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Anchored p90 Ribosomal S6 Kinase 3 is Required for Cardiac Myocyte Hypertrophy

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Abstract

Rationale—Cardiac myocyte hypertrophy is the main compensatory response to chronic stress on the heart. p90 Ribosomal S6 Kinase (RSK) family members are effectors for extracellular signal-regulated kinases that induce myocyte growth. Although increased RSK activity has been observed in stressed myocytes, the functions of individual RSK family members have remained poorly defined, despite being potential therapeutic targets for cardiac disease.

Objective—To demonstrate that type 3 RSK (RSK3) is required for cardiac myocyte hypertrophy.

Methods and Results—RSK3 contains a unique N-terminal domain that is not conserved in other RSK family members. We show that this domain mediates the regulated binding of RSK3 to the muscle A-kinase anchoring protein (mAKAP) scaffold, defining a novel kinase anchoring event. Disruption of both RSK3 expression using RNA interference and RSK3 anchoring using a competing mAKAP peptide inhibited the hypertrophy of cultured myocytes. In vivo, RSK3 gene deletion in the mouse attenuated the concentric myocyte hypertrophy induced by pressure overload and catecholamine infusion.

Conclusions—Taken together, these data demonstrate that anchored RSK3 transduces signals that modulate pathologic myocyte growth. Targeting of signaling complexes that contain select kinase isoforms should provide an approach for the specific inhibition of cardiac myocyte hypertrophy and for the development of novel strategies for the prevention and treatment of heart failure.

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DISCLOSURES
None

Keywords

RSK; mAKAP; cardiac hypertrophy; concentric; Compartmentalization

INTRODUCTION

Myofibrillar assembly driving non-mitotic growth of the cardiac myocyte is the major response of the heart to increased workload.[1] While 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 down-regulation of signaling pathways through the inhibition of upstream cell membrane receptors and ion channels.[2] Novel drug targets may be revealed through the identification of signaling enzymes that regulate distinct pathways within the hypertrophic signaling network due to isoform specificity or association with unique multimolecular signaling complexes.

p90 ribosomal S6 kinases (RSK) are pleiotropic extracellular signal-regulated kinase (ERK) effectors whose activity is increased in myocytes by most hypertrophic stimuli.[3–5] In addition, increased RSK activity has been detected in explanted hearts from patients with end-stage dilated cardiomyopathy.[6] There are four mammalian RSK family members that are ubiquitously expressed and overlap in substrate specificity.[3] RSKs are unusual in that they contain two catalytic domains, N-terminal (NTKD) and C-terminal (CTKD) kinase domains (Figure 1A).[3] The NTKD phosphorylates RSK substrates and is activated by sequential phosphorylation of the CTKD and NTKD by ERK (1, 2, or 5) and 3′-phosphoinositide-dependent kinase 1 (PDK1), respectively.[3]

By binding scaffold proteins, RSKs may be differentially localized within subcellular compartments, conferring isoform-specific signaling. We first became interested in RSK as a potential substrate for PDK1 bound to the scaffold protein mAKAP muscle A-kinase anchoring protein).[7] 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, MAP-Kinase, Ca^{2+}, and hypoxic signaling by binding a diverse set of enzymes, ion channels, and transcription factors.[8] We now show that type 3 RSK (RSK3) binds mAKAPβ directly via RSK3's unique N-terminal domain, defining a novel enzyme-scaffold interaction. Our evidence supports a model in which anchored RSK3 regulates concentric cardiac myocyte growth, revealing an isoform-specific target for therapeutic intervention in pathologic cardiac hypertrophy.

METHODS

Reagents

Commercial antibodies and oligonucleotides are listed in Online Tables VIII and IX. The commercially available RSK3 antibodies were of varying specificity (Online Figure I). Additional reagents and detailed methods are provided in the Online Supplement.

RSK3$^{-/-}$ mouse

All experiments involving animals were approved by the Institutional Animal Care and Use Committee at the University of Miami. Constitutive RSK3 knock-out mice were backcrossed to C57BL/6 mice over 10 generations. All experiments were performed with

littermate controls and mice that were 8–10 weeks of age at the beginning of the study. Transverse aortic constriction was performed as previously described,[9] 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).

RNA assays

mRNA species were assayed using NanoString technology, a 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. 100 ng of RNA were hybridized in solution to a target-specific codeset overnight at 65 °C, and individual mRNAs were counted using a NanoString Digital Analyzer.

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 Student's *t*-tests and are not corrected for multiple comparisons. Repeated symbols represent *p*-values of different orders of magnitude, i.e., * $p<0.05$, ** $p<0.005$, etc. All datasets involving multiple comparisons for which *p*-values are provided were also significant by ANOVA, $\alpha = 0.05$.

RESULTS

mAKAPβ - A scaffold for RSK

We have previously published that RSK proteins and activity are associated with mAKAPα complexes in brain.[7] We now show that RSK is also associated with mAKAPβ in cardiac myocytes (Figure 1B). In order to determine whether mAKAP preferentially binds a specific RSK isoform, HA-tagged RSK family members were co-expressed with mAKAP in HEK293 cells. In contrast to RSK1 and RSK2, RSK3 robustly mediated the co-immunoprecipitation of both mAKAPα and mAKAPβ (Figure 1C and data not shown). RSK family members are similar in primary sequence with the exception of the extreme N- and C-terminal domains and a small region after the hydrophobic motif (Figure 1A). Consistent with the selective binding of RSK3 to the scaffold, the N-terminal domain of RSK3 bound mAKAPβ (Figure 1D).

RSK3 function in neonatal cardiac myocytes

RSK family members can be activated in most cell types by ERK, but not c-Jun N-terminal kinases (JNK) or p38.[3] ERK phosphorylation is permissive for PDK1 phosphorylation of the RSK NTKD, such that PDK1 phosphorylation of RSK S^{218} is indicative of full activation of the enzyme (Figure 1A). To show that ERK activates RSK in cardiac myocytes, we treated neonatal rat ventricular myocytes with different hypertrophic agonists and MAP-Kinase pathway inhibitors and detected RSK activation using a pan-RSK S^{218} (P-S^{218}) phospho-specific antibody (Online Figures I and II). α-adrenergic stimulation with phenylephrine (PE) induced RSK phosphorylation 3-fold by both MEK1/2 (that activates ERK1/2) and MEK5 (that activates ERK5) dependent mechanisms (Online Figure II). Moreover, MEK1/2 inhibition reduced RSK baseline phosphorylation. JNK and p38 inhibition did not affect PE-activation, and, in fact, variably increased baseline RSK phosphorylation. Fetal bovine serum (FBS) and leukemia inhibitory factor (LIF) also increased the level of activated RSK, but more due to an increase in total RSK protein expression than to ERK phosphorylation. Similar results were found for HA-tagged RSK3 (Figure 2A). Acute PE treatment induced the phosphorylation of HA-RSK3 ERK (S^{360}) and PDK1 (S^{218}) sites through both MEK1/2

and MEK5 dependent signaling. Together, these results confirmed that in cardiac myocytes ERK is responsible for RSK activation.

We have previously demonstrated that mAKAPβ complexes are required for the hypertrophy of cultured myocytes.[10–12] Therefore, we 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 2B). RSK3 siRNA did not induce the apoptosis of myocytes cultured either in the absence or presence of serum (Figure 2C). Importantly, in the presence of α-adrenergic stimulation, RSK3 siRNA inhibited morphologic hypertrophy by 34% and atrial natriuretic factor (ANF) expression completely (Figure 2D–F and Online Figure III). In addition, RSK3 siRNA had smaller, but detectable effects on LIF and FBS-stimulated hypertrophy. The results obtained by RSK3 RNAi were confirmed with a second, distinct RSK3 siRNA (data not shown).

Endogenous RSK3 proteins are expressed at a relatively low level in cardiac myocytes compared to the other RSK family members and are induced in expression by chronic PE treatment (Online Figure IVC). As a result, RSK3 RNAi did not affect the level of total RSK in the myocyte, only diminishing the RSK3 detected following immunoprecipitation with a specific RSK3 antibody (Figure 2B and Online Figure IVC). 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 (Online Figure IVA,B). Remarkably, in these experiments, the cross-section area of unstimulated myocytes was increased by adenoviral-based expression of wildtype HA-RSK3 enzyme at a level comparable to 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, we employed the pan-RSK inhibitors BI-D1870 (Figure 3 and Online Figure V) and SL0101 (Online Figure VI),[13–15] finding that like RSK3 siRNA, these compounds inhibited agonist-induced myocyte hypertrophy.

A high affinity RSK3-binding domain in mAKAP

We considered that the requirement for RSK3 in myocyte hypertrophy was due to the association of RSK3 with specific signaling complexes. In order to address this hypothesis, we defined the mAKAP domain(s) responsible for RSK3 binding. HA-tagged RSK3 was co-expressed in heterologous cells with myc-tagged mAKAPβ fragments and co-immunoprecipitated using a myc-tag antibody (Figure 4A, B). RSK3 preferentially associated with mAKAP amino acid residues 1286–1833, although it also weakly associated with mAKAP 245–587 and 525–1286. Consistent with this result, RSK3 binding to a full-length mAKAPβ protein with an internal deletion of residues 1257–1886 was reduced by >85%. Further mapping showed that the main RSK3 binding domain (RBD) of mAKAP mapped to a fragment encompassing residues 1694–1833 (Figure 4C). Accordingly, RSK3 bound poorly to a full-length mAKAPβ protein with an internal deletion of residues 1701–1800 (Figure 4D). As shown earlier, the unique N-terminal domain of RSK3 bound full-length mAKAPβ (Figure 1D). The mAKAP RBD also bound HA-RSK3 1–42 (Figure 4E), but neither a N-terminally truncated RSK3 mutant (HA-RSK3 DN30) nor HA-tagged full-length RSK2 (Figure 4F). These results imply that the mAKAP RBD is responsible for the selective binding of RSK3 to mAKAP.

We next tested whether mAKAP-RSK3 binding is direct (Figure 4G). The binding of bacterially expressed His-tagged mAKAP 1286–1833 and full-length RSK3 was analyzed by surface plasmon resonance (SPR). The binding was direct and high affinity (nanomolar K_D). We have previously reported that once activated, RSK3 binds mAKAPα less well in cells.[7] Interestingly, prior 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.

Disruption of RSK3 anchoring inhibits neonatal myocyte hypertrophy

The identification of the high affinity mAKAP RSK binding domain (RBD) provided the opportunity to test whether anchoring of RSK3 is important for its function. When expressed in neonatal myocytes, a GFP-mAKAP RBD fusion protein (GFP-RBD) competed the association of endogenous RSK3 and mAKAPβ (Figure 5A). Expression of GFP-RBD markedly inhibited PE-induced hypertrophy (Figure 5B–D), similar to RSK3 siRNA (Figure 2). Together, these results imply that RSK3 anchored to scaffolds through its unique N-terminal domain is required for the hypertrophy of cultured myocytes.

Role of RSK3 in cardiac hypertrophy in vivo

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 knock-out mouse. By homologous recombination, stop codons were inserted into the second exon of the *RSK3* (*Rps6ka2*) gene encoding the ATP-binding motif of the NTKD,[16] resulting in the constitutive absence of RSK3 protein in homozygous null mice (Online Figure VII). In general, $RSK3^{-/-}$ mice appeared normal in morphology, bred according to Mendelian genetics (Online Table I), and exhibited no excess mortality up to six months of age (data not shown). Prior to any stress, the $RSK3^{-/-}$ mice had generally normal cardiac function, with the only measureable difference from wildtype littermates being a slight increase in left ventricular internal dimensions detected by echocardiography (Online Table II and Online Figure VIII).

We tested whether RSK3 is required for compensated cardiac hypertrophy by subjecting the $RSK3^{-/-}$ mice to pressure overload for two weeks (Figure 6A). By echocardiography, transverse aortic constriction (TAC) induced a 36% increase in posterior wall thickness (LPVW;d) in wildtype mice, but only a 16% increase in $RSK3^{-/-}$ mice (Table 1 and Online Figure VIII). The decreased hypertrophy was not accompanied by a change in contractility (fractional shortening). Post-mortem gravimetric analysis showed that the corresponding increase in biventricular weight following TAC was similarly diminished in the knock-out mice (48% for $RSK3^{+/+}$ vs. 26% for $RSK3^{-/-}$ mice; Online Table III). TAC primarily induces concentric growth of cardiac myocytes. Inspection of wheat germ agglutinin–stained heart sections revealed that consistent with these results, RSK3 knock-out attenuated the TAC-induced increase in myocyte transverse cross-section area by ~46% (Figure 6B,C). Proportional results were obtained by morphometric analysis of adult cardiac myocytes isolated from the TAC mice (Figure 6D–G).

In order to characterize the $RSK3^{-/-}$ cardiac phenotype at a molecular level, we surveyed for differences in the cardiac expression of 30 genes encoding proteins involved either in cardiac remodeling or hypertrophic signaling (Table 2). About 2/3 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 ANF expression was dramatically inhibited in $RSK3^{-/-}$ mice, consistent with the results obtained for PE-treated neonatal myocytes. Although after two weeks of pressure overload the small increases in cellular apoptosis and interstitial fibrosis detectable by histology for wildtype mice did not reach significance when compared to sham-operated controls (data not shown), these signs of remodeling tended to be less in the knock-out mice (8.2 ± 2.0 vs. $4.2\pm1.0 \times 10^{-4}$ TUNEL-positive nuclei and 0.49 ± 0.18 vs. 0.29 ± 0.11 % collagen staining for wildtype and $RSK3^{-/-}$ TAC hearts, respectively). Interestingly, two genetic markers of fibrosis that were

significantly induced in TAC wildtype mice, transforming growth factor β2 and collagen VI α1,[17] were attenuated in expression by RSK3 knock-out (Table 2).

To explore further the role of RSK3 in cardiac hypertrophy, we employed a second in vivo pathological stressor, chronic isoproterenol (Iso) infusion via subcutaneous osmotic pumps, as well as a physiological stressor, chronic exercise via swimming. Although Iso infusion resulted in a minor increase in ventricular wall thickness by echocardiography (Online Table IV), at the cellular level, Iso significantly induced myocyte growth in width in a RSK3-dependent manner, both as measured by histology and following myocyte isolation (Online Figure IX). Unlike TAC, Iso infusion also induced eccentric growth, as evidenced by increased myocyte length and ventricular dilation by echocardiography (Online Tables IV and V). This eccentric growth was not inhibited by RSK3 knock-out. Together with the TAC data, these results demonstrate that RSK3 contributes to the induction of concentric myocyte hypertrophy in pathologic conditions.

Finally, *RSK3*$^{-/-}$ mice were exercised by swimming. As expected,[18] after swimming, wildtype mice exhibited a decreased resting heart rate (consistent with improved physical conditioning) and increased left ventricular internal dimensions (Online Table VI and Online Figure VIII). After exercise, there were no significant differences between RSK3 knock-out and wildtype mice detectable by echocardiography, and the cohorts exhibited a similar increase in biventricular weight indexed by body weight (6 and 7%, respectively, Online Table VII).

DISCUSSION

RSK activity is associated with the function of the nervous system, immunity, muscle and cancer.[3] The roles of individual RSK family members, however, remain poorly defined. Human *RSK2* mutations cause X-linked Coffin-Lowry Syndrome that 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 that inhibits all RSKs except RSK3.[19] We now reveal a role for RSK3 in the cardiovascular system, regulation of pathological myocyte hypertrophy.

Cardiac myocytes can grow in both width and length, termed “concentric” and “eccentric” hypertrophy, respectively.[1] 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. We 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. IL-6 cytokines such as LIF and cardiotrophin-1 induce an elongated, eccentric phenotype for cultured neonatal myocytes, in contrast to the symmetric growth stimulated by PE.[20] Interestingly, the growth of the cultured myocytes tended to depend upon RSK3 more when induced by α-adrenergic stimulation than by LIF (Figures 2 and 3). The greater inhibition of PE-induced morphologic hypertrophy was consistent with the more robust activation of RSK by PE than LIF (Online Figure II), as well as the results obtained in vivo.

RSK3 was activated in myocytes by ERKs 1, 2 and 5 (Figure 2A). While 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.[21] These “Rasopathies”

feature developmental delay, dysmorphic features and defects in multiple organ systems, often including a hypertrophic phenotype.[22] 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.[23, 24] Conversely, transgenic expression of dominant negative Raf1 inhibited the hypertrophy due to pressure overload.

Recently, the Molkentin group showed that cardiac myocyte-specific knock-out of all four ERK1/2 alleles resulted in a severe, fatal dilated cardiomyopathy without increased myocyte death.[25] ERK1/2 null myocytes were longer and narrower than those from control animals. *PTPN11* (Shp2) knock-out that decreased ERK1/2 activation also resulted in an elongated myocyte morphology and dilated cardiomyopathy.[26] Conversely, myocytes from constitutively active MEK1 transgenic mice were shorter and wider.[25] In contrast to the ERK1/2 and Shp2 knock-out mice, we found that deletion of the downstream effector RSK3 resulted in a milder phenotype, with the defect in concentric growth significant only following 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.

We 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,[27] although ERK5 may also contribute to concentric growth.[28] Whether RSK3 induces hypertrophy in response to both ERK5 and ERK1/2 will require further investigation, especially since mAKAPβ preferentially binds MEK5 and ERK5.[12]

The data obtained using the RSK knock-out mouse establish a function for RSK3 in pathological remodeling. However, we cannot completely exclude a role for RSK3 in physiologic hypertrophy. For example, the myocytes isolated from unstressed *RSK3*$^{-/-}$ mice tended to be smaller in both width and length (Figure 6 and Online Figure IX). In addition, after swimming, *RSK3*$^{-/-}$ biventricular weight was less than wildtype, albeit not significantly after normalization by body weight (Online Table VII).

It is remarkable that even though RSK3 constitutes a minority of the total RSK in the myocyte (Online Figures IV and X), 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.[3]) 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.[29] Little is known about RSK compartmentation in cells or participation in multimolecular signaling complexes. Upon mitogen stimulation cytosolic RSK1 (and potentially other RSK isoenzymes) can transiently translocate to the plasma membrane, while activated RSK tends to be enriched in the nucleus.[3] In neurons RSKs bind PDZ domain-containing proteins via their conserved C-terminal -STxL peptides, directing the kinases to substrates involved in synaptic transmission.[30] By another mechanism, RSK1 binds type 1 protein kinase A (PKA) and D-AKAP-1, a mitochondrion-localized scaffold.[31, 32] Consistent with the fact that we can only detect RSK3 in myocytes following immunoprecipitation, we have not been able to detect endogenous RSK3 protein by immunocytochemistry. When over-expressed at a low level, HA-RSK3 was enriched at the nuclear envelope (data not shown), the predominant location for mAKAPβ in the cardiac

myocyte.[33] By characterizing in detail the protein-protein interaction between the unique RSK3 N-terminus and mAKAPβ, we have identified a new mechanism by which RSK3 can be specifically anchored by one or more scaffolds that may be targeted to different signaling compartments. We demonstrated the functional significance of this RSK3 anchoring using a competing binding peptide (mAKAP RBD) that inhibited myocyte hypertrophy. Moreover, since the binding of RSK3 to mAKAP was inhibited by RSK3 phosphorylation, it is likely that RSK3 anchoring to this and other scaffolds will be regulated in cells.

RSK3 anchoring by scaffolds should specify the phosphorylation of select substrates. Because RSK3's affinity for mAKAPβ is decreased following its activation, it is possible that mAKAPβ-associated RSK3 is responsible for phosphorylating substrates both adjacent to and at a distance from the scaffold (Online Figure XI). Although our initial screen of known RSK substrates did not identify any that were less phosphorylated in *RSK3*$^{-/-}$ mice (Online Figure X and data not shown), mAKAPβ-associated RSK3 may be important for nuclear phosphorylation events involved in cardiac remodeling due to the scaffold's location. Alternatively, proteins phosphorylated by RSK family members also include those involved in the regulation of translation and metabolism, as well as sarcomeric proteins.[3] Besides co-localizing enzymes and substrates, scaffolds can facilitate cross-talk between different signaling pathways. mAKAPβ also organizes cAMP, Ca^{2+}-dependent, and hypoxic signaling modules and is required for calcineurin-NFAT dependent myocyte hypertrophy.[8] Future work will be required to elucidate which mAKAPβ binding partners are present in individual mAKAPβ signalosomes and whether RSK3 and the other enzymes co-regulate downstream effectors.

The regulation of NHE1 by RSK1/2 has spurred recent interest in using RSK inhibitors to treat heart disease.[34] Our results should encourage that line of research as RSK3 knock-out 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 for acquired diseases such as hypertension (pressure overload) and for the treatment of the aforementioned Rasopathies. Recently, a Noonan syndrome mouse model (Raf1 $L^{613}V$ knock-in) mouse was treated with PD0325901, resulting in the attenuated progression of cardiac hypertrophy cardiomyopathy and other Noonan characteristics.[21] The use of MEK inhibitors in humans is currently being tested in clinical trials for cancer. Results have not entirely been encouraging, however, and trials with PD0325901 have been terminated early due to ophthalmologic and neurologic toxicity.[22] Targeting of RSK3 may be 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 should be explored as potential novel cardiac therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Non-standard Abbreviations

ANF	atrial natriuretic factor
CTKD	C-terminal kinase domain
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
GFP	green fluorescent protein
Iso	isoproterenol
JNK	c-Jun N-terminal kinases
LIF	leukemia inhibitory factor
mAKAP	muscle A-kinase anchoring protein
MAP Kinase	mitogen-activated protein kinase
NTKD	N-terminal kinase domain
PDK1	3′phosphoinositide-dependent kinase 1
PE	phenylephrine
RBD	RSK binding domain
RSK	p90 Ribosomal S6 Kinase
siRNA	small interfering RNA oligonucleotide
SPR	surface plasmon resonance
TAC	transverse aortic constriction

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Novelty and Significance

What Is Known?

- Extracellular signal-regulated kinases (ERKs) regulate cardiac myocyte hypertrophy.
- p90 ribosomal S6 kinase (RSK) activity is increased in hypertrophic myocytes and heart disease.

What New Information Does this Article Contribute?

- RSK type 3 (RSK3) is required for the growth of cardiac myocytes in width, “concentric hypertrophy,” in response to stress on the heart, including pressure overload and catecholamine infusion.
- RSK3 binds with high affinity to the scaffold protein mAKAPβ in myocytes through unique domains in each protein.
- Disruption of RSK3 binding to mAKAPβ blocks hypertrophy of cultured myocytes.

Current treatments preventing maladaptive cardiac remodeling in disease are limited, and the prognosis for heart failure patients remains poor. The ERK pathway has been proposed as a possible drug target, but due to regulation by multiple cellular processes, the ERK enzymes have been difficult to target therapeutically. We found that the ERK effector RSK3 is associated with “signalosomes” that are organized by the scaffold protein mAKAPβ located at the myocyte nuclear envelope. Even though all four RSK family members are expressed in the heart and even though RSK3 is among the least highly expressed, knock-out of only RSK3 significantly attenuated the induction of pathologic hypertrophy in response to stress in mice. Similarly, in cultured neonatal myocytes, we found that RSK3 was required for hypertrophy induced by adrenergic agents. Remarkably, the binding of RSK3 to mAKAPβ was important for its function in cell growth. These findings suggest that RSK3 is an anchored enzyme and its binding to scaffold proteins directs its specific functions. Our study defines a novel role for RSK3 in the regulation of concentric hypertrophy and suggests a novel, potential therapeutic strategy – peptide-based inhibition of RSK3 anchoring and signal transduction, for the treatment of heart failure.

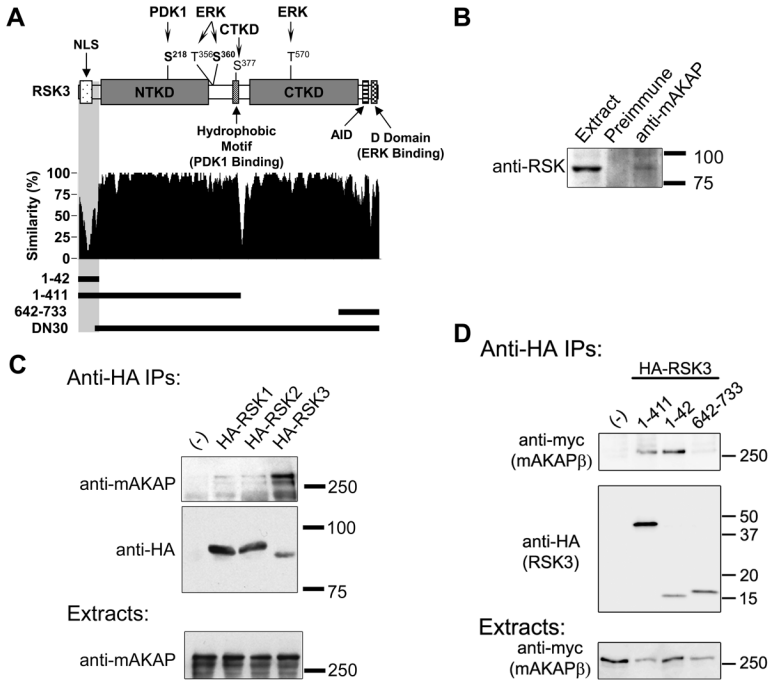


Figure 1. The unique RSK3 N-terminal domain binds mAKAP

A. RSKs contain two catalytic domains, N-terminal (NTKD) and C-terminal (CTKD) kinase domains.[3] Only RSK3 has an N-terminal nuclear localization signal (NLS). In inactive RSK3, the CTKD binds the autoinhibitory domain (AID) αhelix. Pre-bound to the D-domain, when activated, ERK phosphorylates RSK3 residues including the CTKD activation loop (T^{570}). The CTKD then autophosphorylates S^{377}, permitting PDK1 binding and phosphorylation of the NTKD activation loop (S^{218}). The NTKD phosphorylates RSK substrates. RSKs 1–4 were aligned and sequence similarity calculated using Vector NTI AlignX (Invitrogen). RSK3 fragments used for mapping are indicated. B. Rat neonatal myocyte extract (lane 1, 0.2% total extract) was immunoprecipitated with preimmune (lane 2) or anti-VO56 mAKAP (lane 3) sera, and detected using a pan-RSK antibody (C-20, cf. Online Figure I legend). C. HA-tagged kinases and myc-tagged mAKAPα were expressed by co-transfection of HEK293 cells and co-immunoprecipitated with anti-HA antibody. D. HA-tagged RSK3 fragments and myc-tagged mAKAPβ were expressed by co-transfection of COS-7 cells and co-immunoprecipitated with anti-HA antibody. $n \geq 3$ for each panel.

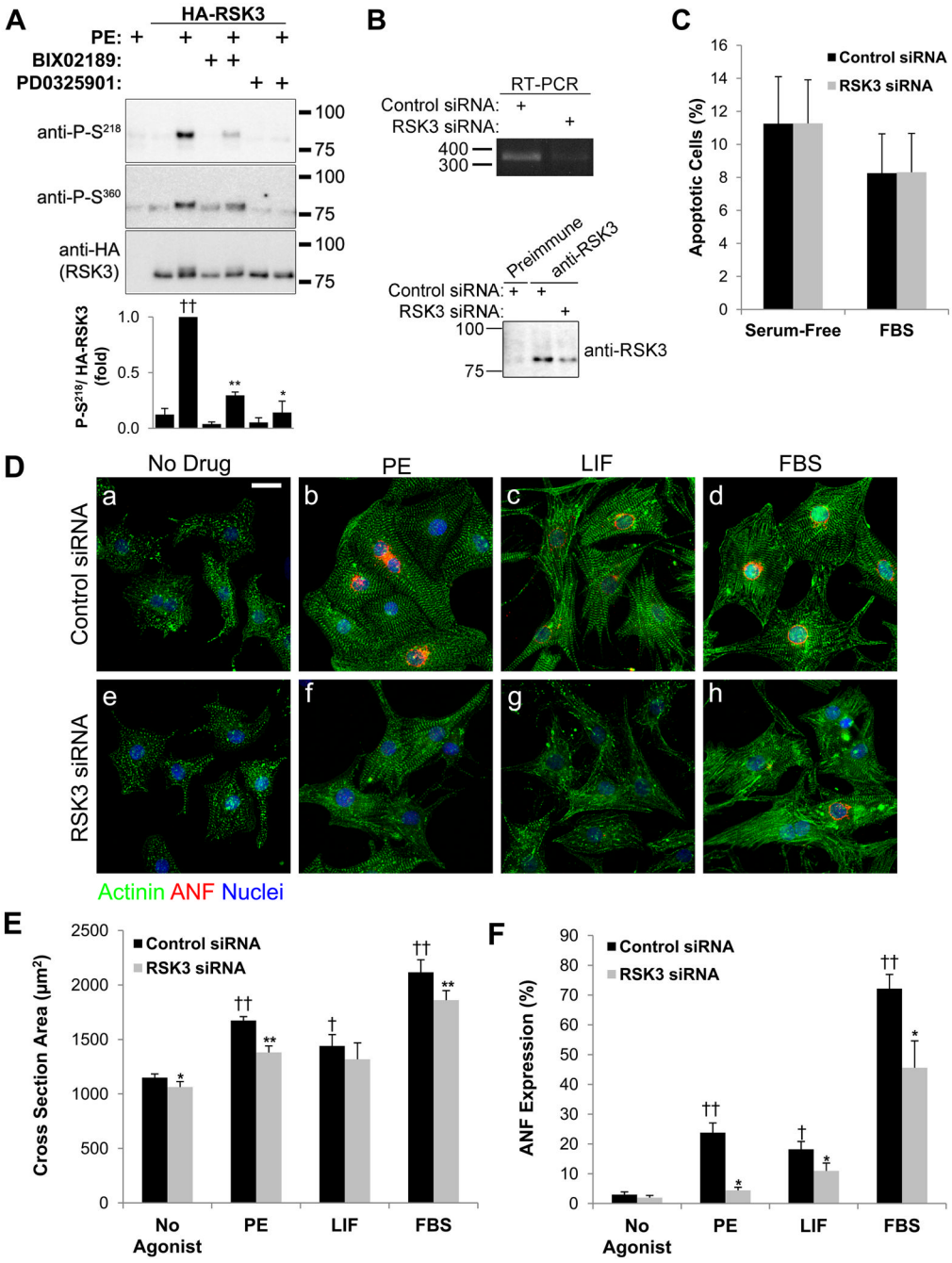


Figure 2. RSK3 signaling is important for neonatal rat ventricular myocyte hypertrophy

A. Neonatal myocytes were infected with Adeno-HA-RSK3 for 1 day in minimal media before being serum-starved for 2 days and then treated for 1 hour with 10 μmol/L PE, 10 μmol/L BIX02189 or 0.1 μmol/L PD0325901. Whole cell extracts were used for immunoblotting. *n*=3. B–F. Myocytes were transfected with control or RSK3 siRNA oligonucleotides and cultured for 2 days ± 10% fetal bovine serum (FBS), 10 μmol/L PE, or 1000 U/mL LIF. B. RSK3 RT-PCR (top panel) and western blot for RSK3 immunoprecipitated with N-16 antibody (bottom panel) using PE-treated myocytes. C. Results obtained by TUNEL staining. *n*=3. D. Immunocytochemistry for α-actinin (green), ANF (red) and Hoechst (blue); bar = 20 μm. Separate ANF and Hoechst channels are

provided in Online Figure III. E. Cross-section area of myocytes. n=4–8. F. Fraction of myocytes expressing ANF. n=4–5. * p-values comparing samples treated with the same hypertrophic agonist. † p-values comparing to no hypertrophic agonist control.

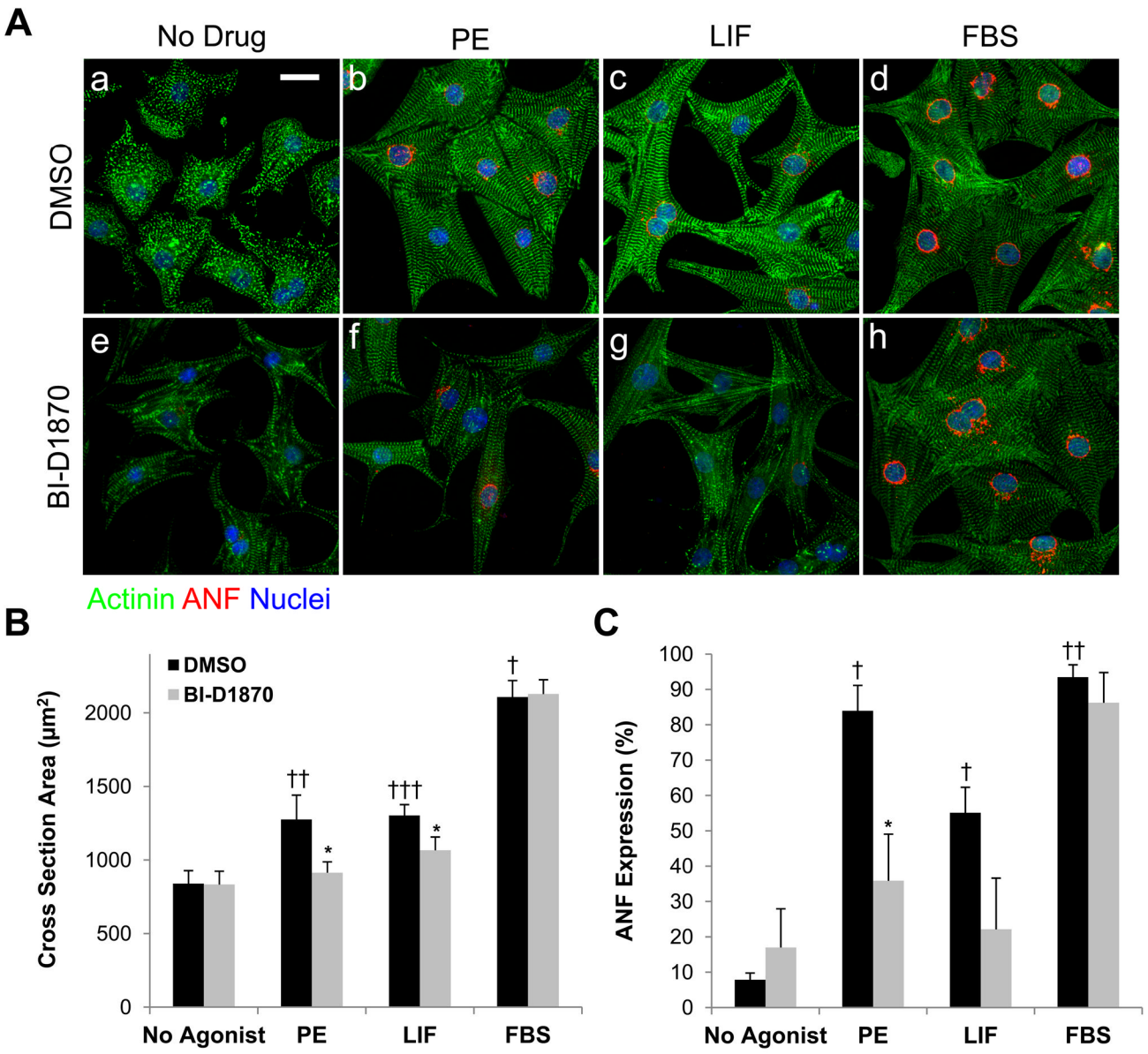


Figure 3. Inhibition of neonatal rat ventricular myocyte hypertrophy with a RSK inhibitor

Myocytes were cultured for 2 days ± 10 μmol/L PE, 1000 U/mL LIF, 10% FBS and/or 10 μmol/L BI-D1870 or 1% DMSO carrier. A. Immunocytochemistry for α-actinin (green), ANF (red) and Hoechst (blue); bar = 20 μm. Separate ANF and Hoechst channels are provided in Online Figure V. B. Cross-section area of myocytes. n=3–7. C. Fraction of myocytes expressing ANF. n=3. * p-values comparing to DMSO. † p-values comparing to no agonist control.

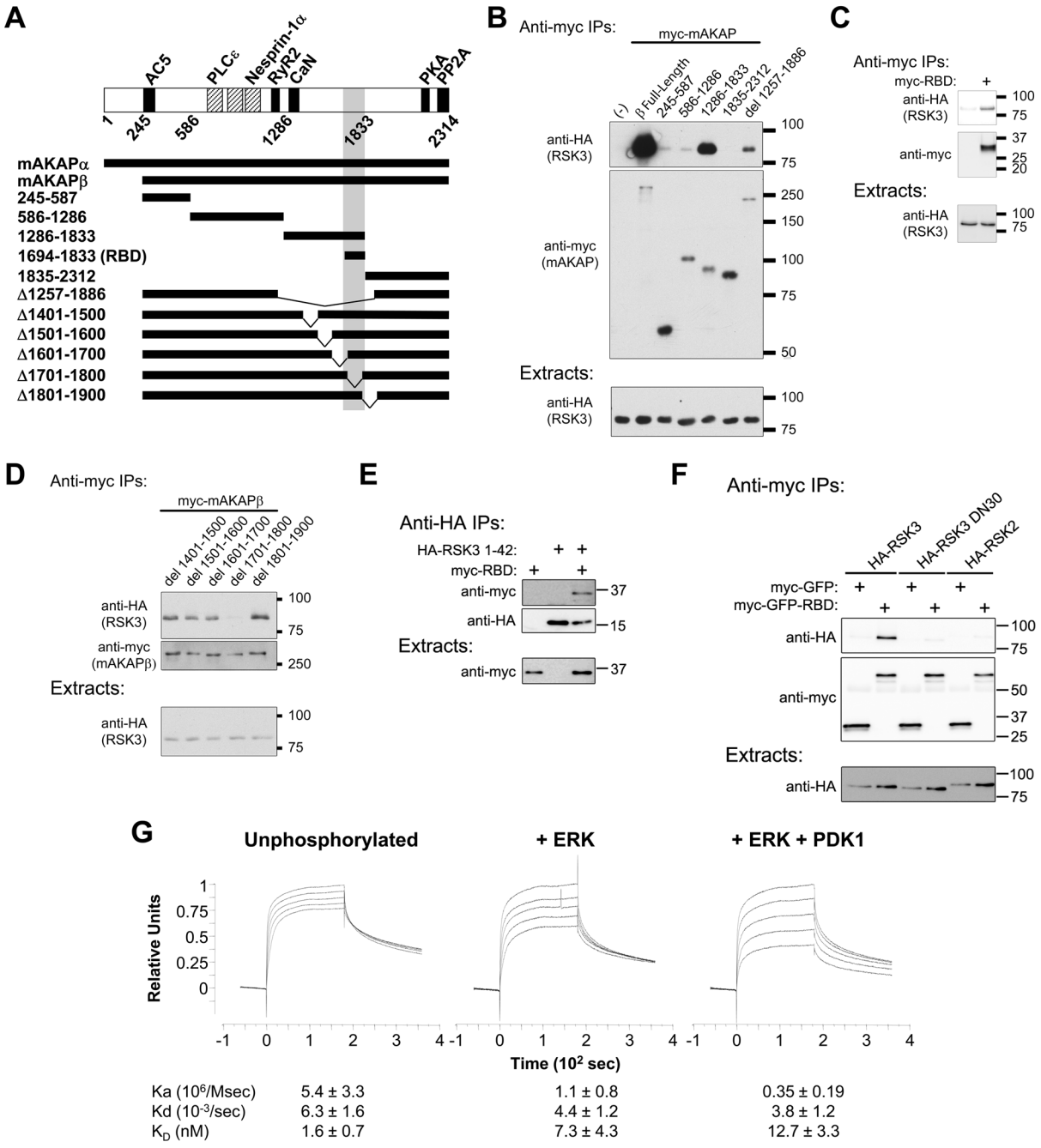
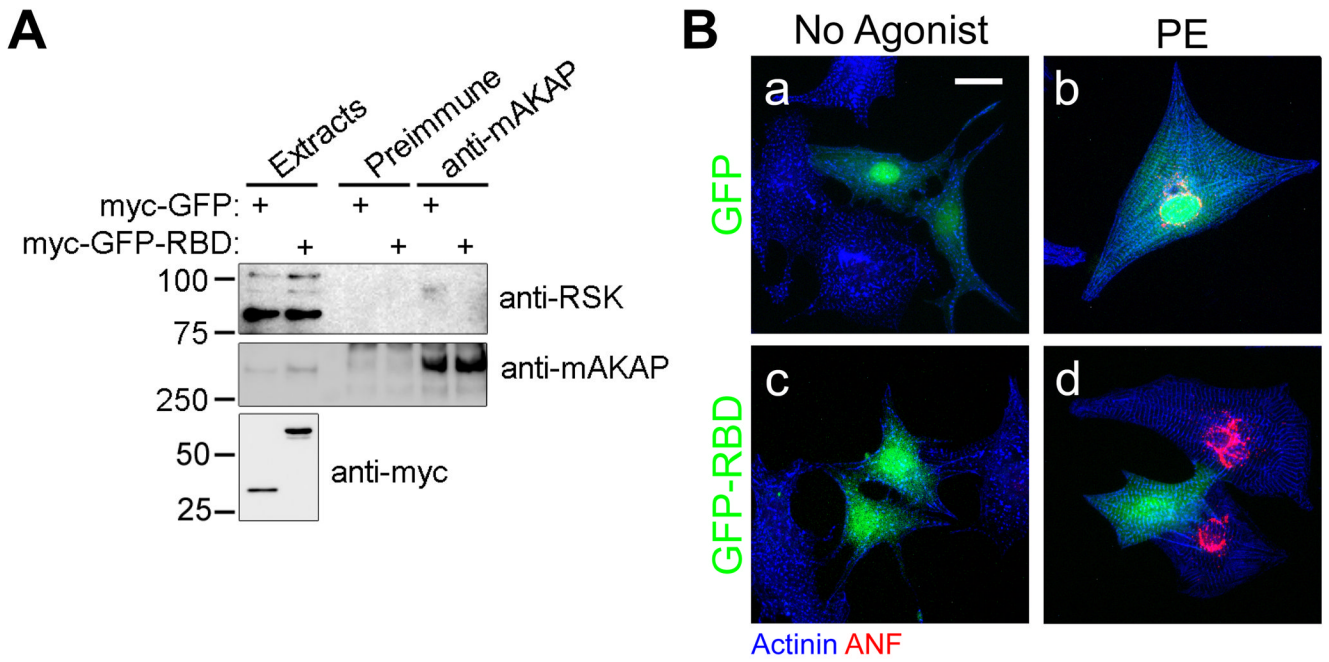


Figure 4. The RSK3 Binding Site within mAKAP

A. mAKAP domain structure. Direct binding partners whose sites have been finely mapped in mAKAPβ are shown.[10, 33, 35–39] mAKAPβ starts at residue 245 of mAKAPα. All fragments are numbered per mAKAPα. The grey bar indicates the RSK3-binding site. B–F. Specific tag antibodies were used to immunoprecipitate myc-tagged mAKAPβ and HA-tagged RSK proteins (cf. Figure 1A) co-expressed in either HEK293 (B) or COS-7 (C–F) cells. n=3 for all panels. G. Surface plasmon resonance using purified, bacterially-expressed proteins. Unphosphorylated (left), ERK-phosphorylated (middle), and ERK and PDK1-phosphorylated (right) His-RSK3 (12.5 – 200 nmol/L in perfusate) were bound to His-

mAKAP 1286–1833 (solid state). Each curve was repeated three times using different protein preparations.



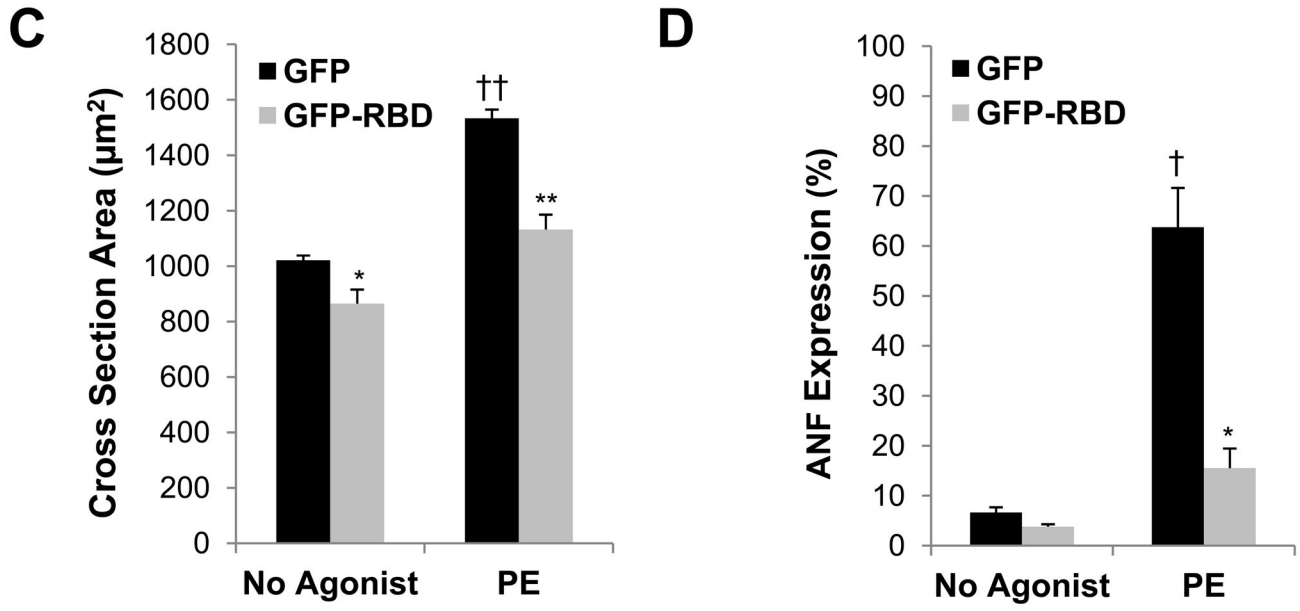


Figure 5. RSK3 anchoring is important for neonatal rat ventricular myocyte hypertrophy

A. mAKAPβ complexes were immunoprecipitated using FL100 mAKAP antiserum from PE-treated, adenovirus-infected 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.

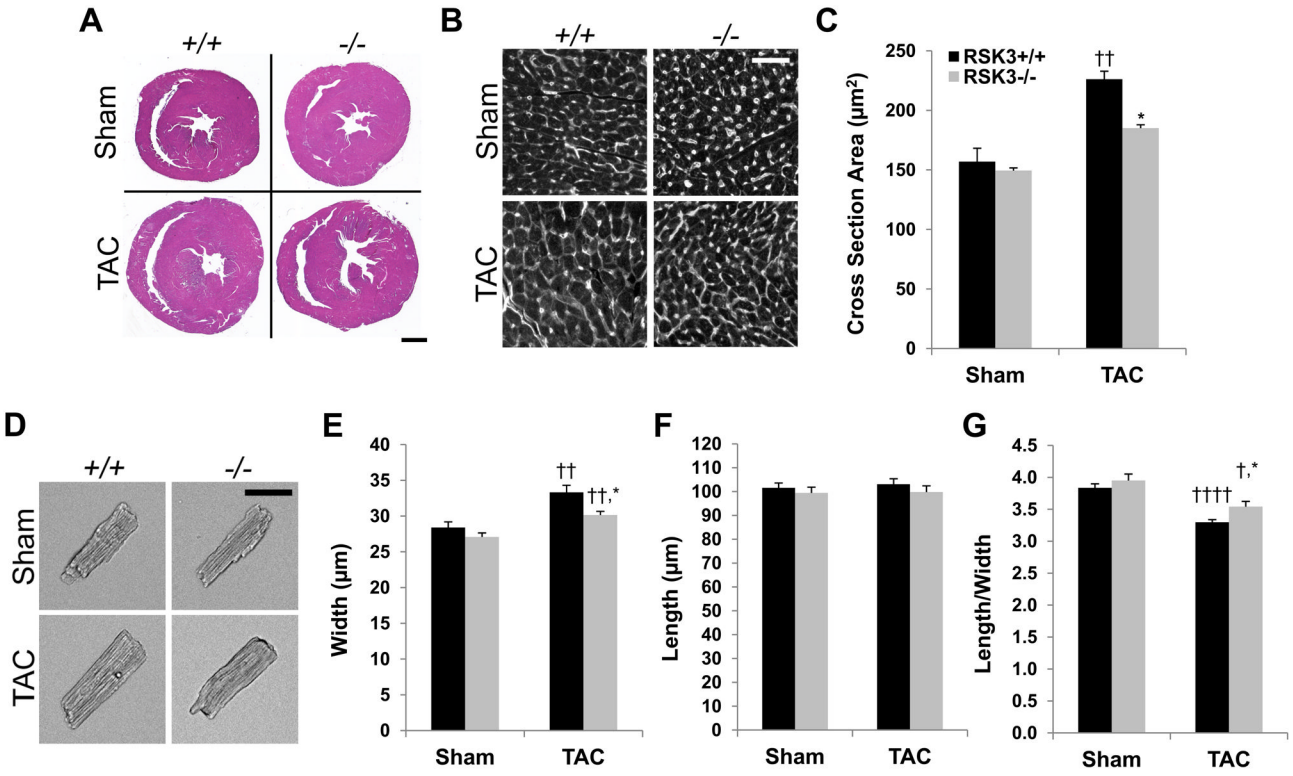


Figure 6. RSK3 knock-out attenuates the effects of pressure overload in vivo

A. Hematoxylin and eosin stained transverse sections. Bar = 1 mm. B. Wheat-germ agglutinin stained sections. Bar = 50 μm. C. Cross-section area of myocytes in tissue sections. *n*=4–9. D. Bright field images of acutely-dissociated adult cardiac myocytes. Bar = 50 μm. Width (E), Length (F) and Length/Width Ratio (G) of isolated myocytes. *n*=5–6. † *p*-values comparing to sham-operated mice of the same genotype; * *p*-values comparing to $RSK3^{+/+}$ TAC mice.

Table 1

Echocardiographic Data for *RSK3*$^{-/-}$ Mice Following TAC

		Sham		TAC	
RSK3		+/+	−/−	+/+	−/−
n		***11***	***11***	***13***	***12***
LVPW;d	mm	0.69 ± 0.03	0.62 ± 0.02	0.94 ± 0.05†††	0.72 ± 0.03$^{\dagger **}$
LVPW;s	mm	1.07 ± 0.04	0.88 ± 0.02**	1.19 ± 0.06	1.01 ± 0.06*
LVAW;d	mm	0.73 ± 0.02	0.64 ± 0.02*	0.97 ± 0.03†††	0.81 ± 0.03$^{\dagger\dagger **}$
LVAW;s	mm	1.00 ± 0.05	0.93 ± 0.04	1.36 ± 0.05†††	1.13 ± 0.05$^{\dagger\dagger **}$
LVID;d	mm	4.19 ± 0.13	4.24 ± 0.07	4.27 ± 0.14	4.26 ± 0.09
LVID;s	mm	3.08 ± 0.14	3.20 ± 0.11	3.26 ± 0.17	3.22 ± 0.10
% FS	%	26.8 ± 1.7	24.5 ± 1.8	24.1 ± 1.9	24.5 ± 1.9
Endocardial Area;d	mm^2	25.4 ± 0.8	25.1 ± 0.5	25.8 ± 0.7	27.0 ± 0.9
Endocardial Area;s	mm^2	18.8 ± 0.6	19.1 ± 0.4	20.5 ± 0.9	20.9 ± 1.0
Endocardial %FAC	%	25.5 ± 1.5	23.8 ± 1.3	20.9 ± 2.0	22.9 ± 1.7
Heart Rate	bpm	473 ± 9	470 ± 8	478 ± 11	471 ± 8
Pressure Gradient	mm Hg			45 ± 3	42 ± 5

M-mode measurements: LVPW, Left ventricular posterior wall thickness; LVAW, Left ventricular anterior wall thickness; LVID, Left ventricular internal diameter; d, diastole; s, systole; % FS, fractional shortening = (LVID;d – LVID;s)/(LVID;d). B-mode measurements: %FAC = (Endocardial Area;d – Endocardial Area;s)/(Endocardial Area;d).

* *p*-values comparing −/− vs. +/+;

† *p*-values comparing TAC vs. Sham-operated for the same genotype.

All data are mean ± sem.

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Table 2

Gene Expression for *RSK3*$^{-/-}$ Mice Following TAC

Gene	Protein	Sham		TAC	
		RSK3$^{+/+}$	***RSK3***$^{-/-}$	***RSK3***$^{+/+}$	***RSK3***$^{-/-}$
Nppa	ANF	1.00 ± 0.05	1.42 ± 0.25	22.42 ± 6.40†	5.70 ± 1.34$^{\dagger *}$
Myh7	β-myosin heavy chain	1.00 ± 0.10	1.07 ± 0.23	15.23 ± 6.35	2.65 ± 0.43†
Acta1	α-skeletal actin	1.00 ± 0.19	1.18 ± 0.18	12.27 ± 3.67†	8.08 ± 1.54††
Nppb	brain naturietic peptide	1.00 ± 0.12	0.84 ± 0.22	4.06 ± 0.68††	3.24 ± 0.88†
Tgfb2	transforming growth factor β2 four and a half LIM domains	1.00 ± 0.09	0.97 ± 0.17	2.77 ± 0.42††	1.37 ± 0.07*
Fhl1	1	1.00 ± 0.12	1.01 ± 0.08	2.05 ± 0.40†	1.22 ± 0.17
Rcan1	regulator of calcineurin 1	1.00 ± 0.09	0.85 ± 0.06	1.91 ± 0.38†	1.22 ± 0.18
Col6a1	collagen VI α1	1.00 ± 0.06	0.90 ± 0.08	1.56 ± 0.08†††	1.28 ± 0.08$^{\dagger *}$
Hif1a	hypoxia inducible factor 1α	1.00 ± 0.06	1.09 ± 0.12	1.43 ± 0.10†	1.34 ± 0.07
Slc8a1	sodium/calcium exchanger 1	1.00 ± 0.13	0.98 ± 0.08	1.07 ± 0.07	0.93 ± 0.08
Mapk3	ERK1	1.00 ± 0.06	0.96 ± 0.07	1.06 ± 0.04	1.09 ± 0.04
Dmd	dystrophin	1.00 ± 0.09	1.09 ± 0.08	1.05 ± 0.08	1.06 ± 0.08
Tnnc1	cardiac troponin C	1.00 ± 0.06	1.10 ± 0.05	1.00 ± 0.10	1.16 ± 0.06
Rps6ka3	RSK2	1.00 ± 0.04	0.91 ± 0.05	0.94 ± 0.07	1.05 ± 0.05
Srf	serum response factor	1.00 ± 0.05	0.97 ± 0.05	0.90 ± 0.06	1.07 ± 0.06
Vcl	vinculin	1.00 ± 0.05	0.96 ± 0.06	0.89 ± 0.04	0.97 ± 0.05
Actc1	α-cardiac actin	1.00 ± 0.05	0.92 ± 0.02	0.86 ± 0.08	0.95 ± 0.05
Akap6	mAKAP	1.00 ± 0.10	0.93 ± 0.06	0.86 ± 0.05	0.95 ± 0.09
Itgb1bp2	melusin	1.00 ± 0.07	1.00 ± 0.03	0.74 ± 0.09†	0.88 ± 0.02†
Tnnt2	cardiac troponin T	1.00 ± 0.06	1.08 ± 0.05	0.73 ± 0.05†	0.85 ± 0.05†
Ryr2	ryanodine receptor	1.00 ± 0.10	0.93 ± 0.09	0.73 ± 0.10	0.85 ± 0.08
Mapk1	ERK2	1.00 ± 0.03	1.03 ± 0.06	0.73 ± 0.06††	0.96 ± 0.03*
Max	myc-associated factor X	1.00 ± 0.04	0.97 ± 0.10	0.70 ± 0.05††	0.88 ± 0.06*
Ckm	creatine kinase	1.00 ± 0.04	0.94 ± 0.05	0.69 ± 0.08†	0.93 ± 0.05*
Myh6	α-myosin heavy chain	1.00 ± 0.12	0.91 ± 0.08	0.62 ± 0.09†	0.77 ± 0.09

Gene	Protein	Sham		TAC	
		RSK3$^{+/+}$	***RSK3***$^{-/-}$	***RSK3***$^{+/+}$	***RSK3***$^{-/-}$
Adra1a	α_{1a}-adrenergic receptor	1.00 ± 0.13	1.14 ± 0.22	0.60 ± 0.10†	1.03 ± 0.11*
Pln	phospholamban	1.00 ± 0.07	1.19 ± 0.09	0.60 ± 0.09†	0.88 ± 0.06$^{\dagger *}$
Mapk7	ERK5	1.00 ± 0.05	0.91 ± 0.03	0.58 ± 0.06††	0.72 ± 0.07†
Atp2a2	SERCA2	1.00 ± 0.10	0.94 ± 0.05	0.54 ± 0.07†	0.77 ± 0.06$^{\dagger *}$
Tnni3	cardiac troponin I	1.00 ± 0.09	1.08 ± 0.06	0.52 ± 0.09††	0.78 ± 0.07$^{\dagger *}$

Total mouse heart RNA was assayed by NanoString technology for the indicated mRNAs and normalized by the data for GAPDH. All data (mean ± s.e.m.) are fold-expression compared to the Sham, +/+ cohort. $n = 5$ for all cohorts.

$^{*}p$-values comparing −/− vs. +/+;

$^{\dagger}p$-values comparing TAC and Sham-operated for the same genotype. Analysis of the complete dataset showed that gene expression for the Sham and the TAC *RSK3*$^{-/-}$ cohorts were highly correlated (Pearson $r \geq 99\%$); in contrast, the TAC *RSK3*$^{+/+}$ dataset diverged from the other three cohorts ($r < 93\%$). ANOVA (two factor with replication) comparing the TAC datasets: $p = 0.05$ for −/− vs. +/+; $p = 3.3 \times 10^{-14}$ for interaction between genes and cohorts.