have discovered various methods of inhibiting that interaction in order to prevent and/or treat cardiac myocyte hypertrophy and/or dysfunction.

[0071] Thus, the present invention includes a method for protecting the heart from damage, by administering to a patient at risk of such damage, a pharmaceutically effective amount of a composition,

which inhibits the interaction of RSK3 and mAKAPβ. It should be appreciated that "a pharmaceutically effective amount" can be empirically determined based upon the method of delivery, and will vary according to the method of delivery.

[0072] The invention also relates to a method of treating heart disease, by administering to a patient a pharmaceutically effective amount of a composition, which inhibits the interaction of RSK3 and mAKAPβ.

[0073] The invention also relates to compositions which inhibit the interaction of RSK3 and mAKAPβ. In particular embodiments, these inhibiting compositions or "inhibitors" include peptide inhibitors, which can be administered by any known method, including by gene therapy delivery. In other embodiments, the inhibitors can be small molecule inhibitors. -

[0074] Specifically, the present invention is directed to methods and compositions for treating or protecting the heart from damage, by administering to a patient at risk of such damage, a pharmaceutically effective amount of a composition which (1) inhibits the interaction of RSK3 and mAKAPβ; (2) inhibits the activity of RSK3 and mAKAPβ; or (3) inhibits the expression of RSK3 and mAKAPβ.

[0075] The invention also relates to methods of treating or protecting the heart from damage, by administering to a patient at risk of such damage, a pharmaceutically effective amount of a composition which inhibits a cellular process mediated by the anchoring of RSK3 through its N-terminal domain.

[0076] In one embodiment, the composition includes an RSK3 peptide. In a preferred embodiment, the RSK3 peptide is obtained from the amino terminus of the RSK3 amino acid sequence. In a particularly preferred embodiment, the RSK3 peptide is amino acids 1-42 of the RSK3 amino acid sequence.

[0077] In another embodiment, the composition includes a small interfering RNA siRNA that inhibits the expression of either or both of RSK3 and mAKAPβ. In a preferred embodiment, the siRNA that inhibits the expression of mAKAPβ is generated in vivo following administration of a short hairpin RNA expression vector or biologic agent (shRNA).

[0078] The composition of the invention can be administered directly or can be administered using a viral vector. In a preferred embodiment, the vector is adeno-associated virus (AAV).

[0079] In another embodiment, the composition includes a small molecule inhibitor. In preferred embodiments, the small molecule is SL0101 or BI-D1870.

[0080] In another embodiment, the composition includes a molecule that inhibits the binding, expression or activity of mAKAPβ. In a preferred embodiment, the molecule is a mAKAPβ peptide. The molecule may be expressed using a viral vector, including adeno-associated virus (AAV).

[0081] In yet another embodiment, the composition includes a molecule that interferes with RSK3-mediated cellular processes. In preferred embodiments, the molecule interferes with the anchoring of RSK3 through its N-terminal domain.

[0082] The invention also relates to diagnostic assays for determining a propensity for heart disease, wherein the binding interaction of RSK3 and mAKAPβ is measured, either directly, or by measuring a downstream effect of the binding of RSK3 and mAKAPβ. The invention also provides a test kit for such an assay.

[0083] In still other embodiments, the inhibitors include any molecule that inhibits the expression of RSK3 and mAKAPβ, including antisense RNA, ribozymes and small interfering RNA (siRNA), including shRNA.

[0084] The invention also includes an assay system for screening of potential drugs effective to inhibit the expression and/or binding of RSK3 and mAKAPβ. In one instance, the test drug could be

administered to a cellular sample with the RSK3 and mAKAPβ, or an extract containing the RSK3 and mAKAPβ, to determine its effect upon the binding activity of the RSK3 and mAKAPβ, by comparison with a control. The invention also provides a test kit for such an assay.

[0085] In preparing the peptide compositions of the invention, all or part of the RSK3 (Figure 2) or mAKAP (Figure 3) amino acid sequence may be used. In one embodiment, the amino-terminal region of the RSK3 protein is used as an inhibitor. Preferably, at least 10 amino acids of the RSK3 amino terminus are used. More preferably, about 18 amino acids of the RSK3 amino terminus are used. Most preferably, amino acids from about 1-42 of the RSK3 amino terminus are used.

[0086] In other embodiments, at least 10 amino acids of the mAKAP sequence are used. More preferably, at least 25 amino acids of the mAKAP sequence are used. Most preferably, peptide segments from amino acids 1694-1833 of mAKAP are used.

[0087] It should be appreciated that various amino acid substitutions, deletions or insertions may also enhance the ability of the inhibiting peptide to inhibit the interaction of RSK3 and mAKAPβ. A substitution mutation of this sort can be made to change an amino acid in the resulting protein in a non-conservative manner (i.e., by changing an amino acid belonging to a grouping of amino acids having a particular size or characteristic to an amino acid belonging to another grouping) or in a conservative manner (i.e., by changing an amino acid belonging to a grouping of amino acids having a particular size or characteristic to an amino acid belonging to the same grouping). Such a conservative change generally leads to less change in the structure and function of the resulting protein. A non-conservative change is more likely to alter the structure, activity or function of the resulting protein. The present invention should be considered to include sequences containing conservative changes, which do not significantly alter the activity, or binding characteristics of the resulting protein.

[0088] The following is one example of various groupings of amino acids:

[0089] Amino acids with nonpolar R groups: Alanine,Valine, Leucine, Isoleucine, Proline, Phenylalanine, Tryptophan, Methionine.

[0090] Amino acids with uncharged polar R groups: Glycine, Serine, Threonine, Cysteine, Tyrosine, Asparagine, Glutamine.

[0091] Amino acids with charged polar R groups (negatively charged at pH 6.0): Aspartic acid, Glutamic acid.

[0092] Basic amino acids (positively charged at pH 6.0): Lysine, Arginine, Histidine (at pH 6.0).

[0093] Another grouping may be those amino acids with phenyl groups: Phenylalanine, Tryptophan, Tyrosine.

[0094] Another grouping may be according to molecular weight (i.e., size of R groups): Glycine (75), Alanine (89), Serine (105), Proline (115), Valine (117), Threonine (119), Cysteine (121), Leucine (131), Isoleucine (131), Asparagine (132), Aspartic acid (133), Glutamine (146), Lysine (146), Glutamic acid (147), Methionine (149), Histidine (at pH 6.0) (155), Phenylalanine (165), Arginine (174), Tyrosine (181), Tryptophan (204).

[0095] Particularly preferred substitutions are:

[0096] - Lys for Arg and vice versa such that a positive charge may be maintained;

[0097] - Glu for Asp and vice versa such that a negative charge may be maintained;

[0098] - Ser for Thr such that a free -OH can be maintained; and

[0099] - Gln for Asn such that a free NH_2 can be maintained.

[00100] Amino acid substitutions may also be introduced to substitute an amino acid with a particularly preferable property. For example, a Cys may be introduced a potential site for disulfide bridges with another Cys. A His may be introduced as a particularly "catalytic" site (i.e., His can act as

an acid or base and is the most common amino acid in biochemical catalysis). Pro may be introduced because of its particularly planar structure, which induces ß-turns in the protein's structure. Two amino acid sequences are "substantially homologous" when at least about 70% of the amino acid residues (preferably at least about 80%, and most preferably at least about 90 or 95%) are identical, or represent conservative substitutions.

[00101] Likewise, nucleotide sequences utilized in accordance with the invention can also be subjected to substitution, deletion or insertion. Where codons encoding a particular amino acid are degenerate, any codon which codes for a particular amino acid may be used. In addition, where it is desired to substitute one amino acid for another, one can modify the nucleotide sequence according to the known genetic code.

[00102] Nucleotides and oligonucleotides may also be modified. U.S. Patent No. 7,807,816, which is incorporated by reference in its entirety, and particularly for its description of modified nucleotides and oligonucleotides, describes exemplary modifications.

[00103] Two nucleotide sequences are "substantially homologous" or “substantially identical” when at least about 70% of the nucleotides (preferably at least about 80%, and most preferably at least about 90 or 95%) are identical.

[00104] Two nucleotide sequences are "substantially complementary" when at least about 70% of the nucleotides (preferably at least about 80%, and most preferably at least about 90 or 95%) are able to hydrogen bond to a target sequence.

[00105] The term "standard hybridization conditions" refers to salt and temperature conditions substantially equivalent to 5 x SSC and 65 C for both hybridization and wash. However, one skilled in the art will appreciate that such "standard hybridization conditions" are dependent on particular conditions including the concentration of sodium and magnesium in the buffer, nucleotide sequence

length and concentration, percent mismatch, percent formamide, and the like. Also important in the determination of "standard hybridization conditions" is whether the two sequences hybridizing are RNA-RNA, DNA-DNA or RNA-DNA. Such standard hybridization conditions are easily determined by one skilled in the art according to well known formulae, wherein hybridization is typically 10-20 C below the predicted or determined T_m with washes of higher stringency, if desired.

[00106] The phrase "pharmaceutically acceptable" refers to molecular entities and compositions that are physiologically tolerable and do not typically produce an allergic or similar untoward reaction, such as gastric upset, dizziness and the like, when administered to a human.

[00107] The phrase "therapeutically effective amount" is used herein to mean an amount sufficient to prevent, and preferably reduce by at least about 30 percent, more preferably by at least 50 percent, most preferably by at least 90 percent, a clinically significant change in a cardiac myocyte feature.

[00108] The preparation of therapeutic compositions which contain polypeptides, analogs or active fragments as active ingredients is well understood in the art. Typically, such compositions are prepared as injectables, either as liquid solutions or suspensions, however, solid forms suitable for solution in, or suspension in, liquid prior to injection can also be prepared. The preparation can also be emulsified. The active therapeutic ingredient is often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof. In addition, if desired, the composition can contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents which enhance the effectiveness of the active ingredient.

[00109] A polypeptide, analog or active fragment, as well as a small molecule inhibitor, can be formulated into the therapeutic composition as neutralized pharmaceutically acceptable salt forms.

Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the polypeptide or antibody molecule) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed from the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

[00110] The therapeutic compositions of the invention are conventionally administered intravenously, as by injection of a unit dose, for example. The term "unit dose" when used in reference to a therapeutic composition of the present invention refers to physically discrete units suitable as unitary dosage for humans, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required diluent; i.e., carrier, or vehicle.

[00111] The compositions are administered in a manner compatible with the dosage formulation, and in a therapeutically effective amount. The quantity to be administered depends on the subject to be treated, capacity of the subject's immune system to utilize the active ingredient, and degree of inhibition of RSK3-mAKAPβ binding desired. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner and are peculiar to each individual. However, suitable dosages may range from about 0.1 to 20, preferably about 0.5 to about 10, and more preferably one to several, milligrams of active ingredient per kilogram body weight of individual per day and depend on the route of administration. Suitable regimes for initial administration and booster shots are also variable, but are typified by an initial administration followed by repeated doses at one or more hour intervals by a subsequent injection or other administration. Alternatively, continuous intravenous infusion sufficient to maintain concentrations of ten nanomolar to ten micromolar in the blood are contemplated.

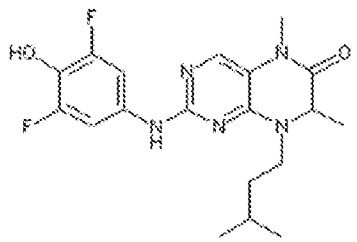
[00112] Because of the necessity for the inhibitor to reach the cytosol, a peptide in accordance with the invention may need to be modified in order to allow its transfer across cell membranes, or may need to be expressed by a vector which encodes the peptide inhibitor. Likewise, a nucleic acid inhibitor (including siRNAs, shRNAs and antisense RNAs) can be expressed by a vector. Any vector capable of entering the cells to be targeted may be used in accordance with the invention. In particular, viral vectors are able to "infect" the cell and express the desired RNA or peptide. Any viral vector capable of "infecting" the cell may be used. A particularly preferred viral vector is adeno-associated virus (AAV).

[00113] siRNAs inhibit translation of target mRNAs via a process called RNA interference. When the siRNA is perfectly complementary to the target mRNA, siRNA act by promoting mRNA degradation. shRNAs, as a specialized type of siRNA, have certain advantages over siRNAs that are produced as oligonucleotides. siRNA oligonucleotides are typically synthesized in the laboratory and are delivered to the cell using delivery systems that deliver the siRNA to the cytoplasm. In contrast, shRNAs are expressed as minigenes delivered via vectors to the cell nucleus, where following transcription, the shRNA are processed by cellular enzymes such as Drosha and Dicer into mature siRNA species. siRNAs are usually 99% degraded after 48 hours, while shRNAs can be expressed up to 3 years. Morover, shRNAs can be delivered in much lower copy number than siRNA (5 copies vs. low nM), and are much less likely to produce off-target effects, immune activation, inflammation and toxicity. While siRNAs are suitable for acute disease conditions where high doses are tolerable, shRNAs are suitable for chronic, life threatening diseases or disorders where low doses are desired. (http://www.benitec.com/technology/sirna-vs-shrna)

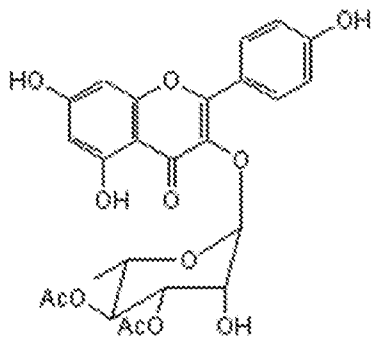
[00114] Guidelines for the design of siRNAs and shRNAs can be found in Elbashir (2001) and at various websites including https://www.thermofisher.com/us/en/home/references/ambion-tech-support/rnai-sirna/general-articles/-sirna-design-guidelines.html and

http://www.invivogen.com/review-sirna-shrna-design, all of which are hereby incorporated by reference in their entireties. Preferably, the first nucleotide is an A or a G. siRNAs of 25-29 nucleotides may be more effective than shorter ones, but shRNAs with duplex length 19-21 seem to be as effective as longer ones. siRNAs and shRNAs are preferably 19-29 nucleotides. Loop sequences in shRNAs may be 3-9 nucleotides in length, with 5, 7 or 9 nucleotides preferred.

[00115] With respect to small molecule inhibitors, any small molecule that inhibits the interaction of RSK3 and mAKAPβ may be used. In addition, any small molecules that inhibit the activity of RSK3 and/or mAKAPβ may be used. Particularly preferred small molecules include BI-D1870, available from Enzo Life Sciences



and SL0101, available from Millipore:



[00116] Small molecules with similar structures and functionalities can likewise be determined by rational and screening approaches.

[00117] Likewise, any small molecules that inhibit the expression of RSK3 and/or mAKAPβ may be used.

[00118] In yet more detail, the present invention is described by the following items which

represent preferred embodiments thereof:

1. A composition comprising a vector encoding a small hairpin ribonucleic acid ("shRNA") against human mAKAP.
2. The composition of item 1, wherein the shRNA inhibits the interaction between human RSK3 and human mAKAP.
3. The composition of item 1, wherein a therapeutically effective amount of said composition administered to a patient in need thereof protects the patient from heart damage.
4. The composition of item 1, wherein the vector is a viral vector.
5. The composition of item 4, wherein the viral vector is an adeno-associated virus vector (AAV).
6. The composition of item 1, wherein the shRNA comprises a sense copy of a human mAKAP mRNA sequence and an antisense copy of a human mAKAP mRNA sequence.
7. The composition of item 6, wherein the sense and antisense copies of the human mAKAP mRNA sequence are separated by a loop sequence.
8. The composition of item 5, wherein the sense and antisense copies of the human mAKAP mRNA sequence are 19 nucleotides in length.
9. The composition of item 1, wherein the vector comprises a sequence substantially complementary to one of the following nucleotide sequences: GGTTGAAGCTTTGAAGAAA (SEQ ID NO:7), GCTAAGAGATACAGAGCTT (SEQ ID NO:8) or GGAGGAAATAGCAAGGTTA (SEQ ID NO:9).
10. The composition of item 9, wherein the vector comprises one of the following nucleotide sequences: GGTTGAAGCTTTGAAGAAA (SEQ ID NO:7),

GCTAAGAGATACAGAGCTT (SEQ ID NO:8) or GGAGGAAATAGCAAGGTTA (SEQ ID NO:9).

11. The composition of item 10, wherein the vector comprises the following nucleotide sequence: GGAGGAAATAGCAAGGTTA (SEQ ID NO:9).
12. The composition of item 4, wherein the viral vector comprises human microRNA (miR)-30a sequences.
13. The composition of item 4, wherein the viral vector comprises a shRNA minigene, wherein the minigene is flanked by a complete AAV2 inverted terminal repeat (ITR) on the 5' end and a deleted AAV2 ITR on the 3' end.
14. The composition of item 4, wherein the viral vector comprises a chicken cardiac troponin T promoter.
15. The composition of item 4, wherein the viral vector comprises a SV40 polyadenylation site.
16. The composition of item 5, wherein the AAV is an AAV serotype 9.
17. A method of inhibiting the expression of human mAKAPß, comprising contacting mRNA encoding mAKAPß with the composition of item 1.
18. A method of protecting the heart from damage, comprising administering to a patient at risk of such damage, a pharmaceutically effective amount of the composition of item 1.
19. A method of treating or preventing heart disease, comprising administering to a patient in need thereof, a pharmaceutically effective amount of the composition of item 1.
20. A method of treating or preventing myocardial infarction, comprising administering to a patient at risk of such damage, a pharmaceutically effective amount of the composition of item 1.

[00119] The following examples are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

[00120] **EXAMPLES:**

[00121] The compositions and processes of the present invention will be better understood in connection with the following examples, which are intended as an illustration only and not limiting of the scope of the invention. Various changes and modifications to the disclosed embodiments will be apparent to those skilled in the art and such changes and modifications including, without limitation, those relating to the processes, formulations and/or methods of the invention may be made without departing from the spirit of the invention and the scope of the appended claims.

[00122] **Example 1**

[00123] **Methods**

[00124] **Reagents**

[00125] Commercial antibodies and oligonucleotides are listed in Figure 19 and Figure 20 of U.S. Patent No. 9,132,174. The commercially available RSK3 antibodies were of varying specificity (Figure 21 of U.S. Patent No. 9,132,174). Additional reagents and detailed methods are provided.

[00126] **RSK3$^{-/-}$ Mouse**

[00127] All experiments involving animals were approved by the Institutional Animal Care and Use Committee at the University of Miami. Constitutive RSK3 knockout mice were backcrossed to C57BL/6 mice over 10 generations. All experiments were performed with littermate controls and mice that were 8 to 10 weeks of age at the beginning of the study. Transverse aortic constriction was performed as previously described (Rockman 1991), and isoproterenol infusion was via Alzet 2002 osmotic pumps (Durect). Echocardiography was performed under isoflurane anesthesia on a Vevo 770

High-Resolution Imaging System (VisualSonics).

[00128] RNA Assays

[00129] mRNA species were assayed using NanoString technology, a direct and multiplexed measurement of gene expression without amplification, using fluorescent molecular bar codes and single-molecule imaging to identify and count multiple transcripts in a single reaction; 100ng of RNA was hybridized in solution to a target-specific code set overnight at 65°C, and individual mRNAs were counted using a NanoString Digital Analyzer.

[00130] Statistics

[00131] For all experiments, n refers to the number of individual mice or individual myocyte preparations. All data are expressed as mean ± SEM. *P* values were calculated using Student *t* tests and are not corrected for multiple comparisons. Repeated symbols represent *P* values of different orders of magnitude (i.e., $P<0.05$, $P<0.005$, and others). All datasets involving multiple comparisons for which *P* values are provided also were significant by ANOVA ($\alpha=0.05$).

[00132] Results

[00133] mAKAPβ: A Scaffold for RSK

[00134] The inventors have previously published that RSK proteins and activity are associated with mAKAPα complexes in the brain (Michel 2005). The inventors now show that RSK also is associated with mAKAPβ in cardiac myocytes (Figure 4B of U.S. Patent No. 9,132,174). To determine whether mAKAP preferentially binds a specific RSK isoform, hemagglutinin (HA)- tagged RSK family members were co-expressed with mAKAP in HEK293 cells. In contrast to RSK1 and RSK2, RSK3 robustly mediated the coimmunoprecipitation of both mAKAPα and mAKAPβ (Figure 4C of U.S. Patent No. 9,132,174). RSK family members are similar in primary sequence with the exception of the extreme N-terminal and C-terminal domains and a small region after the hydrophobic motif

(Figure 4A of U.S. Patent No. 9,132,174). Consistent with the selective binding of RSK3 to the scaffold, the N-terminal domain of RSK3 bound mAKAPβ (Figure 4D of U.S. Patent No. 9,132,174).

[00135] RSK3 Function in Neonatal Cardiac Myocytes

[00136] RSK family members can be activated in most cell types by ERK, but not c-Jun N-terminal kinases or p38 (Anjum 2008). ERK phosphorylation is permissive for PDK1 phosphorylation of the RSK N-terminal kinase domain, such that PDK1 phosphorylation of RSK S^{218} is indicative of full activation of the enzyme (Figure 4A of U.S. Patent No. 9,132,174). To show that ERK activates RSK in cardiac myocytes, the inventors treated neonatal rat ventricular myocytes with different hypertrophic agonists and mitogen-activated protein kinase pathway inhibitors and detected RSK activation using a pan-RSK S^{218} phosphor-specific antibody (Figures 21 and 22 of U.S. Patent No. 9,132,174). The α-adrenergic stimulation with phenylephrine (PE) induced RSK phosphorylation 3-fold by both MEK1/2- dependent (that activates ERK1/2) and MEK5-dependent (that activates ERK5) mechanisms (Figure 22 of U.S. Patent No. 9,132,174). Moreover, MEK1/2 inhibition reduced RSK baseline phosphorylation. c-Jun N-terminal kinase and p38 inhibition did not affect PE activation and, in fact, variably increased baseline RSK phosphorylation. Fetal bovine serum and leukemia inhibitory factor also increased the level of activated RSK, but that occurred more so because of an increase in total RSK protein expression than because of ERK phosphorylation.

[00137] Similar results were found for HA-tagged RSK3 (Figure 5A of U.S. Patent No. 9,132,174). Acute PE treatment induced the phosphorylation of HA-RSK3 ERK (S^{360}) and PDK1 (S^{218}) sites through both MEK1/2-dependent and MEK5-dependent signaling. Together, these results confirmed that in cardiac myocytes ERK is responsible for RSK activation.

[00138] The inventors have previously demonstrated that mAKAPβ complexes are required for the hypertrophy of cultured myocytes (Li 2010, Pare 2005, Dodge-Kafka 2005). Therefore, the

inventors proposed the hypothesis that RSK3 signaling is a major determinant of cardiac myocyte growth. Neonatal myocytes were transfected with small interfering RNA oligonucleotides (siRNA) that diminished RSK3 mRNA and protein levels by >75% (Figure 5B of U.S. Patent No. 9,132,174). RSK3 siRNA did not induce the apoptosis of myocytes cultured either in the absence or in the presence of serum (Figure 5C of U.S. Patent No. 9,132,174). Importantly, in the presence of α-adrenergic stimulation, RSK3 siRNA inhibited morphologic hypertrophy by 34% and atrial natriuretic factor expression completely (Figure 5D–5F and Figure 23 of U.S. Patent No. 9,132,174). In addition, RSK3 siRNA had smaller, but detectable, effects on leukemia inhibitory factor and fetal bovine serum–stimulated hypertrophy. The results obtained by RSK3 RNA interference were confirmed with a second distinct RSK3 siRNA.

[00139] Endogenous RSK3 proteins are expressed at a relatively low level in cardiac myocytes compared with the other RSK family members and are induced in expression by long-term PE treatment (Figure 24C of U.S. Patent No. 9,132,174). As a result, RSK3 RNA interference did not affect the level of total RSK in the myocyte, only diminishing the RSK3 detected after immunoprecipitation with a specific RSK3 antibody (Figure 5B and Figure 24C of U.S. Patent No. 9,132,174). In control experiments, the inhibition of PE-induced hypertrophy by the RSK3 siRNA was rescued by the expression of recombinant HA-tagged human RSK3, but not by an inactive HA-RSK3 $S^{218}A$ mutant (Figure 24A and 24B of U.S. Patent No. 9,132,174). Remarkably, in these experiments, the cross-section area of unstimulated myocytes was increased by adenoviral-based expression of wild-type HA-RSK3 enzyme at a level comparable with that of endogenous RSK3 in PE-treated cells without affecting total RSK levels. Finally, to confirm that RSK activity was important for neonatal myocyte hypertrophy, the inventors used the pan-RSK inhibitors BI-D1870 (Figure 6 and Figure 25 of U.S. Patent No. 9,132,174) and SL0101 (Figure 26 of U.S. Patent No. 9,132,174) (Smith 2005, Sapkota

2007, Malone 2005), finding that, like RSK3 siRNA, these compounds inhibited agonist-induced myocyte hypertrophy.

[00140] High-Affinity RSK3 Binding Domain in mAKAP

[00141] The inventors considered that the requirement for RSK3 in myocyte hypertrophy was attributable to the association of RSK3 with specific signaling complexes. To address this hypothesis, the inventors defined the mAKAP domains responsible for RSK3 binding. HA-tagged RSK3 was co-expressed in heterologous cells with myc-tagged mAKAPβ fragments and coimmunoprecipitated using a myc-tag antibody (Figure 7A and 7B of U.S. Patent No. 9,132,174). RSK3 preferentially associated with mAKAP amino acid residues 1286 to 1833, although it also weakly associated with mAKAP 245 to 587 and 525 to 1286. Consistent with this result, RSK3 binding to a full-length mAKAPβ protein with an internal deletion of residues 1257 to 1886 was reduced by >85%. Further mapping showed that the main RSK3 binding domain (RBD) of mAKAP mapped to a fragment encompassing residues1694 to 1833 (Figure 7C of U.S. Patent No. 9,132,174). Accordingly, RSK3 bound poorly to a full-length mAKAPβ protein with an internal deletion of residues 1701 to 1800 (Figure 7D of U.S. Patent No. 9,132,174). As shown, the unique N-terminal domain of RSK3 bound full-length mAKAPβ (Figure 4D of U.S. Patent No. 9,132,174). The mAKAP RBD also bound HA-RSK3 1 to 42 (Figure 7E of U.S. Patent No. 9,132,174), but not to the N-terminally truncated RSK3 mutant (HA-RSK3 DN30) or the HA-tagged full-length RSK2 (Figure 7F of U.S. Patent No. 9,132,174). These results imply that the mAKAP RBD is responsible for the selective binding of RSK3 to mAKAP.

[00142] The inventors next tested whether mAKAP-RSK3 binding is direct (Figure 7G of U.S. Patent No. 9,132,174). The binding of bacterially expressed His-tagged mAKAP 1286 to 1833 and full-length RSK3 was analyzed by surface plasmon resonance. The binding was direct and of high affinity (nanomolar K_D). The inventors previously reported that once activated, RSK3 binds mAKAPα less

well in cells (Michele 2005). Interestingly, previous RSK3 phosphorylation by either ERK or both ERK and PDK1 decreased the RSK3 binding affinity for mAKAP 5-fold and 8-fold, respectively, through a decrease in the association rate constant.

[00143] **Disruption of RSK3 Anchoring Inhibits Neonatal Myocyte Hypertrophy** The identification of the high-affinity mAKAP RBD provided the opportunity to test whether anchoring of RSK3 is important for its function. When expressed in neonatal myocytes, a green fluorescent protein–mAKAP RBD fusion protein competed the association of endogenous RSK3 and mAKAPβ (Figure 8A of U.S. Patent No. 9,132,174). Expression of green fluorescent protein–mAKAP RBD fusion protein markedly inhibited PE-induced hypertrophy (Figure 8B–5D of U.S. Patent No. 9,132,174), similar to RSK3 siRNA (Figure 5 of U.S. Patent No. 9,132,174). Together, these results imply that RSK3 anchored to scaffolds through its unique N-terminal domain is required for the hypertrophy of cultured myocytes.

[00144] **Role of RSK3 in Cardiac Hypertrophy In Vivo**

[00145] The results obtained in vitro suggested that active RSK3 contributes to the development of pathologic myocyte hypertrophy. Further evidence supporting this hypothesis was obtained using a new RSK3 knockout mouse. By homologous recombination, stop codons were inserted into the second exon of the *RSK3* (*Rps6ka2*) gene encoding the ATP-binding motif of the N-terminal kinase domain (Hanks 1988), resulting in the constitutive absence of RSK3 protein in homozygous null mice (Figure 27 of U.S. Patent No. 9,132,174). In general, *RSK3*$^{-/-}$ mice appeared normal in morphology, were bred according to Mendelian genetics (Figure 12 of U.S. Patent No. 9,132,174), and exhibited no excess mortality up to 6 months of age. Before any stress, the *RSK3*$^{-/-}$ mice had generally normal cardiac function, with the only measureable difference from wild-type littermates being a slight increase in left ventricular internal dimensions detected by echocardiography (Figures 13 and 28 of U.S. Patent No.

9,132,174).

[00146] The inventors tested whether RSK3 is required for compensated cardiac hypertrophy by subjecting the *RSK3*$^{-/-}$ mice to pressure overload for 2 weeks (Figure 9A of U.S. Patent No. 9,132,174). By echocardiography, transverse aortic constriction (TAC) induced a 36% increase in posterior wall thickness in wild-type mice, but only a 16% increase in *RSK3*$^{-/-}$ mice (Figures 10 and 28 of U.S. Patent No. 9,132,174). The decreased hypertrophy was not accompanied by a change in contractility (fractional shortening). Postmortem gravimetric analysis showed that the corresponding increase in biventricular weight after TAC was similarly diminished in the knockout mice (48% for *RSK3*$^{+/+}$ vs. 26% for *RSK3*$^{-/-}$ mice; Figure 14 of U.S. Patent No. 9,132,174). TAC primarily induces concentric growth of cardiac myocytes.

[00147] Inspection of wheat germ agglutinin–stained heart sections revealed that consistent with these results, RSK3 knockout attenuated the TAC-induced increase in myocyte transverse cross-section area by ~46% (Figure 9B and 9C of U.S. Patent No. 9,132,174). Proportional results were obtained by morphometric analysis of adult cardiac myocytes isolated from the TAC mice (Figure 9D–9G of U.S. Patent No. 9,132,174).

[00148] To characterize the *RSK3*$^{-/-}$ cardiac phenotype at a molecular level, the inventors surveyed for differences in the cardiac expression of 30 genes encoding proteins involved either in cardiac remodeling or in hypertrophic signaling (Figure 11 of U.S. Patent No. 9,132,174). Approximately two-thirds of the genes in our panel were significantly increased or decreased in expression by TAC. In general, the changes in expression were attenuated by RSK3 knockout. For example, TAC-induced atrial natriuretic factor expression was dramatically inhibited in *RSK3*$^{-/-}$ mice, consistent with the results obtained for PE-treated neonatal myocytes. Although after 2 weeks of pressure overload the small increases in cellular apoptosis and interstitial fibrosis detectable by

histology for wild-type mice did not reach significance when compared with sham-operated controls, these signs of remodeling tended to be less in the knockout mice (8.2±2.0 vs. 4.2±1.0×10^{-4} TUNEL-positive nuclei and 0.49%±0.18% vs. 0.29%±0.11% collagen staining for wild-type and *RSK3*$^{-/-}$ TAC hearts, respectively). Interestingly, 2 genetic markers of fibrosis that were significantly induced in TAC wild-type mice, transforming growth factor β2 and collagen VI α1 (Yang 2012), were attenuated in expression by RSK3 knockout (Figure 11 of U.S. Patent No. 9,132,174).

[00149] To further explore the role of RSK3 in cardiac hypertrophy, the inventors used a second in vivo pathological stressor, chronic isoproterenol (Iso) infusion via subcutaneous osmotic pumps, and a physiological stressor, chronic exercise via swimming. Although Iso infusion resulted in a minor increase in ventricular wall thickness by echocardiography (Figure 15 of U.S. Patent No. 9,132,174), at the cellular level Iso significantly induced myocyte growth in width in a RSK3- dependent manner as measured by histology and after myocyte isolation (Figure 29 of U.S. Patent No. 9,132,174). Unlike TAC, Iso infusion also induced eccentric growth, as evidenced by increased myocyte length and ventricular dilation by echocardiography (Figures 15 and 16 of U.S. Patent No. 9,132,174). This eccentric growth was not inhibited by RSK3 knockout. Together with the TAC data, these results demonstrate that RSK3 contributes to the induction of concentric myocyte hypertrophy in pathologic conditions.

[00150] Finally, *RSK3*$^{-/-}$ mice were exercised by swimming. As expected (Perrino 2006), after swimming, wild-type mice exhibited a decreased resting heart rate (consistent with improved physical conditioning) and increased left ventricular internal dimensions (Figures 17 and 28 of U.S. Patent No. 9,132,174). After exercise, there were no significant differences between RSK3 knockout and wild-type mice detectable by echocardiography, and the cohorts exhibited a similar increase in biventricular weight indexed by body weight (6% and 7%, respectively; Figure 18 of U.S. Patent No. 9,132,174).

[00151] Detailed Methods

[00152] *Reagents:* Commercial antibodies are listed in Figure 19 of U.S. Patent No. 9,132,174. Secondary antibodies included horseradish peroxidase (HRP)-conjugated donkey secondary antibodies (Jackson ImmunoResearch) and Alexa dye-conjugated donkey secondary antibodies (Invitrogen). Monoclonal 211, polyclonal VO54, VO56 and OR010 mAKAP antibodies were as previously described and are available through Covance Research Products (Kehat 2010). OR42 and OR43 rabbit anti- RSK3 antisera were generated using bacterially-expressed His-tagged RSK3 (full-length) and affinity purified using antigen-coupled Affigel resin (Biorad). FL099 and FL100 rabbit anti-mAKAP antisera were generated using bacterially-expressed GST-tagged mAKAP 245-340. Oligonucleotides are listed in Figure 20 of U.S. Patent No. 9,132,174. Other reagents included: BIX02189 - Boehringer Ingelheim Pharmaceuticals; PD0325901, SB103580, SL0101, and SP600125 - EMD Chemicals Inc.

[00153] All adenovirus were constructed using the pTRE shuttle vector and the Adeno-X Tet-off System (Clontech) and purified after amplification using Vivapure AdenoPACK kits (Sartorius Stedim). These adenoviruses conditionally express recombinant protein when co-infected with tetracycline transactivator-expressing virus (adeno-tTA for "tet-off" or reverse tTA for "tet-on"). HA-tagged RSK and MSK2 expression plasmids acquired from Dario Alessi and John Blenis (McKinsey 2007, Anjum 2008, Sadoshima 1995) and myc-tagged mAKAP mammalian expression vectors (pCDNA3.1 (-) myc-his) and adenovirus were as previously described (Kodama 2000, Takeishi 2002). GFP-RBD was expressed using a pEGFP-based plasmid. Bacterial expression vectors for mAKAP and RSK3 were constructed using pET30 and pGEX-4T parent vectors, and proteins were purified using His-bind (Novagen) and Glutathione Uniflow Resins (Clontech).

[00154] *Neonatal rat myocytes isolation and culture:* 1-3 day old Sprague-Dawley rats were decapitated and the excised hearts placed in 1x ADS Buffer (116 mmol/L NaCl, 20 mmol/L HEPES, 1

mmol/L NaH_2PO_4, 5.5 mmol/L glucose, 5.4 mmol/L KCl, 0.8 mmol/L $MgSO_4$, pH 7.35). The atria were carefully removed and the blood washed away. The ventricles were minced and incubated with 15 mL 1x ADS Buffer containing 3.3 mg type II collagenase (Worthington, 230 U/mg) and 9 mg Pancreatin (Sigma) at 37^{O}C while shaking at 80 RPM. After 15 minutes, the dissociated cardiac myocytes were separated by centrifugation at 50 x g for 1 minute, resuspended in 4 mL horse serum and incubated 37^{O}C with occasional agitation. The steps for enzymatic digestion and isolation of myocytes were repeated 10-12 times to maximize yield. The myocytes were pooled and spun down again at 50 x g for 2 minutes and resuspended in Maintenance Medium (DMEM:M199, 4:1) supplemented with 10% horse serum and 5% fetal bovine serum. To remove any contaminating fibroblasts, the cells were pre-plated for 1 hour before plating on gelatin-coated tissue culture plasticware. This procedure yields >90% pure cardiac myocytes. After 1 day in culture, the media was changed to maintenance medium containing 0.1 mmol/L bromodeoxyuridine to suppress fibroblast growth.

[00155] Experiments were initiated 1 day after myocyte isolation. Adenoviral infection was performed by addition of adenovirus (multiplicity of infection = 5-50) to the media. Plasmids and siRNA oligonucleotides were transfected using Transfast (Promega) and Dharmafect (Thermofisher), respectively, as recommended by the manufacturers using cells cultured in maintenance medium supplemented with 4% horse serum. Starting the day after gene transduction, the cells were treated for as long as 2 days, as indicated for each experiment.

[00156] *Immunoprecipitations:* HEK293 and COS-7 cells were transfected with Lipofectamine 2000 (Invitrogen) or Polyethylenimine "Max" (Polysciences). Cells (including myocytes) were lysed in buffer (20 mmol/L HEPES, pH 7.4, 150 mmol/L NaCl, 5 mmol/L EDTA, 0.5% Triton, 50 mmol/L NaF, 1 mmol/L sodium orthovanadate, 1 mmol/L DTT, and protease inhibitors). After centrifugation at

10,000 x g for 10 minutes at 4 ^{O}C, the clarified extracts were used for immunoprecipitation using appropriate antibodies (10 μg purified antibody or 1-5 μL whole serum) and 20 μL protein G sepharose (Millipore, Fastflow) for 3 hours to overnight at 4°C. The beads were washed 3-5 times with lysis buffer, and the immunoprecipitated proteins were eluted with 1x Laemmli buffer for western blotting. Western blots were developed using horseradish peroxidase-conjugated donkey secondary antibodies, Supersignal West Chemiluminescent Substrates (Thermo Scientific) and X-ray film or a Fujifilm LAS-3000 imaging system.

[00157] *Immunocytochemistry*: Cultured neonatal cardiomyocytes on plastic coverslips were fixed in 3.7% formaldehyde in PBS, permeabilized with 0.3% Triton X-100 in PBS, and blocked with PBS containing 0.2% BSA and 1% horse serum for 1 hour. The slides were then sequentially incubated for 1 hour with primary and Alexa fluorescent dye-conjugated specific-secondary antibodies (Invitrogen, 1:1000) diluted in blocking buffer. The slips were washed three times with blocking buffer. 1 μg/mL Hoechst 33258 was included in the last wash stop to label nuclei. Slides were sealed in SlowFade Gold antifade buffer (Invitrogen) for fluorescent microscopy. Wide-field images were acquired using a Leica DMI 6000 Microscope.

[00158] *Surface Plasmon Resonance:* SPR analysis was performed using a BIAcore T100. 200 resonance units His-tagged mAKAP 1286-1833 were covalently immobilized using NHS (N-hydroxysuccinamide) and EDC [1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide] (Biacore amine coupling kit) to the surface of a sensor chip (BIAcore type CM5). His-RSK3 analytes (6.25 - 200 nmol/L) in HBS buffer (10 mmol/L Hepes, pH 7.4, 150 mmol/L NaCl, and 0.005% Surfactant P20) were injected at a flow rate of 30 μL/min for 5 minutes, followed by buffer alone for another 5 minutes. Sensorgrams were processed by BIAcore T100 evaluation software.

[00159] For phosphorylated His-RSK3, 20 μg His-RSK3 was phosphorylated with 2 μg ERK2

(Millipore, 14-550) and/or PDK1 (Sigma, P7498) for 5 hours in kinase buffer (20 mmol/L MOPS, pH 7.2, 25 mmol/L β-glycerol phosphate, 5 mmol/L EGTA, 1 mmol/L sodium orthovanadate, 1 mmol/L DTT) with 0.5 mmol/L MgATP. Phospho-His-RSK3 was purified using His-binding beads and concentrated before use.

[00160] *Generation of RSK3$^{-/-}$ mouse:* All experiments involving animals were approved by the Institutional Animal Care and Use Committee at the University of Miami. Constitutive knock-out mice were generated using a targeting vector that inserted into Exon 2 a neomycin resistance gene (PGKneo) flanked by loxp sites (Figure 27 of U.S. Patent No. 9,132,174). Targeted 129SvJ ES cells were injected into C57BL/6J blastocysts. PGKneo was removed by crossing mutant mice with B6.C- Tg(CMV-cre)1Cgn/J (The Jackson Laboratory). RSK$^{+/-}$ Mice were selected for loss of the cre transgene and backcrossed to C57BL/6 mice over 10 generations. All experiments were performed with littermate controls and mice that were 8-12 weeks of age. The numbers of mice in each cohort are listed in the various tables and figures.

[00161] *Isoproterenol infusion:* Alzet 2002 osmotic pumps (Durect) were sterilely loaded with 200 μL saline or saline and isoproterenol to deliver 60 mg/kg/day for 14 days. 8 week old mice were anaesthetized, and the pump was inserted sterilely subcutaneously into the shaved back through a transverse incision made intra-scapulae. The wound was closed with surgical staples and covered with betadine solution. Mice were housed separately after surgery.

[00162] *Transverse Aortic Constriction:* All tools were sterilized with a Germinator 500 Dry Sterilizer and Betadine Solution (10% povidone-iodine topical solution). Anesthesia was induced with 5% isoflurane and maintained with 2% isoflurane and 100% oxygen at a flow rate of 1.5 L/min using a SurgiVet flow regulator via nose cone. Loss of consciousness was verified by toe pinch. Mouse fur over the left chest and sternum was removed with a calcium hydroxide lotion (e.g. Nair), and the

surgical site was sterilized with betadine. The skin was incised exposing the pectoralis muscle and the second left intercostal space. The pectoralis muscle and the second rib were blunt dissected and retracted revealing the thymus within the mediastinum. The lobes of the thymus were retracted to reveal the transverse aortic arch as well as the right innominate and left common carotid arteries. Blunt dissection though the connective tissue between these two arteries and under the aorta allowed for the passage of a 6-0 silk using a modified ligation aid (Fine Science Tools 18062-12). A 27 gauge needle was placed on top of the aorta and the 6-0 silk was tied around the needle. The needle was removed, leaving a constricted aorta. The chest was closed in two layers with 5-0 Polysorb Suture. Isoflurane administration was terminated, and the mice were maintained on 100% oxygen by nose cone until conscious. Immediately post-operatively, buprenorphrine (0.05-0.1 mg/kg s.c.) was administered and then q12 h prn. The mice were allowed to recover under a heat lamp until alert and active. Sham-operated mice that experience all but the placement of the aortic ligature served as controls.

[00163] *Swimming:* 8-10 week old mice were forced to swim in water tanks every day for 4 weeks. The swimming tank measured >225 cm^2, with a depth of 15 cm and a water temperature of 30-32°C. Mice were continuously observed to avoid any drowning. The first day of training consisted of two 10-min sessions separated by at least 4 h. Sessions were increased by 10 min each day until 90-min sessions were reached. Additional cohorts were housed normally without exercise to serve as a "sham swim" control group. Food and water were provided ad libitum throughout the month period for all mice.

[00164] *Echocardiography:* Mice minimally anesthetized with 1-2% isoflurane were studied using a Vevo 770®, High-Resolution Imaging System (VisualSonics). The pressure gradient following TAC was calculated from the pulse wave Doppler velocity at the point of ligation as follows: $P = 4v^2$; P = the induced pressure gradient (in mmHg) and v = the velocity across the constriction (in m/s).[7]

[00165] *Adult mouse myocytes isolation by Langendorff perfusion:* Mice were anesthetized using Ketamine (80-100 mg/kg) and Xylazine (5-10 mg/kg) IP followed by 200 U heparin IP and cardiac excision. The heart was placed immediately in perfusion buffer (NaCl 120 mmol/L, KCl 5.4 mmol/L, $Na_2HPO_4 \cdot 7H_2O$ 1.2 mmol/L, $NaHCO_3$ 20.0 mmol/L, $MgCl_2.6H_2O$ 1.6 mmol/L, Taurine 5 mmol/L, Glucose 5.6 mmol/L) equilibrated with 95% O_2 and 5% CO_2. The heart was attached via the aorta to the condenser outlet of a Harvard Langendorff apparatus. Ca^{2+}-free perfusion lasted for 5 minutes with a constant rate at 2.2 mL/min at 37°C. The heart was digested by continuous perfusion with 25 mL buffer containing 25 mg type II collagenase (Worthington, 315 U/mg) and 1.3 mg protease (Sigma type XIV). After removal of the atria, the ventricles were then immersed in 5 mL of the same enzyme solution for dissociation by cutting into small pieces and by passing through a large bore pipette. The cell slurry was filtered through a 150 - 200 μm nylon mesh and the myocytes relaxed by incubation for 10 minutes in perfusion buffer containing 10 mmol/L KCl. The cells were fixed in suspension in perfusion buffer containing 3.7% formaldehyde, before morphometric analysis by light microscopy. *Histochemistry:* Heart tissue was fixed in 3.7% formaldehyde. De-paraffinized 5 μm tissue sections were stained using the Picrosirius Red Stain Kit (Polysciences) and Alexa Fluor 555 Wheat Germ Agglutinin conjugate (Invitrogen) as recommended by the manufacturers. The cross-section area of >150 myocytes in >3 distinct regions of the left ventricle were measured per heart using the wheat germ agglutinin sections. Collagen content was assayed using the Picrosirius Red stained sections and polarized light microscopy for >3 5x objective fields per heart. TUNEL staining for both fixed cells and tissue sections was performed using the *In Situ* Cell Death Detection Kit, TMR red (Roche). Morphometrics and collagen content were measured using IPLab microscope software (BD Biosciences).

[00166] *Morphometry:* Morphometric data was acquired using IPLab Software. For neonatal

myocytes, at least 6 separate images, each containing >100 cells, were assayed for cross-section area and perinuclear prepro-ANF staining per condition for each repetition of the experiment. For adult mouse cardiac myocytes, the maximum lengths perpendicular (width) or parallel (length) to the myofibrils were measured for >100 freshly dissociated myocytes per heart.

[00167] *RNA Assays:* Total RNA was quantified with a Nanodrop 8000 Spectrophotometer (Thermo Scientific) and quality controlled using with a Bioanalyzer 2100 and the RNA 6000 Nano kit (Agilent). qRT-PCR was performed using SYBR green.

[00168] The NanoString assay is based on direct, multiplexed measurement of gene expression without amplification, utilizing fluorescent molecular barcodes and single molecule imaging to identify and count multiple transcripts in a single reaction. For Nanostring assay, 100 ng total RNA were hybridized in solution to a target-specific codeset overnight at 65℃. The codeset contained dual, adjacently placed 50 bp oligonucleotide probes against a panel of 30 genes, one set of probes fluorescently bar-coded and the other biotinylated. The hybridization reactions were loaded onto the NanoString Prep station which removes excess oligonucleotides and binds the hybridized mRNA to the Streptavidin-coated cartridge surface. The cartridges were loaded onto the NanoString Digital Analyzer, and 1155 fields of view were fluorescently scanned to count only those individual mRNAs bound to both a biotinylated and fluorescently bar-coded probe. Datasets for each RNA sample were background-subtracted and normalized using *Gapdh*. In validation assays, NanoString counts were directly proportional over 3 orders of magnitude to the mRNA levels obtained by qRT-PCR and had a similar minimum level of detection.

[00169] *Statistics:* For all experiments, *n* refers to the number of individual mice or individual myocyte preparations. All data are expressed as mean ± s.e.m. *p*-values were calculated using two-tailed Student's *t*-tests, paired or un-paired as appropriate, and are not corrected for multiple

comparisons. Repeated symbols represent *p*-values of different orders of magnitude, for example: * $p<0.05$, ** $p<0.005$, *** $p < 0.0005$, etc. All datasets involving multiple comparisons for which *p*-values are provided were also significant by ANOVA, α0.05.

[00170] **Discussion**

[00171] RSK activity is associated with the function of the nervous system, immunity, muscle, and cancer (Anjum 2008). Human *RSK2* mutations cause X-linked Coffin- Lowry syndrome, which includes mental and growth retardation and skeletal and facial anomalies, but rare cardiac abnormality. In the heart, RSK1 and RSK2 can activate the Na^+/H^+ exchanger NHE1, and α-adrenergic-induced NHE1 phosphorylation is blocked by fmk, which inhibits all RSKs except RSK3 (Cuello 2007). The inventors now reveal a role for RSK3 in the cardiovascular system, regulation of pathological myocyte hypertrophy.

[00172] Cardiac myocytes can grow in both width and length, termed concentric and eccentric hypertrophy, respectively (Kehat 2010). Concentric myocyte hypertrophy involves the parallel assembly of contractile units (sarcomeres), increasing potential myocyte tension and wall thickness. In contrast, eccentric myocyte hypertrophy involves the serial assembly of sarcomeres along the axis of contraction, mainly contributing to increased ventricular wall area. The inventors found that RSK3 was required for TAC-induced concentric hypertrophy, as well as for Iso-induced myocyte growth in width in vivo. These differences can be modeled in vitro. Interleukin-6 cytokines such as leukemia inhibitory factor and cardiotrophin-1 induce an elongated and eccentric phenotype for cultured neonatal myocytes, in contrast to the symmetric growth stimulated by PE (Wollert 1996). Interestingly, the growth of the cultured myocytes tended to depend on RSK3 more when induced by α-adrenergic stimulation than by leukemia inhibitory factor (Figures 5 and 6 of U.S. Patent No. 9,132,174). The greater inhibition of PE-induced morphologic hypertrophy was consistent with the more robust

activation of RSK by PE than leukemia inhibitory factor (Figure 22 of U.S. Patent No. 9,132,174), as well as the results obtained in vivo.

[00173] RSK3 was activated in myocytes by ERK1, ERK2, and ERK5 (Figure 5A of U.S. Patent No. 9,132,174). Whereas RSK3 has been absent from the cardiac literature, ERK signaling has been well-studied both in human disease and in animal models. The autosomal-dominant human syndromes Noonan, Costello, cardiofaciocutaneous, and LEOPARD result from mutations in *PTPN11, HRAS, RAF1*, *BRAF*, *MEK1*, and *MEK2* that activate ERK1/2 signaling (Wu 2011). These Rasopathies feature developmental delay, dysmorphic features, and defects in multiple organ systems, often including a hypertrophic phenotype Gelb 2011). In mice, left ventricular hypertrophy has been induced by cardiac myocyte–specific expression of constitutively active H-Ras and MEK1, as well as cardiac-specific deletion of the Ras GTPase-activating protein neurofibromin that inhibits Ras signaling (Rose 2010, Xu 2009). Conversely, transgenic expression of dominant-negative Raf1 inhibited the hypertrophy due to pressure overload.

[00174] Recently, investigators have shown that cardiac myocyte–specific knockout of all 4 ERK1/2 alleles resulted in a severe, fatal dilated cardiomyopathy without increased myocyte death (Kehat 2011). ERK1/2-null myocytes were longer and narrower than those from control animals. *PTPN11* (Shp2) knockout that decreased ERK1/2 activation also resulted in an elongated myocyte morphology and dilated cardiomyopathy (Kontaridis 2008).

[00175] Conversely, myocytes from constitutively active MEK1 transgenic mice were shorter and wider (Kehat 2011). In contrast to the ERK1/2 and Shp2 knockout mice, the inventors found that deletion of the down-stream effector RSK3 resulted in a milder phenotype, with the defect in concentric growth significant only after TAC and Iso infusion. Together, these observations are consistent with the hypothesis that ERK1/2 signaling through RSK3 promotes stress-induced

concentric growth of cardiac myocytes independently of other signaling pathways that regulate eccentric hypertrophy.

[00176] The inventors found that RSK3 was activated in myocytes by not only ERK1/2 but also ERK5. There is evidence that MEK5- ERK5 signaling primarily induces eccentric myocyte hypertrophy (Nicol 2001), although ERK5 also may contribute to concentric growth (Kimura 2010).

[00177] The data obtained using the RSK knockout mouse establish a function for RSK3 in pathological remodeling. Without being bound by any particular theory, it is also possible that RSK3 has a role in physiologic hypertrophy. For example, the myocytes isolated from unstressed *RSK3*$^{-/-}$ mice tended to be smaller in both width and length (Figures 9 and 29 of U.S. Patent No. 9,132,174). In addition, after swimming, *RSK3*$^{-/-}$ biventricular weight was less than that of wild-type mice, albeit not significantly after normalization by body weight (Figure 18 of U.S. Patent No. 9,132,174).

[00178] It is remarkable that even though RSK3 constitutes a minority of the total RSK in the myocyte (Figures 24 and 30 of U.S. Patent No. 9,132,174), RSK3 activity is, nevertheless, required for myocyte growth. The differential anchoring of RSK3 by scaffold proteins provides a mechanism by which RSK3 may specifically function in vivo. Scaffolds are likely to be most important for enzymes such as RSK3 that are low in abundance and that have broad intrinsic substrate specificity. RSK protein kinases catalyze the phosphorylation of RxRxx(S/T) sites and overlap in specificity with other AGC kinases (Anjum 2008). By co-localizing enzymes, their upstream activators, and substrate effectors, scaffolds can accelerate the kinetics of signaling, amplify responses, increase specificity in enzyme catalysis, and direct signaling to specific subcellular compartments (Good 2011). The prior art provides limited guidance with respect to RSK compartmentation in cells or participation in multi-molecular signaling complexes. On mitogen stimulation, cytosolic RSK1 (and potentially other RSK isoenzymes) can transiently translocate to the plasma membrane, whereas activated RSK tends to be enriched in the

nucleus (Anjum 2008). In neurons, RSKs bind PDZ domain–containing proteins via their conserved C-terminal STxL peptides, directing the kinases to substrates involved in synaptic transmission (Thomas 2005). By another mechanism, RSK1 binds type 1 protein kinase A and D-AKAP-1, a mitochondrion-localized scaffold (Chaturvedi 2006, Huang 1997). Consistent with the fact that the inventors can only detect RSK3 in myocytes after immunoprecipitation, the inventors have not been able to detect endogenous RSK3 protein by immunocytochemistry. When overexpressed at a low level, HA-RSK3 was enriched at the nuclear envelope, the predominant location for mAKAPβ in the cardiac myocyte (Pare 2005). By characterizing in detail the protein–protein interaction between the unique RSK3 N terminus and mAKAPβ, the inventors have identified a new mechanism by which RSK3 can be specifically anchored by ≥ 1 scaffolds that may be targeted to different signaling compartments.

[00179] The inventors demonstrated the functional significance of this RSK3 anchoring using a competing binding peptide (mAKAP RBD) that inhibited myocyte hypertrophy.

[00180] The regulation of NHE1 by RSK1/2 has spurred recent interest in using RSK inhibitors to treat heart disease (Avkiram 2008). The inventors show that RSK3 knockout reduced TAC-induced hypertrophy without diminishing cardiac function and while inhibiting the expression of genetic markers for pathological remodeling. RSK inhibition may have multiple applications, including its use in acquired diseases such as hypertension (pressure overload) and for the treatment of the aforementioned Rasopathies. Recently, a Noonan syndrome mouse model (Raf1L^{613}V knock-in) mouse was treated with PD0325901, resulting in the attenuated progression of cardiac hypertrophy cardiomyopathy and other Noonan characteristics (Wu 2011). Targeting of RSK3 offers an alternative approach to avoid some of the harmful side effects of global ERK pathway inhibition. The use of RSK3 inhibitors that either competitively bind the active site or disrupt anchoring are offered as novel cardiac therapies.

[00181] **Example 2**

[00182] Remodeling of the extracellular matrix and the induction of myocardial interstitial fibrosis is an important factor contributing to the development of heart failure in cardiac disease (Spinale 2013, Edgley 2012). Increased deposition of fibrillar collagen and disruption of the normal cellular architecture of the myocardium can result in decreased compliance and both diastolic and systolic dysfunction, as well as arrhythmia due to interference with the electrical conduction system. p90 ribosomal S6 kinases (RSK) are pleiotropic protein kinases that are activated in myocytes in response to many stress-related stimuli (Anjum 2008, Sadoshima 2005, Kodama 2000). The inventors have shown that type 3 RSK (RSK3) is required for the induction of concentric myocyte hypertrophy in mice subjected to pressure overload (Li 2013). Activated by sequential phosphorylation by extracellular signal-regulated kinases (ERKs) and 3'- phosphoinositide-dependent kinase 1, RSK3 is one of four RSK family members expressed in the heart (Anjum 2008). Remarkably, even though RSK3 comprises a minority of RSK enzyme in cardiac myocytes, RSK3 is required for hypertrophy (Li 2013). Due to its role in pathological hypertrophy, the inventors have suggested that RSK3 targeting might be beneficial in the prevention of heart failure. To our knowledge, however, the prior art is deficient in showing whether RSK family members also regulate cardiac fibrosis. In this study, the inventors now show a role for RSK3 in interstitial fibrosis that is independent of its function in hypertrophic signal transduction.

[00183] Hypertrophic cardiomyopathy (HCM) is the most commonly inherited heart defect (1 in 500 individuals) and the leading cause of sudden death in children, accounting for 36% of sudden deaths in young athletes (Maron 2013). HCM is caused by dominant mutations in sarcomeric proteins that typically induce myocyte hypertrophy and disarray and interstitial fibrosis. However, the phenotype and clinical course resulting from HCM mutations can vary such that genotype-positive

patients without left ventricular hypertrophy can display myocardial fibrosis, diastolic dysfunction, and ECG abnormalities (Maron 2013). Studies using transgenic mice also indicate that the phenotype of HCM mutations depends upon genetic background (Prabhakar 2001, Michele 2002). As described below, expression of the HCM mutation Glu180Gly amino acid substitution of the thin filament protein α-tropomyosin (TM180) in mice of a mixed C57BL/6;FVB/N background results in a small left ventricle with interstitial fibrosis. The inventors show that RSK3 is required in this non-hypertrophic HCM model for the development of interstitial fibrosis and the signs of left-sided heart failure.

[00184] Methods

[00185] SUPPLEMENTAL MATERIAL

[00186] *Reagents:* Primary antibodies included mouse 1F6 monoclonal anti-RSK3 (Abnova, cat# H00006196-M01) that detects all RSK family members (Li 2013), OR43 rabbit anti RSK3, and N-16 goat anti-RSK3 (Santa Cruz Biotechnology). Secondary antibodies included horseradish peroxidase (HRP)-conjugated donkey secondary antibodies (Jackson ImmunoResearch). RSK3 immunoprecipitation was performed as previously described (Li 2013).

[00187] *Mice:* All experiments involving animals were approved by the Institutional Animal Care and Use Committee at the University of Miami. The *RSK3*$^{-/-}$ C57BL/6 mouse was mated to the TM180 transgenic FVB/N mouse (Li 2013, Prahakar 2001). All mice studied were littermates from *RSK3*$^{-/+}$ X TM180; *RSK3*$^{-/+}$ breedings, such that the background strain was 50:50 C57BL/6;FVB/N. All four genotypes were present in typical Mendelian proportion. Unless otherwise specifies, all experiments were performed with mice that were 16 weeks of age. Genotyping was performed at weaning by PCR using tail biopsy samples as previously described (Li 2013, Prahakar 2001). *Echocardiography:* A Vevo 770TM High-Resolution In Vivo Imaging System (VisualSonics) with a RMVTM 707B "High Frame" Scan-head was used for imaging. Mice were anesthetized with 1.5%

isoflurane for both B-mode and M-mode imaging.

[00188] *Histochemistry:* Heart tissue was fixed in 3.7% formaldehyde. De-paraffinized 5 μm tissue sections were stained using the Picrosirius Red Stain Kit (Polysciences) and Alexa Fluor 555 Wheat Germ Agglutinin conjugate (Invitrogen) as recommended by the manufacturers. The cross-section area of >150 myocytes in >3 distinct regions of the left ventricle were measured per heart using the wheat germ agglutinin sections. Collagen content was assayed using the Picrosirius Red stained sections and linearly polarized light microscopy for >3 4x objective fields per heart. Note that while linearly polarized light microscopy is a highly specific assay for fibrillar collagen, the values obtained are an underestimate of total collagen content. TUNEL staining for both fixed cells and tissue sections was performed using the In Situ Cell Death Detection Kit, TMR red (Roche). Morphometrics and collagen content were measured using IPLab microscope software (BD Biosciences). All analyses were performed by a blinded investigator.

[00189] *RNA Assay:* Total RNA was quantified with a Nanodrop 8000 Spectrophotometer (ThermoScientific) and quality controlled using with a Bioanalyzer 2100 and the RNA 6000 Nano kit(Agilent). The NanoString assay is based on direct, multiplexed measurement of gene expression without amplification, utilizing fluorescent molecular barcodes and single molecule imaging to identify and count multiple transcripts in a single reaction. Briefly, 100 ng total RNA were hybridized in solution to a target-specific codeset overnight at 65°C. The codeset contained dual, adjacently placed 50 bp oligonucleotide probes against the entire panel of genes, one set of probes fluorescently bar-coded and the other biotinylated. The hybridization reactions were loaded onto the NanoString Prep station which removes excess oligonucleotides and binds the hybridized mRNA to the Streptavidin-coated cartridge surface. The cartridges were loaded onto the NanoString Digital Analyzer, and 1150 fields of view were fluorescently scanned to count only those individual mRNAs bound to both a

biotinylated and fluorescently bar-coded probe. Datasets for each RNA sample were normalized to internal positive controls and background-subtracted. Probe sequences are available upon request.

[00190] *Statistics:* For all experiments, *n* refers to the number of individual mice. All data are expressed as mean ± s.e.m. *p*-values were calculated using two-tailed Student's t-tests, paired or un-paired as appropriate, and are not corrected for multiple comparisons. Repeated symbols represent p-values of different orders of magnitude: * $p<0.05$, ** $p<0.005$, *** $p < 0.0005$.

[00191] Results

[00192] FVB/N TM180 transgenic mice were crossed with C57BL/6 *RSK3* knock-out mice such that all mice were of a mixed 50:50 background. RSK3 expression was slightly higher (~25%, $p = 0.12$) in the TM180 mice, while absent in $RSK3^{-/-}$ mice, with no evidence of compensatory changes in the expression of other RSK family members (Figure 32 of U.S. Patent No. 9,132,174). Expression of the TM180 transgene was evident by the expected change in α- tropomyosin bands detected by total protein stain (Prabhakar 2001). In these mice of mixed lineage, the TM180 transgene induced a small heart phenotype that included a reduced biventricular weight (21%) and left ventricular myocytes with a proportionally smaller cross-section area (Figures 32 and 34 of U.S. Patent No. 9,132,174). By echocardiography, the TM180 mice had reduced left ventricular internal dimensions, but increased contractility, i.e., both increased fractional shortening on M-mode and increased endocardial fractional area shortening on B-mode (Figures 31 and 36 of U.S. Patent No. 9,132,174). That the changes in the TM180 left ventricle were pathologically important were implied by both an increased atrial weight and a significant, albeit small (12%) increase in wet lung weight, consistent with the presence of mild pulmonary edema and left-sided heart failure (Figures 32D and 34 of U.S. Patent No. 9,132,174).

[00193] *RSK3* knock-out had little effect on the heart in the absence of the TM180 transgene. While *RSK3* knock-out did not reverse the small heart phenotype of the TM180 mouse nor prevent the

atrial enlargement (Figures 32B,C and Figure 34 of U.S. Patent No. 9,132,174), the cardiac function of TM180; *RSK3*$^{-/-}$ mice was more like wildtype mice, including a 29% lesser decrease in short axis dimension by echocardiography (Figures 31 and 36 of U.S. Patent No. 9,132,174). Notably, the increase in fractional shortening and endocardial fractional area shortening due to the TM180 transgene were both attenuated by ~50% by *RSK* knock-out. That the more "normal" cardiac function of the TM180; *RSK3*$^{-/-}$ mice was physiologically important was implied by the observation that wet lung weight was no longer increased following *RSK3* knock-out (Figure 32D and Figure 34 of U.S. Patent No. 9,132,174).

[00194] There was no increase in cellular death associated with the TM180 transgene at 16 weeks of age (~10^{-4} TUNEL-positive nuclei for all cohorts, data not shown). However, trichrome staining of the TM180 hearts revealed a patchy interstitial fibrosis in the myocardium not present in wildtype mice that was greatly reduced in the absence of RSK3 (Figure 33A,B of U.S. Patent No. 9,132,174). Likewise, picrosirius red staining showed that fibrillar collagen content was increased by the TM180 transgene only in the presence of RSK3 (Figure 33C of U.S. Patent No. 9,132,174). These results were corroborated by assay of the expression of genes involved in cardiac function and remodeling (Figure 35 of U.S. Patent No. 9,132,174). Notably, genes involved in cardiac fibrosis, including *Col8a1* and *Postn*, (Oka 2007) encoding collagen type α1 and periostin, respectively , were induced by the TM180 transgene in a RSK3-dependent manner (Figure 33D,E of U.S. Patent No. 9,132,174).

[00195] **Discussion**

[00196] When expressed in FVB/N mice, the HCM TM180 mutation results in concentric left ventricular hypertrophy, extensive fibrosis, atrial enlargement, and death within 5 months (Prabhakar 2001). In contrast, expression of the TM180 mutation in C57BL/6 mice resulted in no ventricular

hypertrophy or fibrosis and a lower heart weight (Michele 2002). The inventors found that in a mixed C57BL/6;FVB/N background, the TM180 transgene induced an intermediate phenotype, including decreased ventricular and increased atrial weights, smaller ventricular myocytes, interstitial fibrosis, and increased contractility by echocardiography. The TM180 mutation is thought to induce cardiomyopathy as a result of the increased Ca^{2+} sensitivity and increased maximum tension generation of TM180 filaments (Prabhakar 2001). Hence, increasing Ca^{2+} reuptake through manipulation of phospholamban and the sarco/endoplasmic reticulum Ca^{2+}-ATPase 2A (SERCA2A) can ameliorate the TM180 FVB/N phenotype (Gaffin 2011). The inventors have utilized the TM180 transgenic mouse to investigate the role of RSK3 in cardiac fibrosis. While the inventors and others have found that total heart ERK1/2 is activated in TM180 FVB/N mice (Gaffin 2011), in the mixed background mice, total ERK1/2 and RSK phosphorylation was not increased. Instead, the inventors only noted a slight increase in RSK3 protein levels (Figure 32A of U.S. Patent No. 9,132,174). Importantly, *RSK3* knock-out blocked the TM180 associated induction of fibrotic gene expression and interstitial fibrosis, as well as improving cardiac function both in terms of echocardiographic findings and wet lung weight. These findings complement our previous observation that RSK3 is specifically required for pathological cardiac hypertrophy. Without being bound by a particular theory, the inventors suggest that due to RSK3 anchoring through its unique N-terminal domain to scaffold proteins such as mAKAP (muscle A- kinase anchoring protein), RSK3 serves a unique function in the heart, despite the higher level of expression of other RSK isoenzymes (Li 2013). There are reports that RSK phosphorylation of CEBP/β is involved in pathological fibrosis of the liver and lung, but there is no published data relating to RSK family members in the heart. The inventors suggest that specific RSK3 inhibition should now be considered more broadly as a therapeutic target both in hypertrophic and fibrotic heart diseases.

[00197] **Example 3**

[00198] ***mAKAP* gene structure and the strategy for a conditional *mAKAP* allele.** The *mAKAP* gene contains 12 common (light blue) and 3 alternatively-spliced exons (beige and yellow). A targeting vector containing negative (tk) and positive (neo) selectable markers was designed to conditionally delete the common Exon 9. The inventors obtained 6 targeted ES cell clones as shown by Southern blots. After breeding of targeted mice, the neo cassette was deleted by mating to a FLP recombinase transgenic. Mating to a mouse expressing cre recombinase will result in the deletion of Exon 9 (KO allele), producing a frame shift and introduction of a stop codon (red) in Exon 10. Mouse genotyping is being performed by PCR of genomic DNA with primers 44 and 45. (Fig. 39 of U.S. Patent No. 9,132,174). For the western blot: *mAKAP Ex9*$^{fl/fl}$;Tg(Myh6-cre/Esr1) mice (lanes 2 and 3) and *mAKAP Ex9*$^{fl/fl}$ (lane 1) and Tg(Myh6-cre/Esr1) (lane 4) control mice were fed 500 mg tamoxifen/kg dry food for one week before the hearts were collected to prepare total RNA and protein extracts. RT-PCR was performed using primers located within *mAKAP* exons 4 and 11 which yield a 1022 bp for wildtype (and floxed) mRNA and a 901 bp product for a mAKAP mRNA species lacking exon 9. While control β-actin mRNA was similarly detected for all samples (bottom panel), >90% less PCR product was obtained for CKO mouse hearts (top panel, lanes 2 and 3) compared to that observed with the control hearts (lanes 1 and 4). Western blots were performed using VO54 mAKAP-specific antibody. No mAKAP protein was detectable for heart extracts prepared from CKO mice (lanes 2 and 3, top panel). Equal loading was determined by Ponceau total protein stain (bottom panel).

[00199] *mAKAP Ex9*$^{fl/fl}$;Tg(Myh6-cre/Esr1) mice (fl/fl;MCMTg) and Tg(Myh6-cre/Esr1) (MCM Tg) at 8 weeks of age were fed tamoxifen-containing chow for one week, rested for one week and then subjected for to 2 weeks of Transverse Aortic Constriction before analysis (Fig. 40 of U.S. Patent No. 9,132,174). mAKAP gene deletion inhibited the development of concentric hypertrophy in response to

pressure overload. Subsequent studies showed that mAKAP gene deletion prevented the development of heart failure in response to chronic pressure overload, inhbiting cardiac remodeling and providing a survival benefit (Kritzer 2014).

[00200] *Transverse Aortic Constriction:* All tools were sterilized with a Germinator 500 Dry Sterilizer and Betadine Solution (10% povidone-iodine topical solution). Anesthesia was induced with 5% isoflurane and maintained with 2% isoflurane and 100% oxygen at a flow rate of 1.5 L/min using a SurgiVet flow regulator via nose cone. Loss of consciousness was verified by toe pinch. Mouse fur over the left chest and sternum was removed with a calcium hydroxide lotion (e.g. Nair), and the surgical site was sterilized with betadine. The skin was incised exposing the pectoralis muscle and the second left intercostal space. The pectoralis muscle and the second rib were blunt dissected and retracted revealing the thymus within the mediastinum. The lobes of the thymus were retracted to reveal the transverse aortic arch as well as the right innominate and left common carotid arteries. Blunt dissection though the connective tissue between these two arteries and under the aorta allowed for the passage of a 6-0 silk using a modified ligation aid (Fine Science Tools 18062-12). A 27 gauge needle was placed on top of the aorta and the 6-0 silk was tied around the needle. The needle was removed, leaving a constricted aorta. The chest was closed in two layers with 5-0 Polysorb Suture. Isoflurane administration was terminated, and the mice were maintained on 100% oxygen by nose cone until conscious. Immediately post-operatively, buprenorphrine (0.05-0.1 mg/kg s.c.) was administered and then q12 h prn. The mice were allowed to recover under a heat lamp until alert and active. Sham-operated mice that experience all but the placement of the aortic ligature served as controls.

[00201] *Echocardiography:* Mice minimally anesthetized with 1-2% isoflurane were studied using a Vevo 770®, High-Resolution Imaging System (VisualSonics). The pressure gradient following TAC was calculated from the pulse wave Doppler velocity at the point of ligation as follows: $P = 4v^2$; P

= the induced pressure gradient (in mmHg) and v = the velocity across the constriction (in m/s). (Fig. 40 of U.S. Patent No. 9,132,174).

[00202] **Example 4**

[00203] **RSK3 anchoring is important for rat ventricular myocyte hypertrophy.**

[00204] Fig. 8A of U.S. Patent No. 9,132,174 shows mAKAPβ complexes were immunoprecipitated using FL100 mAKAP antiserum from PE-treated, adenovirus-infected neonatal rat ventricular myocytes expressing myc-GFP or myc-GFP-RBD (mAKAP 1694-1833) and detected with the pan-RSK 1F6 and mAKAP 211 antibodies. B. Transfected myocytes expressing GFP or GFP-RBD (green) were stained with α-actinin (blue) and ANF (red) antibodies. Bar = 20 μm. C. Cross-section area of myocytes. n=5. D. Fraction of myocytes expressing ANF. n=3. * p-values comparing to GFP-expressing samples. † p-values comparing to no agonist control.

[00205] *Neonatal rat myocytes isolation and culture:* 1-3 day old Sprague-Dawley rats were decapitated and the excised hearts placed in 1x ADS Buffer (116 mmol/L NaCl, 20 mmol/L HEPES, 1 mmol/L NaH_2PO_4, 5.5 mmol/L glucose, 5.4 mmol/L KCl, 0.8 mmol/L $MgSO_4$, pH 7.35). The atria were carefully removed and the blood washed away. The ventricles were minced and incubated with 15 mL 1x ADS Buffer containing 3.3 mg type II collagenase (Worthington, 230 U/mg) and 9 mg Pancreatin (Sigma) at 37°C while shaking at 80 RPM. After 15 minutes, the dissociated cardiac myocytes were separated by centrifugation at 50 x g for 1 minute, resuspended in 4 mL horse serum and incubated 37°C with occasional agitation. The steps for enzymatic digestion and isolation of myocytes were repeated 10-12 times to maximize yield. The myocytes were pooled and spun down again at 50 x g for 2 minutes and resuspended in Maintenance Medium (DMEM:M199, 4:1) supplemented with 10% horse serum and 5% fetal bovine serum. To remove any contaminating

fibroblasts, the cells were pre-plated for 1 hour before plating on gelatin-coated tissue culture plasticware. This procedure yields >90% pure cardiac myocytes. After 1 day in culture, the media was changed to maintenance medium containing 0.1 mmol/L bromodeoxyuridine to suppress fibroblast growth.

[00206] Experiments were initiated 1 day after myocyte isolation. Adenoviral infection was performed by addition of adenovirus (multiplicity of infection = 5-50) to the media. Plasmids and siRNA oligonucleotides were transfected using Transfast (Promega) and Dharmafect (Thermofisher), respectively, as recommended by the manufacturers using cells cultured in maintenance medium supplemented with 4% horse serum. Starting the day after gene transduction, the cells were treated for as long as 2 days, as indicated for each experiment.

[00207] *Immunocytochemistry*: Cultured neonatal cardiomyocytes on plastic coverslips were fixed in 3.7% formaldehyde in PBS, permeabilized with 0.3% Triton X-100 in PBS, and blocked with PBS containing 0.2% BSA and 1% horse serum for 1 hour. The slides were then sequentially incubated for 1 hour with primary and Alexa fluorescent dye-conjugated specific-secondary antibodies (Invitrogen, 1:1000) diluted in blocking buffer. The slips were washed three times with blocking buffer. 1 μg/mL Hoechst 33258 was included in the last wash stop to label nuclei. Slides were sealed in SlowFade Gold antifade buffer (Invitrogen) for fluorescent microscopy. Wide-field images were acquired using a Leica DMI 6000 Microscope.

[00208] AAV9 containing the indicated CIS plasmid encoding myc-GFP-mAKAP 1694-1833 were injected into neonatal wildtype mice. 2 week TAC was performed and analyzed as above at 8 weeks of age. (Figs. 41-42 of U.S. Patent No. 9,132,174). Expression of myc-GFP-mAKAP 1694-1833 inhibited concentric hypertrophy in vivo in the adult heart.

[00209] **Example 5**

[00210] The present invention provides a novel biologic agent for mAKAP RNAi in the cardiac myocyte *in vivo* useful in human patients.

[00211] mAKAPβ signalosomes have been studied using a variety of technologies, including (1) the expression of anchoring disruptor peptides that inhibit signaling enzyme association with the scaffold (Kapiloff 2009, Dodge-Kafka 2010, Vargas 2012, Li 2013, Li 2013), (2) by mAKAP (AKAP6) gene knock-out (Kritzer 2014), and (3) by RNA interference (RNAi) of mAKAP expression using both small interfering RNA oligonucleotides (siRNA) or viral and plasmid vector-expressed small hairpin RNA (shRNA) (Pare 2005, Wong 2008).

[00212] A sequence previously disclosed that was targeted by siRNA and shRNA for mAKAP is rat specific and not present in the human gene:

Sequence	NCBI gene	Initial Publication
GACGAACCTTCCTTCCGAA (SEQ ID NO:10)	NCBI Reference Sequence: XM_017594342.1 rat AKAP6 base pairs 7483-7501	(Pare 2005)

[00213] In order to generate a biologic that would target mAKAP expression in humans, three 19 base pair sequences were chosen from NCBI reference sequence XM_017021808.1 for human AKAP6 transcript variant X1 mRNA (Fig. 8):

Seq #	Sequence	Position in Reference Sequence (base pairs)
1	GGTTGAAGCTTTGAAGAAA (SEQ ID NO:7)	3094-3112
2	GCTAAGAGATACAGAGCTT (SEQ ID NO:8)	3316-3334
3	GGAGGAAATAGCAAGGTTA (SEQ ID NO:9)	7807-7825

[00214] These sequences were incorporated into novel, custom-designed shuttle plasmids for self-complementary adeno-associated virus (AAV) that would confer cardiac myocyte-specific shRNA expression in vivo. The "pscA-TnT-mAKAP shRNA" plasmids were designed as follows (Figs. 9 and 10):

1) A cDNA cassette was generated using human mir-30a sequences to confer efficient shRNA expression. The construct was similar, but not identical to a previously published cassette (Silva 2005): 128 bp of human mir30a 5' sequence with 2 mismatches – 2 bp (CG) - AKAP6 sequence in sense orientation – 19 bp loop sequence - AKAP6 sequence in antisense orientation – 2 bp (CT) - 130 bp of human mir30 sequence with 1 mismatch.
2) The cDNA cassette was located 3' to a fragment of the chicken cardiac troponin T promoter that confers cardiac myocyte specific expression (Prasad 2011) and 5' to a SV40-derived polyadenylation sequence (SV40 genome bp 2599-2769).
3) The entire shRNA minigene was flanked by AAV2 ITR sequence (NC_001401.2 bp 4489-4664 in antisense orientation on the 5' end and bp 4559-4662 on the 3' end) in order to direct production of a self-complementary AAV (scAAV) biologic (Wang 2003).

[00215] Testing in cultured rat neonatal rat ventricular myocytes revealed that transfection with these plasmids resulted in decreased expression of mAKAPβ to varying degrees, with the plasmid for target #3 being most effective at removing mAKAPβ (data not shown). Therefore, pscA-TnT-mAKAP shRNA #3 was used to generate scAAV particles with the cardiotropic serotype 9 capsid protein for in vivo experimentation. The scAAV were generated by the University of Pennsylvania Vector Core with support provided by the National Heart, Lung, and Blood Institute Gene Therapy Resource Program. The scAAV virus were tested by tail vein injection into adult mice. Three weeks after injection, hearts were collected and analyzed by western blot. mAKAPβ expression was repressed >90% by a single intravenous (IV) dose of $5x10^{11}$ viral genomes (vg) of scAAV-mAKAP shRNA #3 when compared to mice injected with a scAAV control shRNA virus or non-injected controls (Fig. 11).

[00216] **Example 6**

[00217] Coronary heart disease is a leading cause of heart failure (Writing Group 2016), and an

ability to block heart failure in ischemic disease by single dose scAAV-mAKAP shRNA is desirable. In mice, left coronary artery ligation results in a large, scarred transmural infarct (sparing the ventricular septum) accompanied by ventricular dilatation and remodeling of the remaining myocardium (Kumar 2005). 8 weeks post-MI, control mice are expected to be in heart failure with low ejection fraction and cardiac output (systolic dysfunction), atrial hypertrophy, and pulmonary edema (increased wet lung weight).

[00218] 8-week old C57BL/6 WT mice were subjected to permanent left coronary artery ligation or sham thoracotomy and studied by serial echocardiography until euthanized 8 weeks post-survival surgery (Figure 12A), using the following methods:

[00219] *Method for Ligation of the Left Coronary Artery:* Mice were anesthetized with 5% isoflurane for induction and then 2.5-3% for maintenance. Orotracheal intubation was performed using a 16G catheter, and the mouse was then ventilated mechanically using a minivent ventilator. The skin over the site of left lateral thoracotomy was prepped and draped in sterile fashion using providone-iodine 10% solution. A heating pad was used to keep mice warm during procedures to prevent heat loss. Surgically sterile non-medicated ophthalmic ointment was applied to the eyes preoperatively to prevent corneal drying. Survival surgery was performed under microscope view. Once adequate sedation was achieved, the chest was opened via left lateral thoracotomy at the fourth intercostal space. If muscle bleeding was present, hemostasis was achieved by the using a thermal cauterizer (e.g. fine tip Bovie). A 3 mm retractor was used to separate the ribs. Following pericardiotomy, the left coronary artery was ligated with a 8-0 prolene suture to produce an anterior MI. The chest was closed in 3 layers with 5-0 absorbable suture (muscle), 7-0 (for 2 ligatures in the ribs) and 6-0 for the skin. Buprenorphine slow release (Bup-SR-LAB) 0.5-1 mg/kg s.c. was administered in a single dose immediately after surgery to control pain for 72 hr. Fluid replacement was also administered as needed

immediately after surgery (e.g., sterile saline solution 0.9%, IP). The mice were allowed to recover until alert and active. Sham-operated mice that experience all but the placement of the coronary artery ligature serve as controls.

[00220] *Echocardiography:* Mice minimally anesthetized with 1-2% isoflurane were studied using a Vevo 2100®, High-Resolution Imaging System (VisualSonics). B- and M-mode images were obtained for mice under anesthesia at various time-points. Posterior wall and anterior wall diastolic and systolic thicknesses and left ventricular cavity end-diastolic (LVEDD) and end-systolic diameters (LVESD) were measured, permitting estimation of LV volumes, fractional shortening and ejection fraction.

[00221] Upon confirmation of reduced ejection fraction (EF $\leq$40%) 2 days after Ligation of the Left Coronary Artery survival surgery (MI cohorts), mice were randomized and injected the following day with $5x10^{11}$ vg I.V. of either scAAV-mAKAP-shRNA #3 or scAAV-control-shRNA. The average EF for the MI cohorts before treatment was 31.66% and 32.79% for mAKAP and control shRNA cohorts, respectively. After scAAV treatment, cohorts were as follows: mAKAP-shRNA-MI (n=10), control-shRNA-MI (n=6), mAKAP-shRNA-Sham (n=4), control-shRNA-Sham (n=5). Administration of mAKAP-shRNA post-MI resulted in amelioration of systolic dysfunction as evidenced by normalization of EF from day 2 to weeks 2, 4 and 8 post-MI, while control-shRNA-MI animals displayed a progressive deterioration of EF after ischemic injury (Figure 12B). At 8 weeks post MI, EF in mAKAP-shRNA-MI was not significant different from the mAKAP- and control-shRNA-Sham animals (44.3±3.3 % vs. 54.6±1.7 % & 54.5±2.0 %), while it was higher than in control-shRNA-MI animals (18.0±4.3%, P<0.0001). In addition, 8 weeks after coronary artery ligation, mAKAP-shRNA-MI mice displayed thicker left ventricular (LV) anterior wall in systole (0.9±0.1 mm vs. 0.5±0.1 mm for control-shRNA-MI, P<0.05) and smaller LV end-systolic-volumes, (72.1±8.6 μL vs. 137.3±26.3μL

for control-shRNA-MI; P<0.01) than control-shRNA-MI mice. Consequently, post-mortem analysis of pulmonary edema showed that treatment with scAAV-mAKAP-shRNA #3 attenuated the development of heart failure (Figure 13), such that wet lung weight for mAKAP-shRNA-MI mice was significantly less than for the control-shRNA-MI cohort (7.7±0.2 vs. 10.6±1.2 mg/mm tibial length, P<0.01).

[00222] These results show that inhibition of mAKAPβ expression using the new scAAV-mAKAP shRNA #3 biologic drug as a treatment after myocardial infarction ameliorates cardiac dysfunction, preserving cardiac structure and preventing the development of heart failure in mice.

[00223] The patent and scientific literature referred to herein establishes the knowledge that is available to those with skill in the art. All United States patents and published or unpublished United States patent applications cited herein are incorporated by reference. All published foreign patents and patent applications cited herein are hereby incorporated by reference. All other published references, documents, manuscripts and scientific literature cited herein are hereby incorporated by reference and full citations can be found in U.S. Patent No. 9,132,174. While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims.

[00224] Throughout this specification and the claims that follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

[00225] The reference in this specification to any prior publication (or information derived from it), or to any matter which is known, is not, and should not be taken as an acknowledgment or admission or any form of suggestion that that prior publication (or information derived from it) or known matter forms part of the common general knowledge in the field of endeavour to which this specification relates.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A composition comprising a vector encoding a small hairpin ribonucleic acid ("shRNA") against human mAKAP, and wherein the small hairpin ribonucleic acid comprises the following nucleotide sequence: GGAGGAAATAGCAAGGTTA (SEQ ID NO: 9).
2. A composition comprising a vector encoding a small hairpin ribonucleic acid ("shRNA") against human mAKAP, and wherein the small hairpin ribonucleic acid consists of the following nucleotide sequence: GGAGGAAATAGCAAGGTTA (SEQ ID NO: 9).
3. The composition of claim 1 or claim 2, wherein the vector is a viral vector.
4. The composition of claim 3, wherein the viral vector is an adeno-associated virus vector (AAV).
5. The composition of claim 3, wherein the viral vector comprises human microRNA (miR)-30a sequences.
6. The composition of claim 3, wherein the viral vector comprises a shRNA minigene, wherein the minigene is flanked by a complete AAV2 inverted terminal repeat (ITR) on the 5' end and a deleted AAV2 ITR on the 3' end.
7. The composition of claim 3, wherein the viral vector comprises a chicken cardiac troponin T promoter.
8. The composition of claim 3, wherein the viral vector comprises a SV40 polyadenylation site.
9. The composition of claim 4, wherein the AAV is an AAV serotype 9.
10. A pharmaceutical comprising an amount of the composition of any one of claims 1 to 9, which is therapeutically effective when administered to a patient in need to protect the patient from heart damage.

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11. A method of inhibiting the expression of human mAKAPβ, comprising contacting mRNA encoding mAKAPβ with the composition of any one of claims 1 to 9, or the pharmaceutical composition of claim 10.

12. A method of protecting the heart from damage, comprising administering to a patient at risk of such damage, a pharmaceutically effective amount of the composition of any one of claims 1 to 9, or the pharmaceutical composition of claim 10.

13. A method of treating or preventing heart disease, comprising administering to a patient in need thereof, a pharmaceutically effective amount of the composition of any one of claims 1 to 9, or the pharmaceutical composition of claim 10.

14. A method of treating or preventing myocardial infarction, comprising administering to a patient at risk of such damage, a pharmaceutically effective amount of the composition of any one of claims 1 to 9, or the pharmaceutical composition of claim 10.

15. Use of the composition of any one of claims 1 to 9, or the pharmaceutical composition of claim 10, in the manufacture of a medicament for inhibiting the expression of human mAKAPβ.

16. Use of the composition of any one of claims 1 to 9, or the pharmaceutical composition of claim 10, in the manufacture of a medicament for protecting the heart from damage.

17. Use of the composition of any one of claims 1 to 9, or the pharmaceutical composition of claim 10, in the manufacture of a medicament for treating or preventing heart disease.

18. Use of the composition of any one of claims 1 to 9, or the pharmaceutical composition of claim 10, in the manufacture of a medicament for treating or preventing myocardial infarction.

FIGURE 1

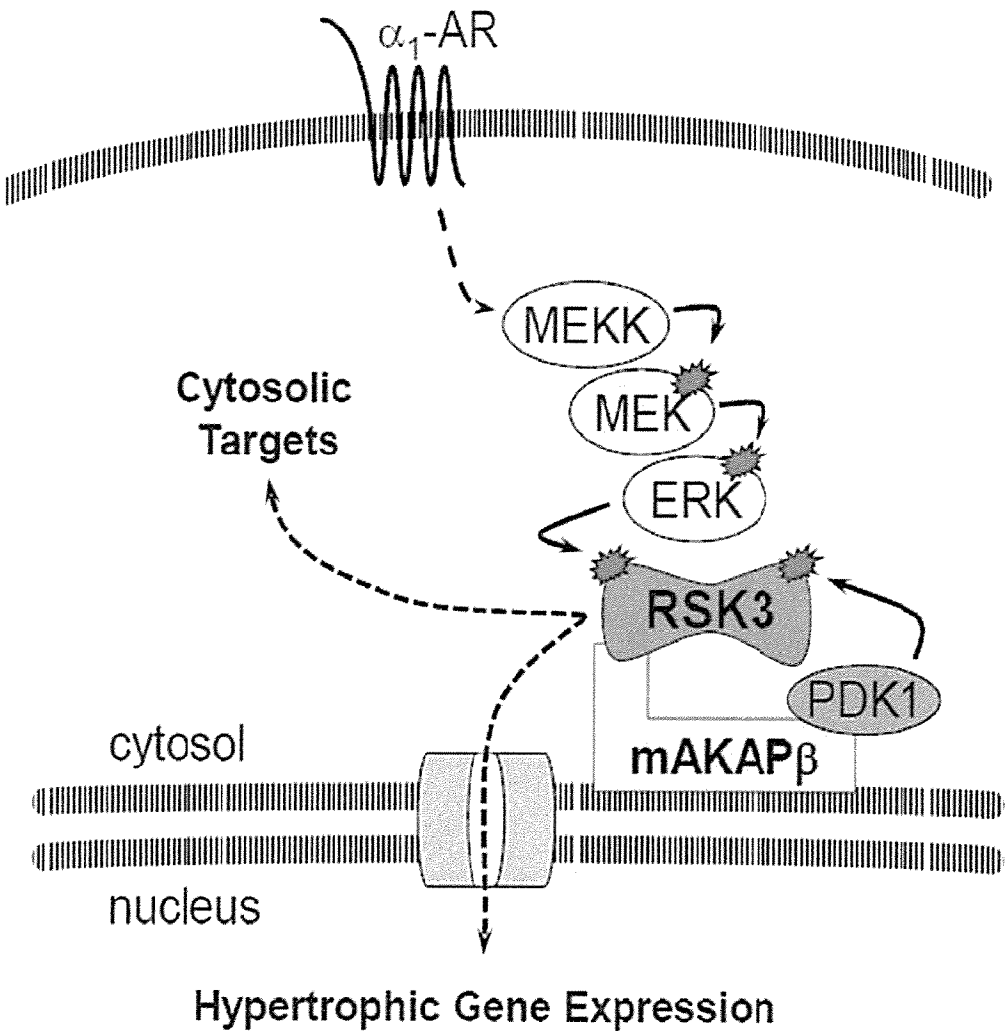
α1-AR
MEKK
MEK
ERK
Cytosolic Targets
RSK3
PDK1
mAKAPβ
cytosol
nucleus
Hypertrophic Gene Expression

FIGURE 2

>h-RSK3 1-42 in yellow
MDLSMKKFAVRRFFSVYLRRKSRSKSSSLSRLEEEGVVKEIDISHHVKEGFEKADPSQFELLKVLGQGSY
GKVFLVRKVKGSDAGQLYAMKVLKKATLKVRDRVRSKMERDILAEVNHPFIVKLHYAFQTEGKLYLILDF
LRGGDLFTRLSKEVMFTEEDVKFYLAELALALDHLHSLGIIYRDLKPENILLDEEGHIKITDFGLSKEAI
DHDKRAYSFCGTIEYMAPEVVNRRGHTQSADWWSFGVLMFEMLTGSLPFQGKDRKETMALILKAKLGMPQ
FLSGEAQSLLRALFKRNPCNRLGAGIDGVEEIKRHPFFVTIDWNTLYRKEIKPPFKPAVGRPEDTFHFDP
EFTARTPTDSPGVPPSANAHHLFRGFSFVASSLIQEPSQQDLHKVPVHPIVQQLHGNNIHFTDGYEIKED
IGVGSYSVCKRCVHKATDTEYAVKIIDKSKRDPSEEIEILLRYGQHPNIITLKDVYDDGKFVYLVMELMR
GGELLDRILRQRYFSEREASDVLCTITKTMDYLHSQGVVHRDLKPSNILYRDESGSPESIRVCDFGFAKQ
LRAGNGLLMTPCYTANFVAPEVLKRQGYDAACDIWSLGILLYTMLAGFTPFANGPDDTPEEILARIGSGK
YALSGGNWDSISDAAKDVVSKMLHVDPHQRLTAMQVLKHPWVVNREYLSPNQLSRQDVHLVKGAMAATYF
ALNRTPQAPRLEPVLSSNLAQRRGMKRLTSTRL

FIGURE 3

```
LOCUS       r-mAKAP\(rattus)         2314 aa                      30-DEC-
1999
ORIGIN
        1 mltmsvtlsp lrsqgpdpma tdaspmainm tptveqeege geeavkaida eqqygkpppl
       61 htaadwkivl hlpeietwlr mtservrdlt ysvqqdadsk hvdvhlvqlk dicedisdhv
      121 eqihallete fslkllsysv nvivdihavq llwhqlrvsv lvlrerilqg lqdangnytr
      181 qtdilqafse ettegrldsl tevddsgqlt ikcsqdylsl dcgitafels dyspsedllg
      241 glgdmttsqa ktksfdswsy semekefpel irsvglltva tepvpsscge anedssqasl
      301 sddhkgehge dgapvpgqql dstvgmssld gtlanaaehp setakqdsts spqlgakktq
      361 pgpceittpk rsirdcfnyn edsptqptlp krglflketq knerkgsdrk gqvvdlkpel
      421 srstpslvdp pdrsklclvl qssypsspsa asqsyeclhk vglgnleniv rshikeisss
      481 lgrltdchke klrlkkphkt laevslcrip kqgggsgkrs estgssagps mvspgapkat
      541 mrpetdsast asgglchqrn rsgqlpvqsk asssppcshs sesslgsdsi kspvpllskn
      601 ksqkssppap chatqngqvv eawygsdeyl alpshlkqte vlalkleslt kllpqkprge
      661 tiqdiddwel semnsdseiy ptyhikkkht rlgtvspsss sdiasslges iesgplsdil
      721 sdedlclpls svkkftdeks erpsssekne shsatrsali qklmhdiqhq enyeaiweri
      781 egfvnkldef iqwlneamet tenwtppkae tdslrlylet hlsfklnvds hcalkeavee
      841 eghqllelvv shkaglkdtl rmiasqwkel qrqikrqhsw ilraldtika eilatdvsve
      901 deegtgspka evqlchletq rdaveqmslk lyseqytsgs krkeefanms kahaegsngl
      961 ldfdseyqel wdwlidmesl vmdshdlmms eeqqqhlykr ysvemsirhl kksellskve
     1021 alkkgglslp ddilekvdsi nekwellgkt lrekiqdtia ghsgsgprdl lspesgslvr
     1081 qlevrikelk rwlrdtelfi fnsclrqeke gtsaekqlqy fkslcreikq rrrgvasilr
     1141 lcqhllddrd tcnlnadhqp mqliivnler rweaivmqav qwqtrlqkkm gkesetlnvi
     1201 dpglmdlngm sedalewdet disnklisvh eesndldqdp epmlpavkle ethhkdsgye
     1261 eeagdcggsp ytsnitapss phiyqvyslh nvelhedsht pflksspkft gttqptvltk
     1321 slskdssfss tkslpdllgg sglvrpysch sgdlsqnsgs esgivsegdn emptnsdmsl
     1381 fsmvdgspsn petehpdpqm gdaanvleqk fkdngesikl ssvsrasvsp vgcvngkagd
     1441 lnsvtkhtad clgeelqgkh dvftfydysy lqgsklklpm imkqpqseka hvedpllggf
     1501 yfdkksckak hqasesqpda ppherilasa phemgrsayk ssdiektftg iqsarqlsll
     1561 srsssvesls pggdlfglgi fkngsdslqr stsleswlts yksnedlfsc hssgdisvss
     1621 gsvgelskrt ldllnrleni qspseqkikr svsdmtlqss sqkmpfagqm sldvassine
     1681 dspasltels ssdelslcse divlhknkip esnasfrkrl nrsvadesdv nvsmivnvsc
     1741 tsactddedd sdllssstlt lteeelclkd edddssiatd deiyeesnlm sgldyiknel
     1801 qtwirpklsl trekkrsgvt deikvnkdgg gnekanpsdt ldieallngs irclsenngn
     1861 gktpprthgs gtkgenkkst ydvskdphva dmengniest pererekpqg lpevsenlas
     1921 nvktisesel seyeavmdgs edssvarkef cppndrhppq mgpklqhpen qsgdckpvqn
     1981 pcpgllseag vgsrqdsngl kslpndapsg arkpagccll eqneteesas issnasccnc
     2041 kpdvfhqkdd edcsvhdfvk eiidmastal ksksqpesev aaptsltqik ekvlehshrp
     2101 ihlrkgdfys ylslsshdsd cgevtnyide ksstplppda vdsglddked mdcffeacve
     2161 depvneeagl pgalpnesai edgaeqkseq ktasspvlsd ktdlvplsgl spqkgaddak
     2221 egddvshtsq gcaesteptt psgkanaegr srmqgvsatp eenaasakpk iqafslnakq
     2281 pkgkvamryp spqtltckek lvnfhedrhs nmhr
//
1694-1833 in yellow is RSK-binding domain
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```
                                                                        ooo
                                                     -174  CCCGGCGCGGCCTGCCCTTTG TGA CCGCAGCTCGCGCCCACGCCCCGCGCCCAT     -120
GGCCGCCGTGCCGGGCTCCCTGGCCACGCGTGCCCCCCCGCGGACCTGAGCCCCGCGCCTGGGATGCCGGGGATGCGCGTCCCCCGGCCCTGCGGCTGCTCCGGGCTGCCCGCGGCCG       -1
A ATG GAC CTG AGC ATG AAG AAG TTC GCC GTG CGC AGG TTC TTC TCT GTG TAC CTG CGC AGG AAG TCG CGC TCC AAG AGC TCC AGC CTG AGC    90
   M   D   L   S   M   K   K   F   A   V   R   R   F   F   S   V   Y   L   R   R   K   S   R   S   K   S   S   S   L   S    30
  CGG CTC GAG GAA GAA GGT GTC GTG AAG GAG ATA GAC ATC AGC CAT CAT GTG AAG GAG GGC TTT GAG AAG GCA GAT CCT TCC CAG TTT GAG   180
   R   L   E   E   E   G   V   V   K   E   I   D   I   S   H   H   V   K   E   G   F   E   K   A   D   P   S   Q   F   E    60
  CTG CTG AAG GTT TTA GGA CAA GGA TCC TAT GGA AAG GTG TTC CTG GTG AGG AAG GTG AAG GGG TCC GAC GCT GGG CAG CTC TAC GCC ATG   270
   L   L   K   V   L   G   Q   G   S   Y   G   K   V   F   L   V   R   K   V   K   G   S   D   A   G   Q   L   Y   A   M    90
  AAG GTC CTT AAG AAA GCC ACC CTA AAA GTT CGG GAC CGA GTG AGA TCG AAG ATG GAG AGA GAC ATC TTG GCA GAA GTG AAT CAC CCC TTC   360
   K   V   L   K   K   A   T   L   K   V   R   D   R   V   R   S   K   M   E   R   D   I   L   A   E   V   N   H   P   F   120
  ATT GTG AAG CTT CAT TAT GCC TTT CAG ACG GAA GGA AAG CTC TAC CTG ATC CTG GAC TTC CTG CGG GGA GGG GAC CTC TTC ACC CGG CTC   450
   I   V   K   L   H   Y   A   F   Q   T   E   G   K   L   Y   L   I   L   D   F   L   R   G   G   D   L   F   T   R   L   150
  TCC AAA GAG GTC ATG TTC ACG GAG GAG GAT GTC AAG TTC TAC CTG GCT GAG CTG GCC TTG GCT TTA GAC CAT CTC CAC AGC CTG GGG ATC   540
   S   K   E   V   M   F   T   E   E   D   V   K   F   Y   L   A   E   L   A   L   A   L   D   H   L   H   S   L   G   I   180
  ATC TAC AGA GAT CTG AAG CCT GAG AAC ATC CTC CTG GAT GAA GAG GGG CAC ATT AAG ATC ACA GAT TTC GGC CTG AGT AAG GAG GCC ATT   630
   I   Y   R   D   L   K   P   E   N   I   L   L   D   E   E   G   H   I   K   I   T   D   F   G   L   S   K   E   A   I   210
  GAC CAC GAC AAG AGA GCG TAC TCC TTC TGC GGG ACG ATC GAG TAC ATG GCA CCC GAG GTG GTG AAC CGG CGA GGA CAC ACG CAG AGT GCC   720
   D   H   D   K   R   A   Y   S   F   C   G   T   I   E   Y   M   A   P   E   V   V   N   R   R   G   H   T   Q   S   A   240
  GAC TGG TGG TCC TTC GGC GTG CTC ATG TTT GAG ATG CTC ACG GGG TCC CTG CCG TTC CAG GGG AAG GAC AGG AAG GAG ACC ATG GCT CTC   810
   D   W   W   S   F   G   V   L   M   F   E   M   L   T   G   S   L   P   F   Q   G   K   D   R   K   E   T   M   A   L   270
  ATC CTC AAA GCC AAG CTG GGG ATG CCG CAG TTC CTC AGT GGG GAG GCA CAG AGT TTG CTG CGA GCT CTC TTC AAA CGG AAC CCC TGC AAC   900
   I   L   K   A   K   L   G   M   P   Q   F   L   S   G   E   A   Q   S   L   L   R   A   L   F   K   R   N   P   C   N   300
  CGG CTG GGT GCT GGC ATT GAC GGA GTG GAG GAA ATT AAG CGC CAT CCC TTC TTT GTG ACC ATA GAC TGG AAC ACG CTG TAC CGG AAG GAG   990
   R   L   G   A   G   I   D   G   V   E   E   I   K   R   H   P   F   F   V   T   I   D   W   N   T   L   Y   R   K   E   330
  ATC AAG CCA CCG TTC AAA CCA GCA TTG GGC AGG CCT GAG GAC ACC TTC CAC TTT GAC CCC GAG TTC ACA GCG CGG ACG CCC ACA GAC TCT  1080
   I   K   P   P   F   K   P   A   L   G   R   P   E   D   T   F   H   F   D   P   E   F   T   A   R   T   P   T   D   S   360
  CCT GGC GTC CCC CCG AGT GCA AAC GCT CAT CAC CTG TTT AGA GGA TTC AGC TTT GTG GCC TCA AGC CTG ATC CAG GAG CCC TCA CAG CAA  1170
   P   G   V   P   P   S   A   N   A   H   H   L   F   R   G   F   S   F   V   A   S   S   L   I   Q   E   P   S   Q   Q   390
  GAT CTG CAC AAA GTC CCA GTT CAC CCA ATC GTG CAG CAG TTA CAC GGG AAC AAC ATC CAC TTC ACC GAT GGC TAC GAG ATC AAG GAG GAC  1260
   D   L   H   K   V   P   V   H   P   I   V   Q   Q   L   H   G   N   N   I   H   F   T   D   G   Y   E   I   K   E   D   420
  ATC GGG GTG GGC TCC TAC TCA GTG TGC AAG CGA TGT GTG CAT AAA GCC ACA GAC ACC GAG TAT GCC GTG AAG ATC ATT GAT AAG AGC AAG  1350
   I   G   V   G   S   Y   S   V   C   K   R   C   V   H   K   A   T   D   T   E   Y   A   V   K   I   I   D   K   S   K   450
  AGA GAC CCC TCG GAA GAG ATT GAG ATC CTC CTG CGG TAC GGC CAG CAC CCG AAC ATC ATC ACC CTC AAG GAT GTC TAT GAT GAT GGC AAG  1440
   R   D   P   S   E   E   I   E   I   L   L   R   Y   G   Q   H   P   N   I   I   T   L   K   D   V   Y   D   D   G   K   480
  TTT GTG TAC CTG GTA ATG GAG CTG ATG CGT GGT GGG GAG CTC CTG GAC CGC ATC CTC CGG CAG AGA TAC TTC TCG GAG CGC GAA GCC AGT  1530
   F   V   Y   L   V   M   E   L   M   R   G   G   E   L   L   D   R   I   L   R   Q   R   Y   F   S   E   R   E   A   S   510
  GAC GTC CTG TGC ACC ATC ACC AAG ACC ATG GAC TAC CTC CAT TCC CAG GGG GTT GTT CAT CGA GAC CTG AAG CCG AGT AAC ATC CTG TAC  1620
   D   V   L   C   T   I   T   K   T   M   D   Y   L   H   S   Q   G   V   V   H   R   D   L   K   P   S   N   I   L   Y   540
  AGG GAT GAG TCG GGG AGC CCA GAA TCC ATC CGA GTC TGC GAC TTC GGC TTT GCC AAG CAG CTG CGC GCG GGG AAC GGG CTG CTC ATG ACA  1710
   R   D   E   S   G   S   P   E   S   I   R   V   C   D   F   G   F   A   K   Q   L   R   A   G   N   G   L   L   M   T   570
  CCC TGC TAC ACG GCC AAT TTC GTG GCC CCG GAG GTC CTG AAG CGT CAA GGC TAT GAT GCG GCG TGT GAC ATC TGG AGT TTG GGG ATC CTG  1800
   P   C   Y   T   A   N   F   V   A   P   E   V   L   K   R   Q   G   Y   D   A   A   C   D   I   W   S   L   G   I   L   600
  TTG TAC ACC ATG CTG GCA GGA TTT ACC CCT TTT GCA AAT GGG CCA GAC GAT ACC CCT GAG GAG ATT CTG GCG CGG ATC GGC AGT GGG AAG  1890
   L   Y   T   M   L   A   G   F   T   P   F   A   N   G   P   D   D   T   P   E   E   I   L   A   R   I   G   S   G   K   630
  TAT GCC CTT TCT GGG GGA AAC TGG GAC TCG ATA TCT GAC GCA GCT AAA GAC GTC GTG TCC AAG ATG CTC CAC GTG GAC CCT CAT CAG CGC  1980
   Y   A   L   S   G   G   N   W   D   S   I   S   D   A   A   K   D   V   V   S   K   M   L   H   V   D   P   H   Q   R   660
  CTG ACG GCG ATG CAA GTG CTC AAA CAC CCG TGG GTG GTC AAC AGA GAG TAC CTG TCC CCA AAC CAG CTC AGC CGA CAG GAC GTG CAC CTG  2070
   L   T   A   M   Q   V   L   K   H   P   W   V   V   N   R   E   Y   L   S   P   N   Q   L   S   R   Q   D   V   H   L   690
  GTG AAG GGC GCG ATG GCC GCC ACC TAC TTT GCT CTA AAC AGA ACA CCT CAG GCC CCG CGG CTG GAG CCC GTG CTG TCA TCC AAC CTG GCT  2160
   V   K   G   A   M   A   A   T   Y   F   A   L   N   R   T   P   Q   A   P   R   L   E   P   V   L   S   S   N   L   A   720
  CAG CGC AGA GGC ATG AAG AGA CTC ACG TCC ACG CGG CTG TAG CGGGTGGGACCCTGGCCCCAGCGTCCCCTGCCAGCATCCTCGTGGGCTCACAGACCCCGGCCT  2265
   Q   R   R   G   M   K   R   L   T   S   T   R   L   *   733

  CGGAGCCCCGTCTGGCACCCAGAGTGACCACAAGTCCAGCAGGGAGGCGGCGCCGCCTCGCCGTGTCCGTGTTTTCTTTTTCAGCCCCGGAGAGGTCCTGACCTGGGGGCTTCTCCAAGC  2384
  CTCACTGCGCCACGCTCCCCGCCCCCTCTCTTTTCTCCCAAGCGAAACCAAATGCCCCCCTTCACCTCGCGTGCCCGTGCCGAGGCCGGGGCCTTCTTTCAGAGCCCCGCGGGTCCTCTCA  2503
  TACATGGCTTCTGTGTCTGCCGAGAGATCTGTTTTCCAATTATGAAGCCGGTCGGTTTGGTCAGACTCCCGACACCCACGTCCAGGTACCCGGTGGAAAGTGGCAGTGCGAGGG  2617
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FIGURE 4

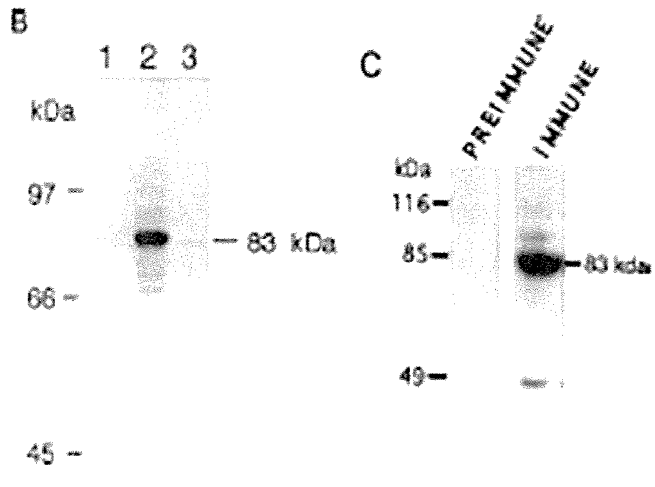

FIGURE 5

```
LOCUS       hRSK3        5817 bp    DNA     linear   PRI 22-JAN-2009
DEFINITION  Homo sapiens ribosomal protein S6 kinase, 90kDa, polypeptide 2
(RPS6KA2), transcript variant 1, mRNA.
BASE COUNT     1285 a      1585 c      1591 g      1356 t
ORIGIN
        1 gcggagaagg aggcggaggg agcgattgtg gccccggccg cggtggccgg cgcggcctgc
       61 cctttgtgac cgcagctcgc gccccacgcc ccgcgcccat ggccgccgtg ccgggctccc
      121 tggccacgcg tgcccgcccg cggacctgag ccccgcgcct gggatgccgg ggatgcgcgt
      181 ccccccggccc tgcggctgct ccgggctggg cgcggggcga tggacctgag catgaagaag
      241 ttcgccgtgc gcaggttctt ctctgtgtac ctgcgcagga agtcgcgctc caagagctcc
      301 agcctgagcc ggctcgagga agaaggcgtc gtgaaggaga tagacatcag ccatcatgtg
      361 aaggagggct ttgagaaggc agatccttcc cagtttgagc tgctgaaggt tttaggacaa
      421 ggatcctatg gaaaggtgtt cctggtgagg aaggtgaagg ggtccgacgc tgggcagctc
      481 tacgccatga aggtccttaa gaaagccacc ctaaaagttc gggaccgagt gagatcgaag
      541 atggagagag acatcttggc agaagtgaat caccccttca ttgtgaagct tcattatgcc
      601 tttcagacgg aaggaaagct ctacctgatc ctggacttcc tgcggggagg ggacctcttc
      661 acccggctct ccaaagaggt catgttcacg gaggaggatg tcaagttcta cctggctgag
      721 ctggccttgg ctttagacca tctccacagc ctggggatca tctacagaga tctgaagcct
      781 gagaacatcc tcctggatga agaggggcac attaagatca cagatttcgg cctgagtaag
      841 gaggccattg accacgacaa gagagcgtac tccttctgcg ggacgatcga gtacatggcg
      901 cccgaggtgg tgaaccggcg aggacacacg cagagtgccg actggtggtc cttcggcgtg
      961 ctcatgtttg agatgctcac ggggtccctg ccgttccagg ggaaggacag gaaggagacc
     1021 atggctctca tcctcaaagc caagctgggg atgccgcagt tcctcagtgg ggaggcacag
     1081 agtttgctgc gagctctctt caaacggaac ccctgcaacc ggctgggtgc tggcattgac
     1141 ggagtggagg aaattaagcg ccatcccttc tttgtgacca tagactggaa cacgctgtac
     1201 cggaaggaga tcaagccacc gttcaaacca gcagtgggca ggcctgagga caccttccac
     1261 tttgaccccg agttcacagc gcggacgccc acagactctc ctggcgtccc cccgagtgca
     1321 aacgctcatc acctgtttag aggattcagc tttgtggcct caagcctgat ccaggagccc
     1381 tcacagcaag atctgcacaa agtcccagtt cacccaatcg tgcagcagtt acacgggaac
     1441 aacatccact tcaccgatgg ctacgagatc aaggaggaca tcggggtggg ctcctactca
     1501 gtgtgcaagc gatgtgtgca taaagccaca gacaccgagt atgccgtgaa gatcattgat
     1561 aagagcaaga gagacccctc ggaagagatt gagatcctcc tgcggtacgg ccagcacccg
     1621 aacatcatca ccctcaagga tgtctatgat gatggcaagt ttgtgtacct ggtaatggag
     1681 ctgatgcgtg gtggggagct cctggaccgc atcctccggc agagatactt ctcggagcgc
     1741 gaagccagtg acgtcctgtg caccatcacc aagaccatgg actacctcca ttcccagggg
     1801 gttgttcatc gagacctgaa gccgagtaac atcctgtaca gggatgagtc ggggagccca
     1861 gaatccatcc gagtctgcga cttcggcttt gccaagcagc tgcgcgcggg gaacgggctg
     1921 ctcatgacac cctgctacac ggccaatttc gtggccccgg aggtcctgaa gcgtcaaggc
     1981 tatgatgcgg cgtgtgacat ctggagtttg gggatcctgt tgtacaccat gctggcagga
     2041 tttacccctt ttgcaaatgg gccagacgat acccctgagg agattctggc gcggatcggc
     2101 agtgggaagt atgccctttc tgggggaaac tgggactcga tatctgacgc agctaaagac
     2161 gtcgtgtcca agatgctcca cgtggaccct catcagcgcc tgacggcgat gcaagtgctc
     2221 aaacacccgt gggtggtcaa cagagagtac ctgtccccaa accagctcag ccgacaggac
     2281 gtgcacctgg tgaagggcgc gatggccgcc acctactttg ctctaaacag aacacctcag
     2341 gccccgcggc tggagcccgt gctgtcatcc aacctggctc agcgcagagg catgaagaga
     2401 ctcacgtcca cgcggctgta gcgggtggga ccctggcccc agcgtcccct gccagcatcc
     2461 tcgtgggctc acagaccccg gcctcggagc ccgtctggca cccagagtga ccacaagtcc
     2521 agcagggagg cggcgcccgc cctcgccgtg tccgtgtttt cttttcagc cccggagagg
     2581 gtcctgacct gggggcttct ccaagcctca ctgcgccagc ctccccgccc gctctctttt
     2641 ctcccaagcg aaaccaaatg cgccccttca cctcgcgtgc ccgtgcgagg ccgggggctt
     2701 ctttcagagc ccgcgggtcc tctcatacat ggcttctgtt tctgccgaga gatctgtttt
     2761 ccaattatga agccggtcgg tttggtcaga ctcccgacac ccacgtccca ggtacccggt
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FIGURE 5 (continued)

```
2821  gggaaagtgg  cagtgcgagg  gcgcagccat  tggtggttgc  agggccccag  agggctgggg
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FIGURE 6

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COMMENT     VNTNAME|r-mAKAP|
COMMENT     VNTAUTHORNAME|Michael Kapiloff|
COMMENT     VNTAUTHORTEL|305-243-7863|
COMMENT     VNTAUTHOREML|mkapiloff@med.miami.edu|
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FIGURE 6 (continued)

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FIGURE 6 (continued)

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FIGURE 6 (continued)

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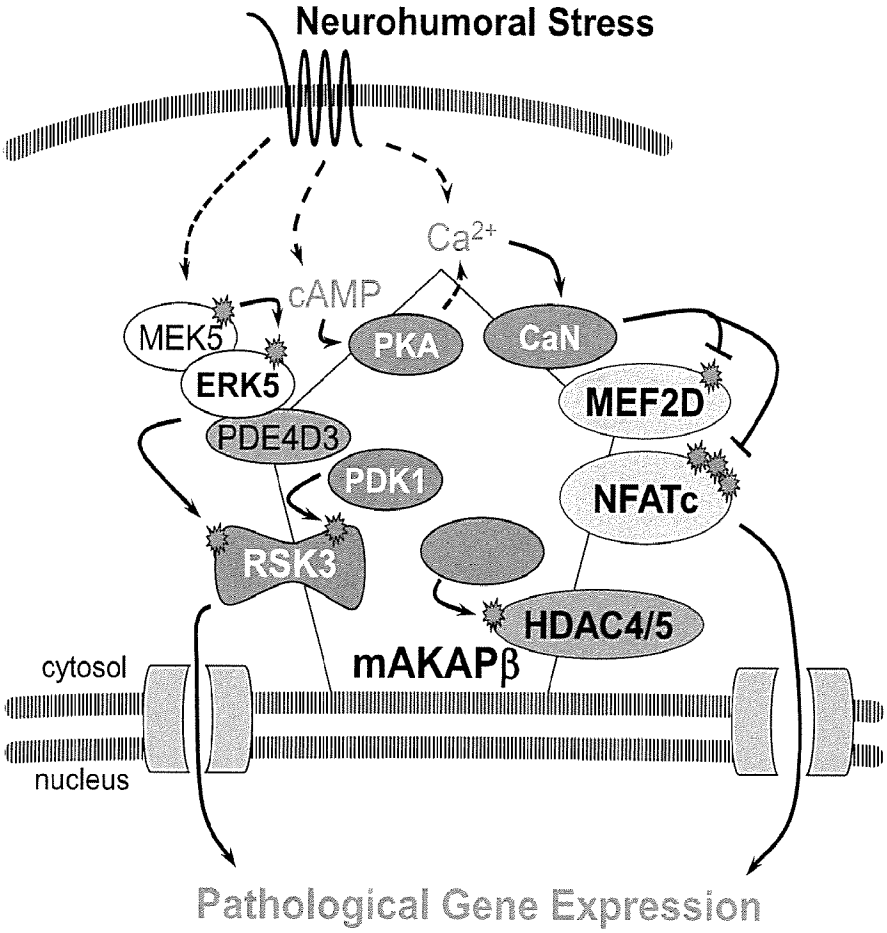
FIGURE 7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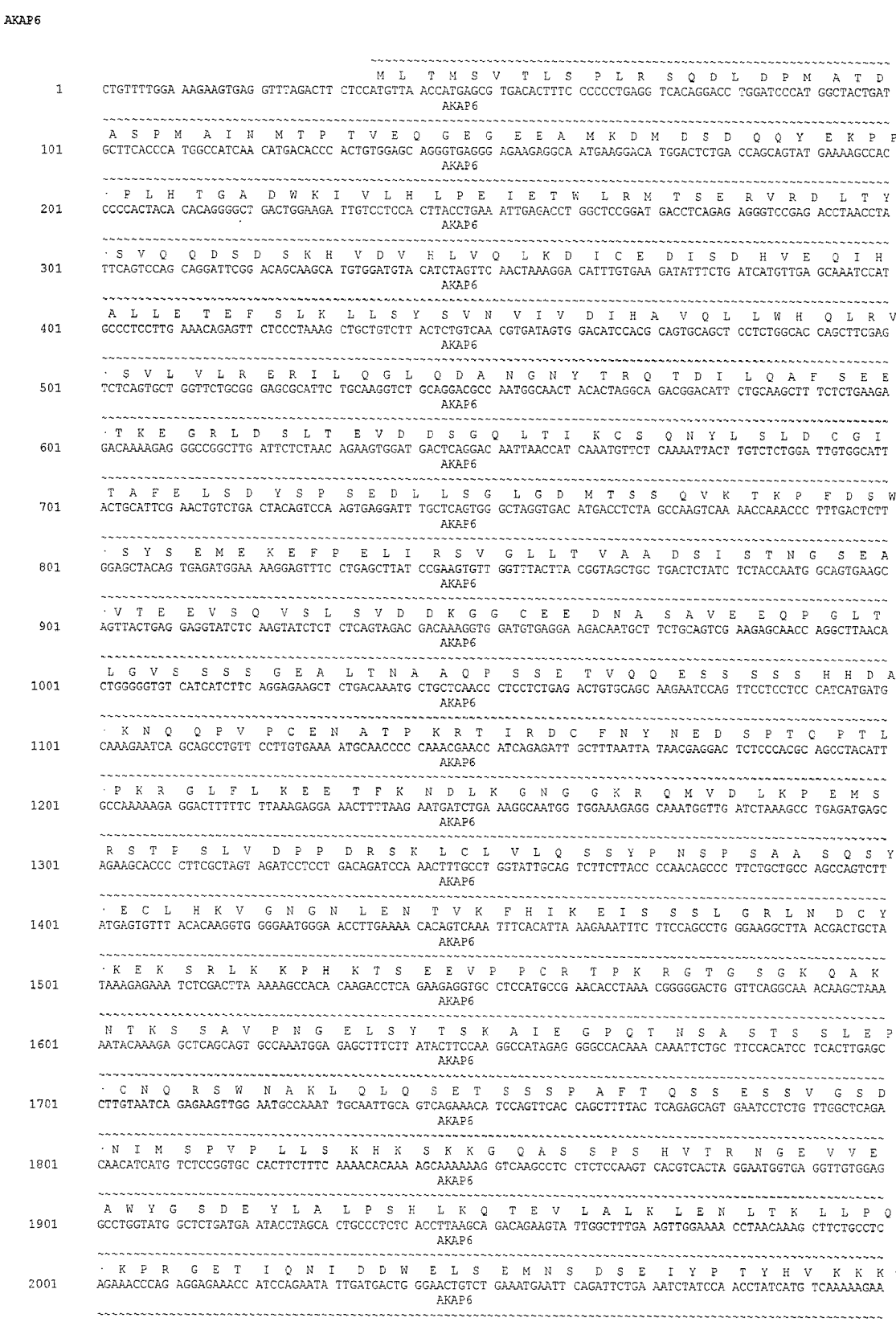
mAKAPβ
cytosol
nucleus
Pathological Gene Expression

FIGURE 8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A S P M A I N M T P T V E Q G E G E E A M K D M D S D Q Q Y E K P P
101 GCTTCACCCA TGGCCATCAA CATGACACCC ACTGTGGAGC AGGGTGAGGG AGAAGAGGCA ATGAAGGACA TGGACTCTGA CCAGCAGTAT GAAAAGCCAC
P L H T G A D W K I V L H L P E I E T W L R M T S E R V R D L T Y
201 CCCCACTACA CACAGGGGCT GACTGGAAGA TTGTCCTCCA CTTACCTGAA ATTGAGACCT GGCTCCGGAT GACCTCAGAG AGGGTCCGAG ACCTAACCTA
S V Q Q D S D S K H V D V H L V Q L K D I C E D I S D H V E Q I H
301 TTCAGTCCAG CAGGATTCGG ACAGCAAGCA TGTGGATGTA CATCTAGTTC AACTAAAGGA CATTTGTGAA GATATTTCTG ATCATGTTGA GCAAATCCAT
A L L E T E F S L K L L S Y S V N V I V D I H A V Q L L W H Q L R V
401 GCCCTCCTTG AAACAGAGTT CTCCCTAAAG CTGCTGTCTT ACTCTGTCAA CGTGATAGTG GACATCCACG CAGTGCAGCT CCTCTGGCAC CAGCTTCGAG
S V L V L R E R I L Q G L Q D A N G N Y T R Q T D I L Q A F S E E
501 TCTCAGTGCT GGTTCTGCGG GAGCGCATTC TGCAAGGTCT GCAGGACGCC AATGGCAACT ACACTAGGCA GACGGACATT CTGCAAGCTT TCTCTGAAGA
T K E G R L D S L T E V D D S G Q L T I K C S Q N Y L S L D C G I
601 GACAAAAGAG GGCCGGCTTG ATTCTCTAAC AGAAGTGGAT GACTCAGGAC AATTAACCAT CAAATGTTCT CAAAATTACT TGTCTCTGGA TTGTGGCATT
T A F E L S D Y S P S E D L L S G L G D M T S S Q V K T K P F D S W
701 ACTGCATTCG AACTGTCTGA CTACAGTCCA AGTGAGGATT TGCTCAGTGG GCTAGGTGAC ATGACCTCTA GCCAAGTCAA AACCAAACCC TTTGACTCTT
S Y S E M E K E F P E L I R S V G L L T V A A D S I S T N G S E A
801 GGAGCTACAG TGAGATGGAA AAGGAGTTTC CTGAGCTTAT CCGAAGTGTT GGTTTACTTA CGGTAGCTGC TGACTCTATC TCTACCAATG GCAGTGAAGC
V T E E V S Q V S L S V D D K G G C E E D N A S A V E E Q P G L T
901 AGTTACTGAG GAGGTATCTC AAGTATCTCT CTCAGTAGAC GACAAAGGTG GATGTGAGGA AGACAATGCT TCTGCAGTCG AAGAGCAACC AGGCTTAACA
L G V S S S S G E A L T N A A Q P S S E T V Q Q E S S S S S H H D A
1001 CTGGGGGTGT CATCATCTTC AGGAGAAGCT CTGACAAATG CTGCTCAACC CTCCTCTGAG ACTGTGCAGC AAGAATCCAG TTCCTCCTCC CATCATGATG
K N Q Q P V P C E N A T P K R T I R D C F N Y N E D S P T Q P T L
1101 CAAAGAATCA GCAGCCTGTT CCTTGTGAAA ATGCAACCCC CAAACGAACC ATCAGAGATT GCTTTAATTA TAACGAGGAC TCTCCCACGC AGCCTACATT
P K R G L F L K E E T F K N D L K G N G G K R Q M V D L K P E M S
1201 GCCAAAAAGA GGACTTTTTC TTAAAGAGGA AACTTTTAAG AATGATCTGA AAGGCAATGG TGGAAAGAGG CAAATGGTTG ATCTAAAGCC TGAGATGAGC
R S T P S L V D P P D R S K L C L V L Q S S Y P N S P S A A S Q S Y
1301 AGAAGCACCC CTTCGCTAGT AGATCCTCCT GACAGATCCA AACTTTGCCT GGTATTGCAG TCTTCTTACC CCAACAGCCC TTCTGCTGCC AGCCAGTCTT
E C L H K V G N G N L E N T V K F H I K E I S S S L G R L N D C Y
1401 ATGAGTGTTT ACACAAGGTG GGGAATGGGA ACCTTGAAAA CACAGTCAAA TTTCACATTA AAGAAATTTC TTCCAGCCTG GGAAGGCTTA ACGACTGCTA
K E K S R L K K P H K T S E E V P P C R T P K R G T G S G K Q A K
1501 TAAAGAGAAA TCTCGACTTA AAAAGCCACA CAAGACCTCA GAAGAGGTGC CTCCATGCCG AACACCTAAA CGGGGGACTG GTTCAGGCAA ACAAGCTAAA
N T K S S A V P N G E L S Y T S K A I E G P Q T N S A S T S S L E P
1601 AATACAAAGA GCTCAGCAGT GCCAAATGGA GAGCTTTCTT ATACTTCCAA GGCCATAGAG GGGCCACAAA CAAATTCTGC TTCCACATCC TCACTTGAGC
C N Q R S W N A K L Q L Q S E T S S S P A F T Q S S E S S V G S D
1701 CTTGTAATCA GAGAAGTTGG AATGCCAAAT TGCAATTGCA GTCAGAAACA TCCAGTTCAC CAGCTTTTAC TCAGAGCAGT GAATCCTCTG TTGGCTCAGA
N I M S P V P L L S K H K S K K G Q A S S P S H V T R N G E V V E
1801 CAACATCATG TCTCCGGTGC CACTTCTTTC AAAACACAAA AGCAAAAAAG GTCAAGCCTC CTCTCCAAGT CACGTCACTA GGAATGGTGA GGTTGTGGAG
A W Y G S D E Y L A L P S H L K Q T E V L A L K L E N L T K L L P Q
1901 GCCTGGTATG GCTCTGATGA ATACCTAGCA CTGCCCTCTC ACCTTAAGCA GACAGAAGTA TTGGCTTTGA AGTTGGAAAA CCTAACAAAG CTTCTGCCTC
K P R G E T I Q N I D D W E L S E M N S D S E I Y P T Y H V K K K
2001 AGAAACCCAG AGGAGAAACC ATCCAGAATA TTGATGACTG GGAACTGTCT GAAATGAATT CAGATTCTGA AATCTATCCA ACCTATCATG TCAAAAAGAA

FIGURE 8 (continued)

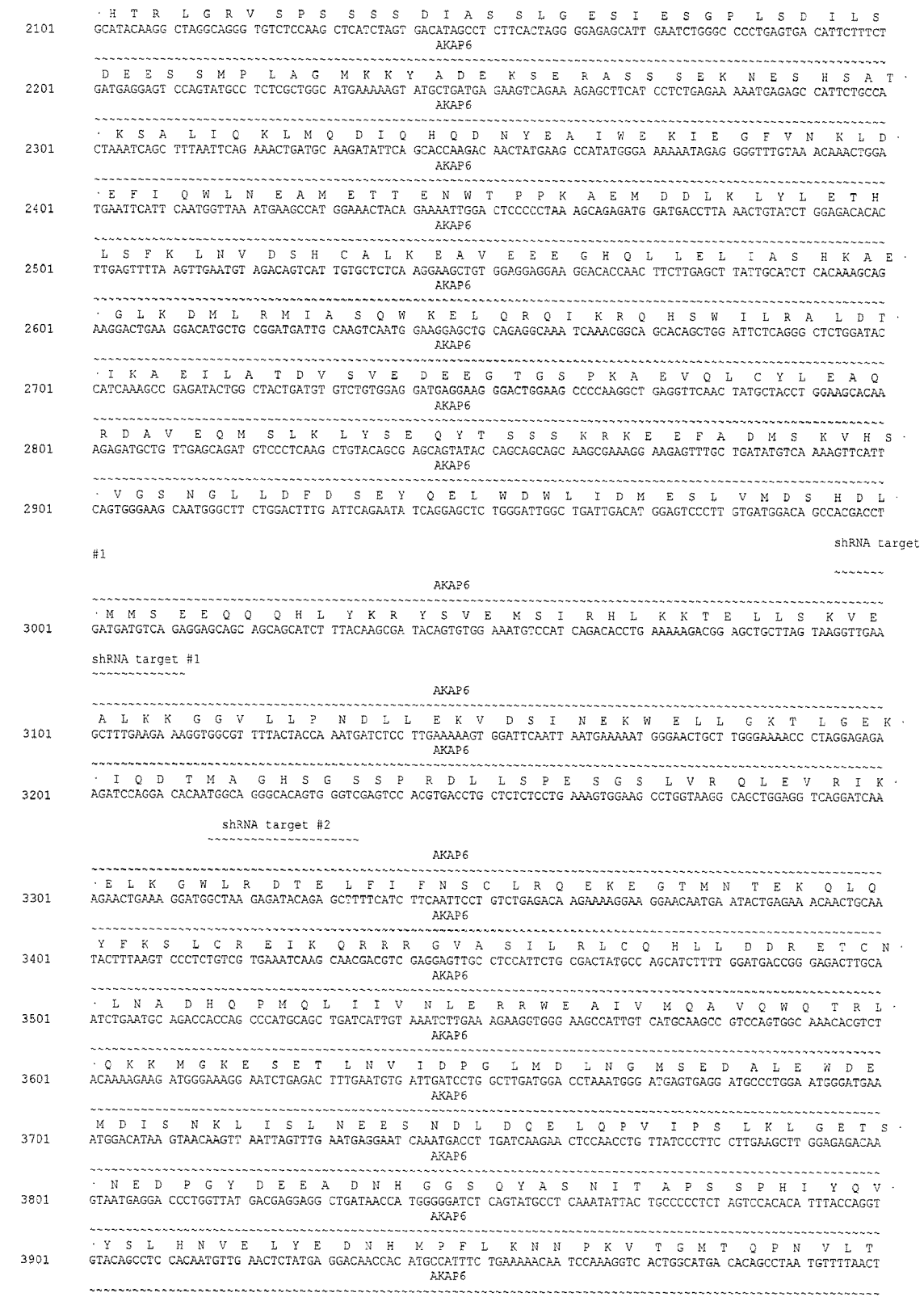

FIGURE 8 (continued)

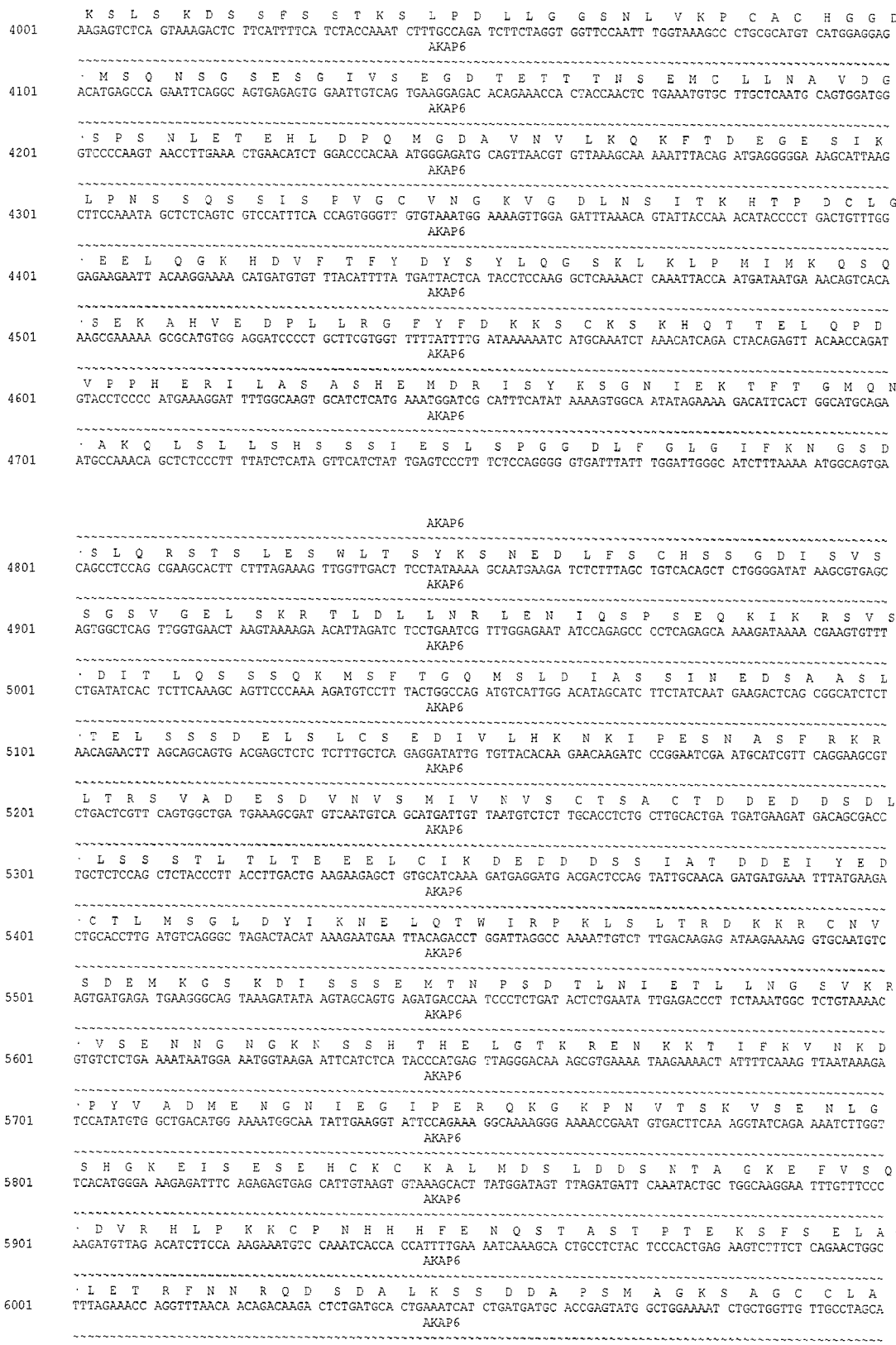

FIGURE 8 (continued)

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           L  E  Q  N   G  T  E   E  N  A   S  I  S  N   I  S  C   C  N  C   E  P  D  V   F  H  Q   K  D  A   E  D  C  S ·
6101       CTTGAACAAA ACGGAACAGA GGAAAATGCT TCTATCAGCA ACATTTCCTG TTGCAACTGT GAGCCAGATG TTTTCCATCA AAAAGATGCC GAAGATTGTT
                                                      AKAP6
           ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
           · V  H  N   F  V  K   E  I  I  D   M  A  S   T  A  L   K  S  K  S   Q  P  E   N  E  V   A  A  P  T   S  L  T ·
6201       CAGTACACAA CTTTGTTAAG GAAATCATTG ACATGGCTTC GACAGCCCTA AAAAGTAAAT CTCAACCTGA AAACGAGGTG GCTGCTCCTA CTTCATTAAC
                                                      AKAP6
           ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
           ·Q  I  K   E  K  V  L   E  H  S   H  R  P   I  Q  L  R   K  G  D   F  Y  S   Y  L  S  L   S  S  H   D  S  D
6301       TCAAATCAAG GAGAAAGTGT TGGAGCATTC TCACCGGCCC ATCCAGCTGA GAAAAGGGGA CTTTTATTCG TACTTATCTC TCTCATCTCA TGACAGTGAT
                                                      AKAP6
           ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
           C  G  E  V   T  N  Y   I  E  E   K  S  S  T   P  L  P   L  D  T   T  D  S  G   L  D  D   K  E  D   I  E  C  F ·
6401       TGTGGGGAGG TCACCAATTA CATAGAAGAG AAAAGCAGCA CTCCATTGCC ACTAGACACC ACTGACTCGG GCTTAGATGA CAAGGAAGAT ATTGAATGCT
                                                      AKAP6
           ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
           · F  E  A   C  V  E   G  D  S  D   G  E  E   P  C  F   S  S  A  P   P  N  E   S  A  V   P  S  E  A   A  M  P ·
6501       TTTTTGAGGC CTGTGTTGAG GGTGACTCTG ATGGAGAGGA GCCTTGTTTC TCTAGTGCTC CTCCAAATGA ATCTGCAGTT CCCAGCGAAG CTGCAATGCC
                                                      AKAP6
           ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
           ·L  Q  A   T  A  C  S   S  E  F   S  D  S   S  L  S  A   D  D  A   D  T  V   A  L  S  S   P  S  S   Q  E  R
6601       ACTACAAGCA ACAGCATGTT CTTCTGAGTT CAGTGATAGT TCTCTTTCAG CTGATGATGC AGATACAGTG GCTCTTTCAA GTCCTTCCTC TCAGGAAAGA
                                                      AKAP6
           ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
           A  E  V  G   K  E  V   N  G  L   P  Q  T  S   S  G  C   A  E  N   L  E  F  T   P  S  K   L  D  S   E  K  E  S ·
6701       GCTGAGGTTG GAAAGGAAGT GAATGGTTTG CCCCAAACTT CCAGTGGCTG TGCAGAAAAC TTAGAGTTTA CTCCTTCAAA GCTTGACAGT GAAAAGGAAA
                                                      AKAP6
           ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
           · S  G  K   P  G  E   S  G  M  P   E  E  H   N  A  A   S  A  K  S   K  V  Q   D  L  S   L  K  A  N   Q  P  T ·
6801       GTTCCGGAAA ACCAGGTGAA TCTGGAATGC CAGAAGAACA TAATGCTGCT TCAGCCAAAT CTAAAGTTCA AGACCTCTCC TTGAAGGCAA ATCAGCCAAC
                                                     AKAP6
           ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
           ·D  K  A   A  L  H  P   S  P  K   T  L  T   C  E  E  N   L  L  N   L  H  E   K  R  H  R   N  M  H   R  *
6901       AGACAAGGCC GCATTGCATC CCAGCCCCAA AACTTTAACC TGTGAAGAAA ATCTTCTAAA CCTTCATGAA AAACGACATA GAAATATGCA TAGGTAGAAT
7001       GTACCCCCTC CCCAAGCATG AAAATCATCT CACTGAAAGA TACGCCTGGC TGCAACTCAG GGGTGGCCTC ATCCTCCCGC CCTGGGCTGG CCTCTGGTTC
7101       CATCACGTTT GTCACTGCCG TTTATTACAT TGACTTCTCC CAAGATGAAT CTTCCTTCCA AATGTGTTTT CTCCACACAA GCCTTGTGAT CTGAATGTGT
7201       GCGCTGGTTC TCTTTAGGTG ATCGTCTTTG AAGTTCAGCA AAGCTGCTTG TTCTCCCATG GATTCCTGTC CCAAGCTACC TCTACCAACC CTCTCTCTCC
7301       AGCTAGACTT TTCTCTTTGC CTCCTCCCTT CCCTTCCACT CTTTAAAGTT CTGCAGTTCA CCAACTGGTA GTCCATTAAA TTCTCCTGTC TAGAATGACC
7401       CCCCCACCAG TACTTGACCA ATTTCATGTA TCAATCTGGA TTTTTTTTTA ACGGTATAAT GACTGTGTTT ATTGAAAGAG TTTTACCTAA AAAGCCAACA
7501       TTTGAATTGG TTGCAGCATA GAGAAGAAAC ACTGGTCCTT CTTTCAAAAT TAAGCAACTA TTAAAAGCGC CATTTTATTT ATTTCATTTA AAAAATAATC
7601       TATGCAGCAT TTCAAGAAAC AACCATATGG TGTTGTATAT TATAAACTGG TGACATTCTA CTATTGAATT ATGTACAACA TTTTCATTTT TTATGCTTCT
7701       TGAGGTGGTA ATGAGAAAAA AGTTTTTTAA AAAAGTGTGC CTTGCTGTAT TTCTTATACC ATTTATTAAA AAGCTGCTTT CACGGTAAAA TTATGTTGGT
                  shRNA target #3
                 ~~~~~~~~~~~~~~~~~~~~~

7801       TTGAAAGGAG GAAATAGCAA GGTTAAGATG TGTGAATAAT TTCTGTATAT ATGTATAACC AAGTACAAAC ATTGATGTAT AATGACAGTA TAAAATGCTT
7901       TCATGTTTGT GATGTCTAGT GATGTGGAAA ATATAAGCCT TAAATCCATT AGATTGCATG GTAATTAAAA TTGGCATAAT AAACACAGAT TATTGGGGGA
8001       AAAGGAAAAT TAGTGATCTC TTCTACTATG TTCTTTACCA AATTGTTGCA TCTGGTTCTG AAAAAGTATA GCATGTAGCA GCTTCCAAAC ATATTCATAT
8101       TGCTTAAGAG GCTTAACATT ACCTAAACTA GAGACTAGAC GTAAAGCCTT CAGTTTTCAA AATCTTTCTG GTCACTATAA AGATCTTGGA ACAGCAAATG
8201       ATTAAATGTC AGTTCCCCTA AACCAATAAA CATTTATACT AGATTTTTTA TTTCCACTTA TCATTAATGA TTTAATGTTG GATTTCAGGT ACCTTGTATG
8301       TCTTAATTTA TTTTAAATAT TTATTTTGAA TGAGTTTGAT AGAAAGCTAG TAGAAAAGTA CAGAAAATTT GACTATTATT TATAGATTTC AGGTATATTT
8401       ATATGTGTAA AAGAAATTGA CAAAGAAATA TTTCATCTGG CCTTTACTGA CTCCTGTTAA ATGCAGTTTT AAATTTATAT CGTAACACCT ACTTAAGTGC
8501       CTGACACAGT AGGTATTCAA TAAAAATTTA CTGAATTAAA GGATTAAATT AGGTGACATG GTGACATCTA TCCCTTTATT TTGACACTAA AACATGGACA
8601       CAACTAGAAA GAGGTACAAT GCAATATAAA GTCACAATAG ATAATATATA TCAAATTTCT AAAAGGTAAA GAATGTTGTG GGTTCATGCA GTCACAGGAA
8701       TGACAATCAT TCAACAGATA GTTCAGAAAC ACTTTTTATC TGCAAGGCAC TATTCTAGAT CCAGAAGATG CAATGTTGAA CAAACAGACA AAGCCCTGCC
8801       CTCAGAAGGC TGTCCTGCAT TAGGAACAAG TGAACACGCA AATGACATGA AGTATTTGTT GCAGAGCTGA GGAACAGAGC AAATGTAGTG ATAGAAGCGC
8901       AATGAGAGAA GCAGCAGTGG GTACAAGGAG GAAGAAAAAG GGCTTGCAGA GAGTGGAAAG TTAGTGGAAT ATTCATGAAA CTTCATTGCA GGGGTAATAG
9001       AAGAAAAAGT AAATTGGGAG GACTTAATGG AAGGTCTTTT AAAAAGTTAA CTTGGAGCTT CTGTATGTAA AATGCTAGGT AATAAGGACA CTTTGTACAG
9101       GCTGTTTTGC ACCTGATTTT ATTTATCATT AGTGCCACGC CAAGATCATT TAGACGATGC TTATCTGTAA TTCTACCACT TTAATAACTA TTTGTATTTT
9201       TATGCCCCTT CTGATCTTTT CCATATGTAT TTCTAAATGG ATAAATTATT CTAGGCTTCT TAATAGGTAG TAATTTGTTC AAAAGCGGTT TTAGCCAGAC
9301       ATCTAGTTGC AGTGTTCAAG AGGATTATGG GGGAAAGAGA TTAGAGATAA TTGTCTAGTT AGGGGGCAGC TGGAGAAAAT AAGCTAAGTT TGCAATAACA
9401       GAGTACACAA GTATAGTGGC CCAGGATGTA GTGAAAGAAC AAATCCTAGA GTCTTTGAAA TTTCTAAGGG CATTCTAGAC CTCTGTTGGG ATATGGTATT
9501       ATTTTACATA CTGACACAAC CTAAATTTTC TTTGGGTAGT AACTAATGTC AAGTCTACAT CGACTGGTAA AACATTCAAA GAACAAACTG ACAATGATGT
9601       TCTACCTACT TGTTACATGC TCATGGAAGA CCGTGCAGTA TTGAAAGTAT TTGTTAATTA TCTGCTTAGT ATTAACACTA AATTTGTAGA ATGACTTTCA
9701       GGTTTGTTGA ACAATGCCTT TTCAGGTTGG AAGAAGAAAA ATAGCCTCAA TCTCCCACCC CATGTAGGCA CTACCTCCCC AATTACCCTT AGAAAATGAT
9801       CACACCAACT CTGCCTACAC ACTTCCAGTG ATAGTGGCTC ATTGTCTGTT AAGGCAAACT GTTCCACTGT TGGGCATATC TCTTTGTTAG AAAGTTCTTT
9901       CTTAGGTTGC TAAAATCTGC CTAGTACCCC GCTACCCTGT TCTGTCTTAT GGAGCAGCCC AGATTATCTT TACTCCCTCT TTCTCATGGC AACCCTGAAG
10001      ATAATCAAGG CCAGTTACTC ATCATCTCCC AACCACTGTT TCCTCAACTG CCCTTCATAT GTCATGGTTT TCAGATCCAT TCCAACCTGA CTGAATGTTA
10101      ACAGACAGAA TTCTTCACAT TAAGGAACTG TCTTCATCAT CATACATGTA GAAAAGAATC TGAACATTTA AGTGCGAAGT TTTCTCTAGA AATATATTCA
10201      AGATATGTTT ATTCTATTAT TGTAAATTTC AAACAATAAA TAAATAAGAA TCCATGACTT CCTTCAGTGG CCCAGTCCAG TGCCTAAGTC ATCTGGAATC
10301      TTCTCCTTAC ATCGAATACA AACCTAACCT GTTTCCAAGT AGGGCACTCC CTCCGCTTAT TTATTCATTT ATTCAGCCAT TCAGCAAACA TTTATTAAAT
10401      GGCTACCTAT GTGAGGCACT ATATTTGGCA TTAGGTATAT AAAGGCAACT AACACATGGT ATCTGTCTCC AAAGGTTTAC AGCTTTTCTT CTGAAAGTGC
10501      CTTTTGTGTC CTTGAATAGA GTGATTATGA CTTTTGTCTC AATTAAATGA TGACCTCATT TAGTTTCTGA TGAAGTTTCT CTACATTTAA GAAAATCTAG
10601      TGAGCAAAAC GGTATACAAA AAATCATCTG GATCTGACTA CCAGAAAAAG TAAAGTGGTT TTAATTACAG AAAGAAAAAT ATTTGTTAGA TTTCTGGTCA
10701      CTGAATAAAT ATGCATCTTA TACAACACAG AACAACCTAA ATTTAGGGCT TCTGGAAACC TGAGTTTGAT GACTTTGTTG TGTTTGGTTC ATCCGGACTA
10801      GGTTTCACCC ATTCTATAAT TCCTAGAAAC AAACTAGATG AAAATATCAA TGAAATGATC CAGGTCTGTC TTACAAGTCA ATTTGCTTTA GCTTCCAAAA
10901      GAAGTTGAAA TTTTTTAGAA TTTTAAAGAA AAACTCACTT TTCAAGTGTC ATATAATTCT TATATTTTAA ATATAAATCG AACCAACTAA ATTTACCGCT
11001      ATATTTTTTT CTACATATTC TTTTTTCAAT TTTCATTTTG AAAAAATTCA GACCTATGTA AAAGTTGAGA GTAGAACAAA GATACTTGGT ACTCATCCCT
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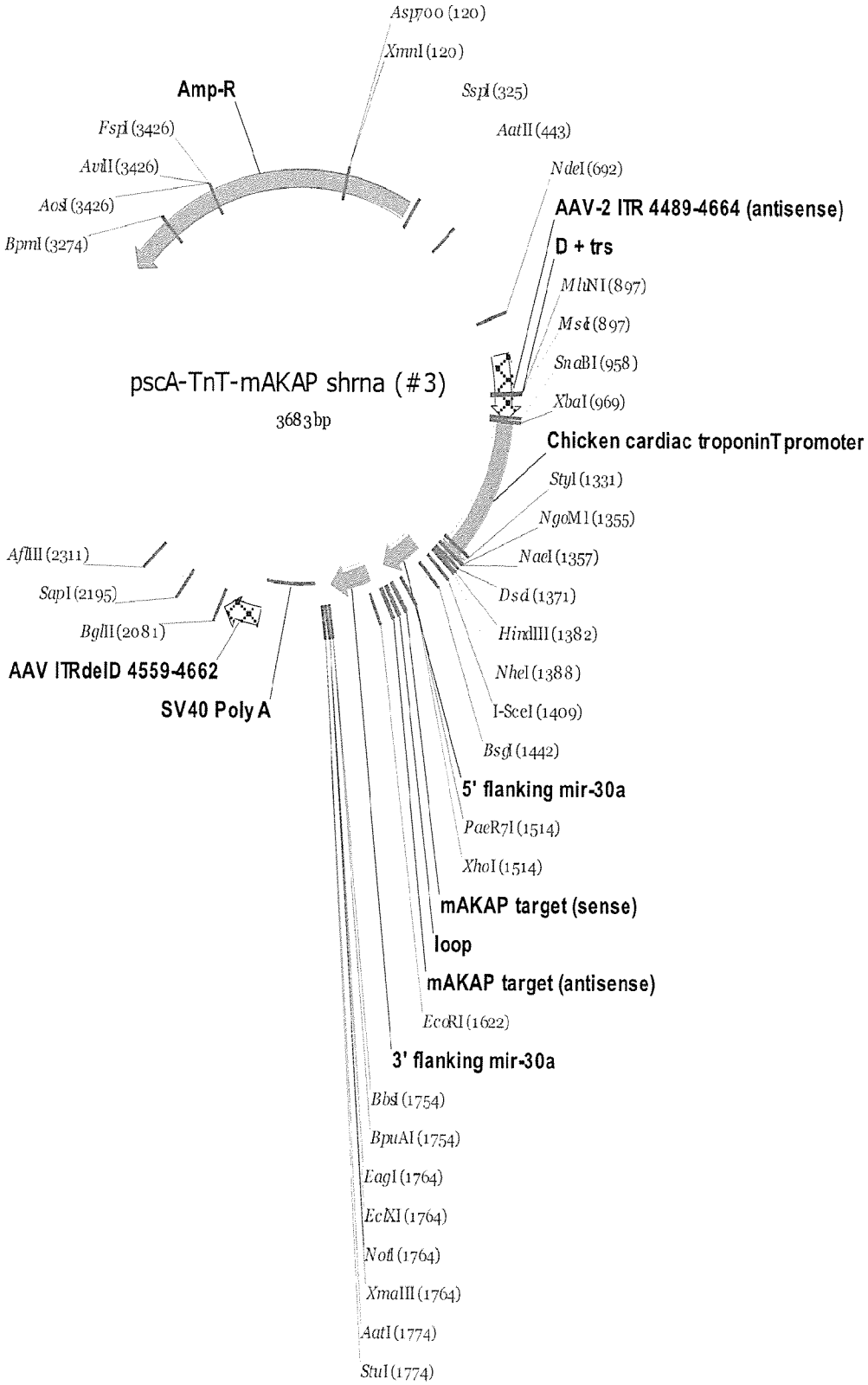
FIGURE 9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XmnI (120)
Amp-R
SspI (325)
FspI (3426)
AatII (443)
AvrII (3426)
NdeI (692)
AosI (3426)
AAV-2 ITR 4489-4664 (antisense)
BpmI (3274)
D + trs
MluNI (897)
MscI (897)
pscA-TnT-mAKAP shrna (#3)
SnaBI (958)
3683bp
XbaI (969)
Chicken cardiac troponinT promoter
StyI (1331)
NgoMI (1355)
AflIII (2311)
NaeI (1357)
SapI (2195)
DsaI (1371)
BglII (2081)
HindIII (1382)
AAV ITRdelD 4559-4662
NheI (1388)
SV40 Poly A
I-SceI (1409)
BsgI (1442)
5' flanking mir-30a
PaeR7I (1514)
XhoI (1514)
mAKAP target (sense)
loop
mAKAP target (antisense)
EcoRI (1622)
3' flanking mir-30a
BbsI (1754)
BpuAI (1754)
EagI (1764)
EclXI (1764)
NotI (1764)
XmaIII (1764)
AatI (1774)
StuI (1774)

FIGURE 10

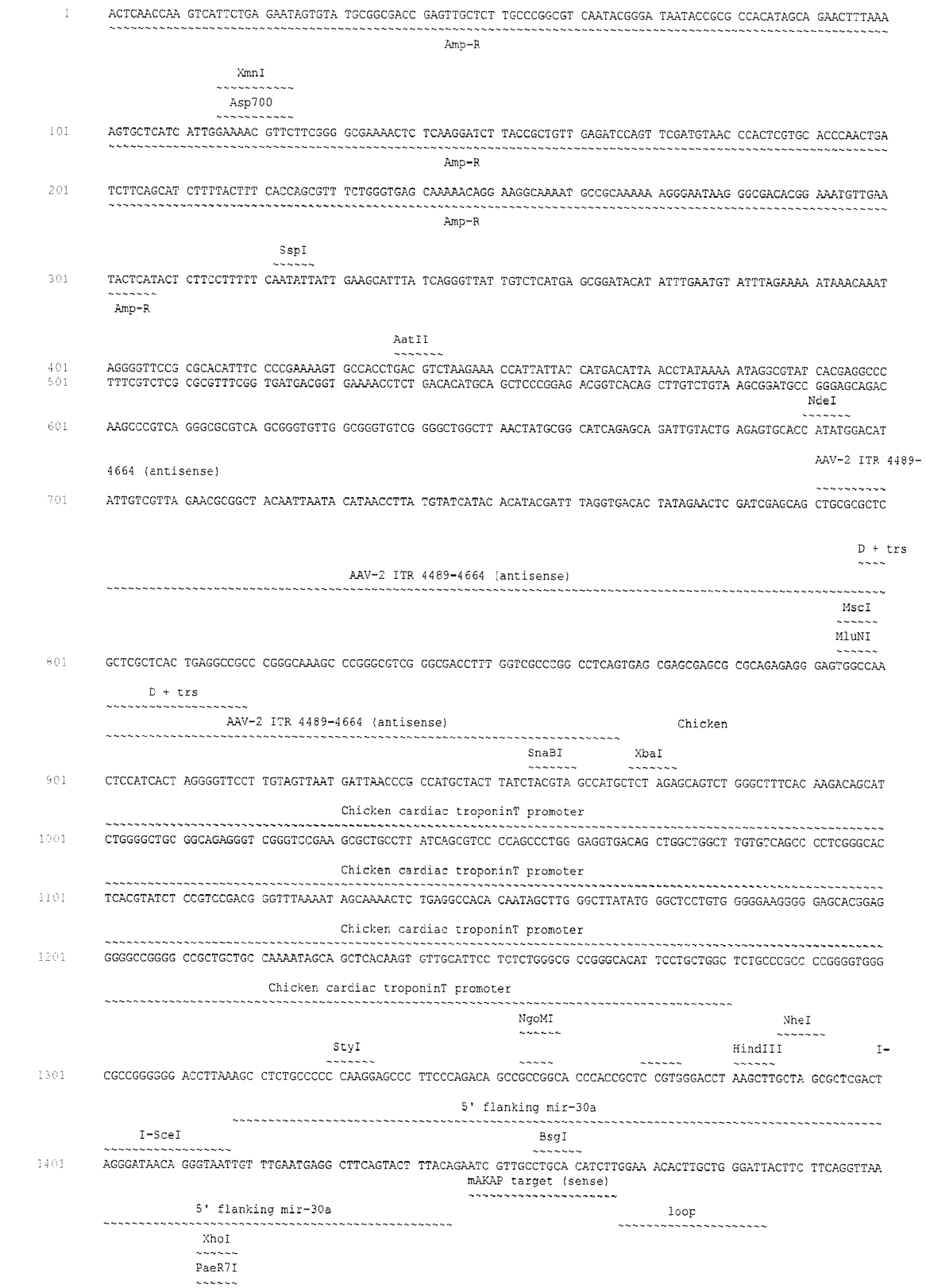

Amp-R
XmnI
Asp700
Amp-R
Amp-R
SspI
Amp-R
AatII
NdeI
AAV-2 ITR 4489-4664 (antisense)
D + trs
AAV-2 ITR 4489-4664 (antisense)
MscI
MluNI
D + trs
AAV-2 ITR 4489-4664 (antisense)
Chicken
SnaBI
XbaI
Chicken cardiac troponinT promoter
Chicken cardiac troponinT promoter
Chicken cardiac troponinT promoter
Chicken cardiac troponinT promoter
NgoMI
NheI
StyI
HindIII
I-
5' flanking mir-30a
I-SceI
BsgI
mAKAP target (sense)
5' flanking mir-30a
loop
XhoI
PaeR7I

FIGURE 10 (continued)

FIGURE 11

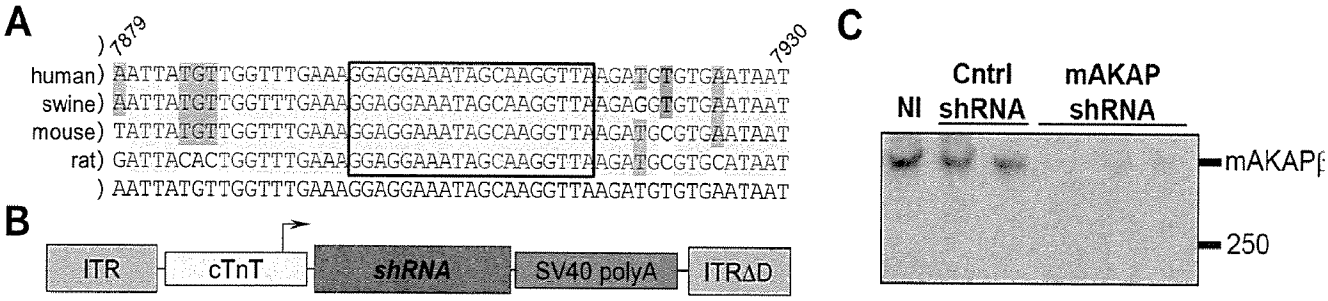

A
) 7879
7930
human) AATTATGTTGGTTTGAAAGGAGGAAATAGCAAGGTTAAGATGTGTGAATAAT
swine) AATTATGTTGGTTTGAAAGGAGGAAATAGCAAGGTTAAGAGGTGTGAATAAT
mouse) TATTATGTTGGTTTGAAAGGAGGAAATAGCAAGGTTAAGATGCGTGAATAAT
rat) GATTACACTGGTTTGAAAGGAGGAAATAGCAAGGTTAAGATGCGTGCATAAT
) AATTATGTTGGTTTGAAAGGAGGAAATAGCAAGGTTAAGATGTGTGAATAAT
B
ITR
cTnT
shRNA
SV40 polyA
ITRΔD
C
NI
Cntrl shRNA
mAKAP shRNA
mAKAPβ
250

FIGURE 12

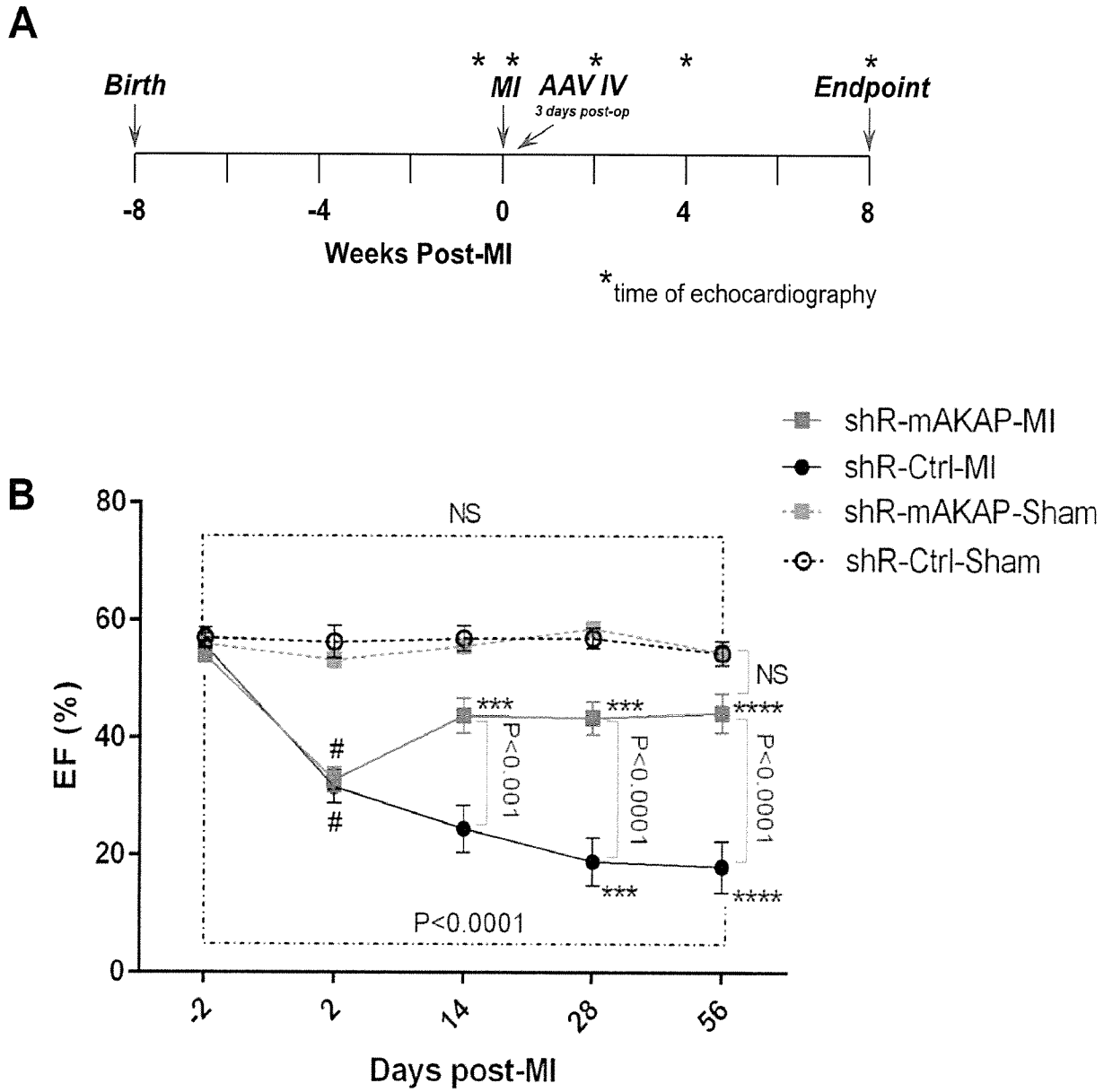
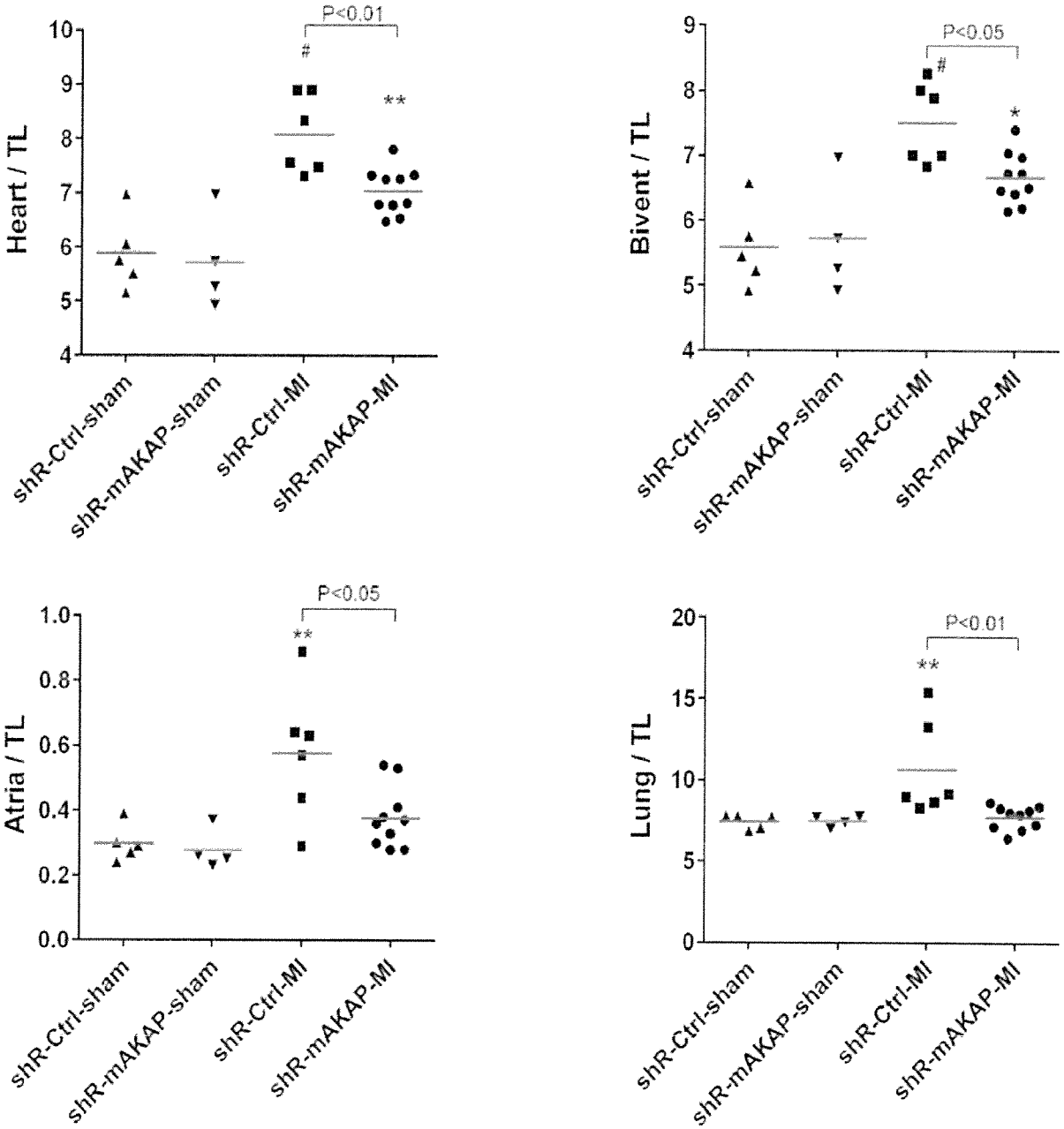

A
Birth
MI
AAV IV
3 days post-op
Endpoint
-8
-4
0
4
8
Weeks Post-MI
*time of echocardiography
B
shR-mAKAP-MI
shR-Ctrl-MI
shR-mAKAP-Sham
shR-Ctrl-Sham
EF (%)
Days post-MI
NS
P<0.0001
P<0.001
P<0.0001
P<0.0001

FIGURE 13


Heart / TL
P<0.01
#
**
shR-Ctrl-sham
shR-mAKAP-sham
shR-Ctrl-MI
shR-mAKAP-MI
Bivent / TL
P<0.05
#
*
Atria / TL
P<0.05
**
Lung / TL
P<0.01
**
*P<0.05 vs. sham
**P<0.01 vs. sham
#P<0.0001 vs. sham