NIH Public Access
Author Manuscript
J Mol Cell Cardiol. Author manuscript; available in PMC 2012 August 26.

Published in final edited form as:
J Mol Cell Cardiol. 2009 October ; 47(4): 520–527. doi:10.1016/j.yjmcc.2009.06.006.

Hic-5 is required for fetal gene expression and cytoskeletal organization of neonatal cardiac myocytes.

Erin Yund[1], **Joseph A. Hill**[2], and **Rebecca S. Keller**[1,3]
[1]Center for Cardiovascular Science, Albany Medical College, Albany, NY
[2]Departments of Internal Medicine (Cardiology) and Molecular Biology, University of Texas Southwestern Medical Center, Dallas

Introduction

Pathological hypertrophy is one of the earliest predictors of heart failure. While it is often the result of sustained mechanical load as in hypertension, it can also be induced or worsened by elevations in circulating levels of angiotensin II (Ang-II) or endothelin-1 (ET-1), increased sympathetic nerve activity, or addition of exogenous adrenergic agonists such as phenylephrine (PE). Pathological hypertrophy is associated with an increase in the size and protein content of cardiomyocytes, as well as re-expression of a fetal gene program, which includes upregulation of atrial naturetic factor (ANF), α-skeletal actin and β-myosin heavy chain genes.

While many signaling pathways have been reported integral to this process, an increasing number of studies have implicated cardiac costameres as signaling hotspots in the regulation of hypertrophy and fetal gene reprogramming [1,2]. Costameres are the site of physical linkage of the extracellular matrix to the contractile sarcomere at the z-disc. They are rich in proteins including β1 integrin, focal adhesion kinase (FAK), integrin linked kinase (ILK), talin, vinculin, and paxillin. Knockdown and inhibitory studies of costmeric proteins have indicated a critical role for these proteins in the maintenance of structural integrity [3-5] as well as in the regulation of cardiac hypertrophy [6-9] and function [10,11].

The mechanism by which integrins and their associated proteins affect cardiac hypertrophy is an active area of investigation. The scaffolding protein, paxillin, has been reported to be phosphorylated and localize to cardiac costameres with PE treatment [9]. Paxillin also has been shown to reorganize in a sarcomeric pattern in transgenic mouse models of dilated cardiac hypertrophy including tropomodulin overexpression [12] and constitutively active rac [13]. While paxillin would appear to be a potential candidate for the regulation of cardiac hypertrophy, a direct role has not been tested.

Paxillin is a member of a superfamily of proteins, which also includes hic-5 and leupaxin. Leupaxin is thought to be exclusively expressed in leukocytes, but paxillin and hic-5 have been identified in a variety of tissues [14]. Unlike paxillin, hic-5 has been reported to be

© 2009 Elsevier Ltd. All rights reserved.

[3]To whom to address correspondence Address: Rebecca S. Keller, PhD 47 New Scotland Ave. MC8 Center for Cardiovascular Science Albany Medical College Albany, NY 12208 (518)262-8105 (518)262-8101 (fax) kellerre@mail.amc.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

absent from adult human cardiac muscle according to one study [15], however it has been identified in the developing mouse embryonic heart [16]. Paxillin and hic-5 share 55% amino acid identity and 72% similarity and are thought to have both analogous and antagonistic functions [17]. Both proteins have been shown to interact with numerous proteins through leucine rich LD motifs and/or double zinc finger LIM domains [14]. Paxillin has five LD and four LIM domains and has been shown to have multiple binding partners, including cytoskeleton associated molecules (vinculin, actopaxin), and signaling molecules (FAK, Pyk2, ILK, PIX/PKL/PAK and ERK). Hic-5 shares four of the five LD domains, and has been shown to have similar binding partners as paxillin.

Based on reports suggesting altered paxillin localization and phosphorylation with hypertrophic stimuli [9,12,13], we hypothesized that paxillin family members play a role in the regulation of the hypertrophic process. We report for the first time expression of hic-5 in neonatal rat cardiac myocytes localized at the ends of actin fibers and cardiac z-discs. In addition, we demonstrate upregulation of hic-5, not paxillin after PE treatment. Our results demonstrate that hic-5 overexpression is sufficient to induce cytoskeletal organization and required for PE induced regulation of both fetal gene expression and cytoskeletal organization.

Materials and Methods

Neonatal Rat Ventricular Myocytes (NRVM)

Cell cultures consisting of >95% ventricular myocytes were obtained by enzymatic digestion of neonatal rat hearts from 1 day old Sprague-Dawley pups. Digestion was followed by discontinuous Percoll gradient as previously described [6]. Freshly isolated NRVM were plated overnight in Dulbeccos Modified Eagles Medium (DMEM) containing 10% fetal bovine on fibronectin (10 μg/ml) coated slides at a density of 100 cells/mm^2 or 6-well plates at a density of 250 cells/mm^2. These procedures conform to the *Guide for the Care and Use of Laboratory* Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996).

siRNA Transfection

NRVM were washed in Opti-MEM media (Invitrogen). siRNA duplexes were allowed to incubate with Oligofect-AMINE transfection reagent (Invitrogen) in Opti-MEM for 10 minutes before addition to cells. siRNA/Oligofect-AMINE mixture was added to cells at a final concentration of 10 nM. Post transfection (24 hr) media was changed to serum free DMEM for an additional 48 hours. Pxn is a siGENOME ON-TARGETplus SMARTpool (11-14) for rat paxillin from Dharmacon. Hic-5 is also a siGENOME ON-TARGETplus SMARTpool (11-14) for rat TGFB1I1 (Hic-5) (9-12) from Dharmacon.

Hic-5 Adenovirus

Cardiac myocytes were infected with GFP or hic-5 adenovirus (MOI 2) for 48 hours in DMEM. Hic-5 adenovirus was a gift from Drs. David Tumbarello and Chris Turner.

RNA Extraction and cDNA Synthesis

NRVM were lysed in Trizol (Invitrogen) and stored at -80°C overnight. RNA was isolated following the Invitrogen Trizol protocol. RNA was subsequently treated with DNAse (6.65U/μL, 30 min, 37°C) and heat inactivated at 65°C (30 min). cDNA was synthesized using Ready-to-go beads (Amersham) and Random Hexamer Primers (Invitrogen).

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Analysis of Fetal Gene Expression

Changes in expression of ANF α-skeletal actin and β-MyHC mRNA in NRVM were measured using quantitative RT-PCR Bio-Rad iCYCLER system. GAPDH (GDH) was used as a housekeeping gene for normalization. Mastermixes contain SYBER-Green (2x, Bio-Rad), and specific primers for ANF f(CAACACAGATCTGATGGATT), r(TTCAAGAGGGCAGATCTATCG), β-MyHC f(GGCCTGAATGAAGAGTAGAT), r(GTGTTTCTGCCTAAGGTGCT), α-skeletal actin f(CGTCACCAGGGTGTCATGG, r(TGTAGAAGGTGTGGTGCCAGAT) and GAPDH f(TGATGACATCAAGAAGGTGG), r(TGTCATACCAGGAAATGAGC) were used. ANF, α-skeletal actin, β-MyHC and GAPDH levels were extrapolated off a standard curve. ANF α-skeletal actin and β-MyHC levels were normalized to GAPDH.

Western Blot Analysis

Protein expression was validated by western blot analysis. NRVM were lysed in RIPA buffer (156.5mM NaCl, 10mM Tris, 1mM EGTA, 0.1% SDS, 1% Na-deoxycholate, 1% TritonX-100, pH 7.2) containing Complete Protease Inhibitors (Roche) and HALT phosphatase inhibitors (Thermo Scientific). Proteins were separated on 10% SDS-PAGE, transferred to nitrocellulose and blocked in 5% milk/TBST (Tris-buffered saline, 1% Tween-20) or 5% BSA/TBST solution. Antibodies used for western blotting include paxillin (clone 349) and hic-5 (clone 34) (BD-Transduction), α-actinin (Sigma), ERK1/2, phospho-ERK1/2, histone-3 (Cell Signaling Technology), and paxillin (Y118) (Chemicon).

Immunofluorescence (IF)

NRVM were washed and fixed (15 min) in Cytoskeletal Stabilizing Buffer (10mM MES, 138mM KCl, 3mM MgCl, 2mM EGTA, 12% sucrose, 4% paraformaldehyde) for 15 minutes. Cells were washed, and blocked in blocking buffer (137mM NaCl, 3mM KCl, 20mM Na_2HPO_4, 2mM KH_2PO_4 0.05%, goat serum, 0.003% Triton-X 100) for 1 hour. Again cells were washed and incubated with primary antibody in antibody dilution buffer (137mM NaCl, 3mM KCl, 20mM Na_2HPO_4 2mM KH_2PO_4, 1% BSA, 0.003% triton-X 100) and incubated overnight (4°C). Cells were then washed in PBS, and incubated in secondary Alexa 488 or 594 antibodies (Molecular Probes, 1hr). Rhodamine-Phallodin stain was then added (20 minutes) for staining of actin. Coverslips were mounted using Vectashield Hard Set Mounting Media with DAPI. Slides were imaged using Zeiss Axio Observer microscope (Figure 2A, 3A) or Zeiss LSM 510 META confocal microscope (Figure 2B-D). Antibodies used for IF include paxillin clone PCX-10, and alpha-actinin (Sigma), hic-5 (clone 34) (BD-transduction), paxillin (Y118) (Chemicon), and ANF (a gift from Joe Loftus, Mayo Clinic, Arizona).

Pressure Overload Model

This model has been previously described in detail [18]. Briefly, male C57BL6 mice (6–8 wk old) were subjected to pressure overload by thoracic aortic constriction (TAC) using protocols approved by the University of Texas Southwestern Animal Care and Use Committee. The constriction was placed in the transverse aorta between the innominate and left common carotid arteries. TAC and sTAC were induced to 27 and 28-gauge stenosis, respectively. It has been previously shown that constriction to a 27-gauge stenosis induces moderate hypertrophy (≈40% increase in heart mass) without clinical signs of heart failure or malignant ventricular arrhythmia and that severe, decompensated hypertrophy (sTAC) is induced by banding the thoracic aorta to a 28-gauge diameter [18]. At 3 wk, integrity of aortic banding was confirmed by inspection of the surgical constriction and by visualization of marked differences in caliber of the right and left carotid arteries. Protein was isolated from 6 hearts; sham (2), TAC(2), sTAC(2) for western blot analysis. Approximately 100 mg

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

of cardiac tissue from the left ventricular free wall was homogenized in RIPA buffer (1 ml) containing protease inhibitors. Protein content was assessed and protein (50 μg) was used for western blot analysis.

Statistics

Data presented in Figure 2, 4 and 6 were analyzed by a one sample t-test. “*” indicates $p<0.05$ between groups. Data presented in Figure 3 was analyzed by t-test, where “*” indicates $p<0.05$. Data presented in Figures 5 and 7 were analyzed by 2-way ANOVA. “*” $p<0.05$ (effect of phenylephrine) “#” indicates $p<0.05$ (effect of siRNA) using Bonferroni post-test.

Results

Hic-5 co-localizes with paxillin and α-actinin in NRVM

Western blot analysis indicated the presence of paxillin family members, paxillin and hic-5, in neonatal rat ventricular myocyte (NRVM) lysates. As this is the first time hic-5 has been identified in neonatal cardiac myocytes, we examined its localization by immunofluorescence. Hic-5 was present in sarcomeric patterns throughout the cells and co-localized with the ends of many actin filaments (arrows) (Figure 1A). To determine if the sarcomeric hic-5 immunostaining was specifically localized to the z-disc, we overexpressed GFP tagged hic-5 in NRVM by adenoviral infection, and immunostained GFP-hic-5 expressing cells with an antibody to the z-disc protein, α-actinin. α-Actinin and hic-5 were co-localized, confirming the presence of GFP-hic-5 in the z-discs (Figure 1B). As sarcomeric staining of paxillin also has been reported in cardiac myocytes, in the context of hypertrophic stimulation [2,9,12], we hypothesized that paxillin and hic-5 might be co-localized. To test this, we co-stained NRVM with antibodies to hic-5 and phospho-Y118 paxillin (pY118-pxn). The pY118-pxn antibody was chosen for specificity in the detection of paxillin and not hic-5 (Supplemental Data 1), with the limitation that non-Y118 phosphorylated paxillin would not be detected. By confocal microscopy, we determined that pY118-pxn and hic-5 were co-localized in a sarcomeric pattern (Figure 1C). We validated pY118 paxillin localization at the z-disc by co-staining NRVM with antibodies recognizing pY118-pxn and α-actinin. Our results indicated that pY118-pxn and α-actinin were in fact co-localized (Figure 1D). Together, these findings implicate the presence of both paxillin and hic-5 at the z-disc.

PE upregulates hic-5, not paxillin, in NRVM

Stimulation of NRVM with PE (48 hours) results in enhanced cytoskeletal organization and upregulation of fetal genes. In addition, PE treatment results in increased expression of many proteins involved in hypertrophy. We examined the effect of PE on both hic-5 and paxillin levels. We found that PE treatment (100 μM, 48 hours) resulted in increased expression of hic-5 levels as compared to untreated cells (Figure 2A). We examined paxillin levels using the paxillin antibody (clone 349), which has been found to cross-react with hic-5 in various cell types [15,19-21]. We further validated this crossreactivity by overexpressing and knocking down paxillin and hic-5 in NRVM (Supplemental Data 2 and Figure 4). The paxillin antibody (clone 349) detected two bands in NRVM lysates; one at 68 kDa, the expected molecular weight of paxillin, and one band slightly lower (between 50 and 60 kDa) (Figure 2C). We found that expression of the upper band (paxillin) was not affected by PE treatment (Figure 2D). In contrast, the lower band (hic-5) increased expression after PE treatment (Figure 2E). This data suggests that hic-5, but not paxillin is specifically upregulated with PE treatment.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Hic-5 overexpression increases cytoskeletal organization

Based on the close association of hic-5 with z-disc proteins and actin filaments, in addition to the increased hic-5 expression observed with PE treatment, we hypothesized that overexpression of hic-5 might be sufficient to induce cytoskeletal organization. To test this, cells plated overnight were infected with GFP alone or GFP tagged hic-5 adenovirus for 48 hours, and then fixed and stained with phalloidin antibody to visualize actin organization. Representative cells are shown (Figure 3A). The percentage of cells with organized actin cytoskeleton was quantified (Figure 3B). We found that hic-5 overexpression increased the number of cells that were organized as compared to GFP control.

Validation of hic-5 or paxillin knockdown in NRVM by siRNA transfection

To determine the endogenous role of hic-5 and paxillin in NRVM, we individually knocked down paxillin and hic-5 by transfection with siGenome On-Target Plus Smartpool (10 nM, 72 hours). siRNA to firefly luciferase (luc, 10 nM) was used as a control. To confirm paxillin and hic-5 knockdown, NRVM lysates were immunoblotted for both paxillin and hic-5 using paxillin antibody (clone 349). Transfection with paxillin siRNA (pxn) resulted in a decrease in intensity of the upper band (paxillin) (Figure 4 A and B) but had no effect on the intensity of the lower band (hic-5) detected by the paxillin antibody (Figure 4 A and C). Similarly, transfection with hic-5 siRNA resulted in a decrease in the intensity of the lower band detected with paxillin antibody (clone 349), without having a significant effect on the intensity of the upper band (Figure 4 A, B and C). Hic-5 knockdown was also validated in these samples using a specific hic-5 antibody (Figure 4 D and E). These data further implicate paxillin antibody (clone 349) crossreactivity with hic-5. In addition, these results demonstrate specific knockdown of paxillin or hic-5 in NRVM by siRNA transfection.

Hic-5 not paxillin regulates PE induced cytoskeletal organization and fetal gene expression

To determine if endogenous hic-5 or paxillin was required for PE induced changes in cytoskeletal organization and hypertrophic gene expression, cells were plated overnight, and hic-5 or paxillin siRNA was transfected. Seventy-two (72) hours post-transfection, cells were subsequently treated with PE (100 μM, 48 hrs). Cells were immunostained with antibodies recognizing ANF (a hypertrophic marker) and phalloidin to visualize actin organization and imaged by confocal microscopy. Representative myocytes are shown (Figure 5A). The percentage of cells with an organized cytoskeleton in each group was quantified (Figure 5B and C). PE resulted in an increase in the percentage of cells with organized actin in luc siRNA transfected cells. Paxillin knockdown had no effect on PE induced cytoskeletal organization. In contrast, hic-5 knockdown significantly reduced the ability of PE to organize the actin cytoskeleton. We also assessed the effect of hic-5 and paxillin knockdown on ANF expression (Figure 5D and F). We found hic-5 knockdown significantly decreased the percentage of ANF positive cells after treatment with PE. However, there was no significant effect of paxillin knockdown on the percentage of cells with ANF positive nuclei after treatment with PE. Finally, to exclude the possibility of a toxic effect of any siRNAs, specifically hic-5, a TUNEL assay was performed. There was no significant difference in the number of TUNEL positive cells in hic-5 or paxillin knockdown cells as compared to luc control (data not shown).

To test whether hypertrophic markers were being affected at a message level, we measured changes in gene expression of ANF, α-skeletal actin and β-myosin heavy chain by quantitative PCR. Our data show that hic-5 knockdown significantly inhibited ANF and α-skeletal actin mRNA induction and had variable or no effect on β-myosin heavy chain mRNA levels (Figure 6).

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

Hic-5 is downstream of ERK1/2 signaling

ERK1/2 has been implicated by a number of groups as a regulator of fetal gene expression and sarcomeric organization [22,23]. Based on our results above, we hypothesized that knockdown of hic-5 would inhibit PE induced ERK1/2 signaling. In support of this hypothesis, hic-5 has been implicated in the regulation of ERK1/2 phosphorylation in other cell types [24-26]. Myocytes transfected with luc or hic-5 siRNA (72 hrs) were treated with PE (100 μM, 10 min) and lysed for protein. Western blot analysis of phospho-ERK1/2 and ERK1/2 showed no significant change in PE stimulated ERK1/2 phosphorylation with hic-5 knockdown (Figure 7A and B), indicating that the effects of hic-5 on hypertrophic phenotype are ERK1/2 independent. To determine if hic-5 was downstream of ERK1/2 signaling, cells were pretreated with the MEK inhibitor U0126 (10 μM), then treated with PE (100 μM, 48 hours). We found that while PE treatment for 48 hours induced hic-5 upregulation, MEK inhibition completely prevented the PE induced increase in hic-5 levels (Figure 7C and 7D). This suggests that ERK1/2 signaling is required for hic-5 upregulation.

Hic-5 is re-expressed in a model of decompensated cardiac hypertrophy

Based on the results obtained using NRVM, we hypothesized that hic-5 expression might be altered in an *in vivo* model of cardiac hypertrophy. We examined paxillin and hic-5 expression levels in two different adult mouse models of pressure overload induced cardiac hypertrophy (described in Materials and Methods). As previously described, three weeks of TAC results in compensated hypertrophy, characterized by a 40% increase in heart mass, while severe TAC (sTAC) results in decompensated dilated hypertrophic phenotype [18]. Equal amounts (50 μg) of protein lysates from sham, TAC and sTAC hearts were immunoblotted for paxillin and hic-5 protein using hic-5 and paxillin antibody (clone 349). Histone-3 was used as a loading control. Using the hic-5 antibody, we detected an increase in hic-5 levels, specifically under sTAC condtions. Immunoblotting with the paxillin antibody revealed an increase in intensity of the upper band (paxillin) with TAC and sTAC, as well as upregulation in expression of the lower band (hic-5) specifically under sTAC conditions.

Discussion

Changes in paxillin localization and phosphorylation have been investigated in both *in vivo* and *in vitro* models of cardiac hypertrophy [9,12-13]. In contrast, expression patterns of the highly homologous paxillin family member hic-5 have not been explored. In this study, we have identified hic-5 in neonatal cardiac myocytes (NRVM) and characterized its subcellular localization. Our results indicate a MEK1/2 dependent upregulation of hic-5 expression by PE. Furthermore, we have discovered a critical role for hic-5, not paxillin, in the regulation of PE induced fetal gene expression and actin organization of NRVM. Finally, we provide evidence of hic-5 re-expression in an adult model of severe, decompensated cardiac hypertrophy.

As this is the first time hic-5 has been identified in neonatal cardiac myocytes, we examined its localization by immunofluorescent microscopy. We found hic-5 distributed in a sarcomeric pattern as well as co-localized at the ends of actin filaments. GFP-hic-5 was co-localized with α-actinin, suggesting the presence of hic-5 at the z-disc. As paxillin also has been described to be sarcomeric, we hypothesized that hic-5 and paxillin were co-localized. Our data shows endogenous co-localization of hic-5 and phospho-Y118 paxillin. Additionally, we found phospho-Y118 paxillin to co-localize with α-actinin, a z-disc protein. Together, this data implies the presence of both hic-5 and paxillin at the z-disc. While paxillin and hic-5 are not thought to interact, they are highly homologous and share many similar binding partners. Their co-expression at the z-disc and cell periphery might

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

even imply that the two proteins compete for binding partners. Future studies will be needed to further understand this relationship.

Phoshpho-Y118 paxillin was chosen for immunofluorescent detection of paxillin based solely on its specificity in detection of paxillin, and not hic-5. We considered paxillin antibody (clone 349) inappropriate for paxillin immunostaining due to the cross-reactivity with hic-5 that was previously reported [15,19-21]. We confirmed this crossreactivity by showing that knockdown of paxillin by siRNA transfection reduces the intensity of the 68 kDa band and knockdown of hic-5 reduced the intensity of the 55 kDa band. Detection of GFP-tagged hic-5 with this antibody (Supplementary Data 1) further implicates a crossreaction with hic-5. Several other paxillin antibodies were tried, including clone PCX-10 which we found detected paxillin in other cell types, but only weakly reacted with paxillin in NRVM (Supplementary Figure 3) and could not be reduced by paxillin siRNA transfection (data not shown).

PE stimulation results in increased expression of numerous proteins that have been shown to enhance or inhibit the hypertrophic process. Our results demonstrate that PE increases hic-5 expression while having no effect on paxillin levels. This suggested to us that hic-5 might play a role in the regulation of changes associated with cardiac hypertrophy. Hic-5 has been shown to be upregulated in response to oxidative stress or TGF-β stimulation in other cell types [27] although the exact mechanism by which this occurs is not fully understood. Recently it was reported that hic-5 upregulation was dependent on a signaling pathway involving RhoA/ROCK [28]. Our results indicate that hic-5 upregulation is downstream of MEK1/2-ERK1/2 activation, suggesting involvement of a common downstream target of both these pathways. Interestingly, algorithms have predicted a SRF binding site in the promoter region of hic-5 [29]. Because SRF target genes have been shown to be downstream of both RhoA and ERK1/2 pathways [30], we hypothesize that SRF would be a likely candidate for the regulation of hic-5, although this has not been tested.

To see if increased expression of hic-5 is sufficient to induce changes in cytoskeletal organization, we expressed a GFP-tagged hic-5 in NRVM. We found that overexpression of hic-5 increased the percentage of cells with organized cytoskeleton. Interestingly not all cells expressing GFP-hic-5 were organized, suggesting that hic-5 requires the help of additional proteins that may not be activated under un-stimulated conditions.

To determine the endogenous roles of paxillin and hic-5 in PE induced hypertrophy, we specifically knocked down hic-5 and paxillin in NRVM. Our data show that hic-5 knockdown significantly impaired PE induced actin organization while paxillin knockdown had no significant effect. There was no significant difference in basal (-PE) percentages of organized cells between groups. In order to rule out a toxic effect of hic-5 knockdown, we assessed whether these cells were undergoing cell death. There was no significant difference in the number of TUNEL positive cells (data not shown).

We also assessed the ability of hic-5 to regulate fetal gene re-expression. Our data show hic-5 is required for the induction of ANF protein expression, while paxillin knockdown did not significantly affect PE induced ANF protein expression. Further analysis of gene expression by qPCR revealed that hic-5 was required for ANF and skeletal actin mRNA upregulation, however did not have a -myosin significant heavy chain induction, indicating that hic-5 is not required for re-expression of all hypertrophic genes.

Mechanistically it is still unclear how hic-5 affects cytoskeletal organization and fetal gene expression of NRVM. In an effort to elucidate the mechanism by which hic-5 affected fetal gene expression and cytoskeletal organization, we assessed ERK1/2 phosphorylation. ERK1/2 phosphorylation has been demonstrated by numerous groups to be an important

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

regulator of fetal genes as well as cytoskeletal organization [22,23]. Despite reports linking hic-5 to the regulation of ERK1/2 phosphorylation in other cell types [24-26], we see no significant effect of hic-5 knockdown on ERK1/2 phosphorylation in response to PE. One interpretation of this data might be that hic-5 affects cytoskeleton and gene expression independent of ERK1/2. That is, the effects of hic-5 and ERK1/2 are additive. Alternatively hic-5 also might be acting downstream of ERK1/2. We demonstrated that the increase in hic-5 protein expression after PE is downstream of ERK signaling using the MEK1/2 inhibitor U0126. U0126 treatment resulted in complete inhibition of hic-5 upregulation by PE. We hypothesize that this increase in hic-5 expression is responsible for cytoskeletal changes mediated by PE. However one can not rule out the possibility that hic-5 has basal effects as well. The proposed abilities of hic-5 to act as a regulator of transcription in the nucleus as well as a scaffold for cytoskeletal proteins leave many possibilities for mechanisms by which hic-5 may exhibit its effects.

Finally, we examined the expression patterns of hic-5 and paxillin in two different adult mouse models of cardiac hypertrophy (induced by TAC and sTAC). We found hic-5 to be re-expressed in a severe, decompensated model of cardiac hypertrophy (sTAC) with no change in hic-5 expression levels in compensatory hypertrophy induced by TAC, suggesting that hic-5 may play a role under more pathological situations. The increase in hic-5 expression correlates nicely with an increase in ERK1/2 phosphorylation previously reported under sTAC specific conditions [18]. Future studies will determine specifically that the upregulation observed under these conditions occurs in the cardiomyocytes of these hearts, and not in another cell type (i.e., cardiac fibroblasts or smooth muscle cells). While our in vitro data suggests that hic-5 is up-regulated in neonatal cardiac myocytes in response to PE, upregulation in other cell types under sTAC conditions cannot be ruled out. In addition, we found that despite the lack of participation of paxillin in neonatal cardiac myocytes in response to PE, paxillin expression increased under both TAC and sTAC conditions. This may suggest a role for paxillin under pressure overload conditions. This brings up several interesting questions regarding existing models of hypertrophy and their mechanisms of hypertrophic development. While PE acts directly on α-adrenergic receptor agonists, TAC works through a number of mechanisms; directly inducing a pressure response on the heart as well as evoking systemic changes in neural and circulating hormonal factors which have been shown to contribute directly to hypertrophy as well. Many of these factors have been shown to be elevated, specifically with sTAC. The stimulus specific upregulation of hic-5 and paxillin may be important clues to understanding how their expression is regulated as well as in our understanding their potential roles in the regulation of distinct phenotypes.

In conclusion, these findings suggest a novel role for hic-5 in the regulation of a number of changes associated with cardiac hypertrophy, including cytoskeletal organization and fetal gene expression. These results, in association with the discovery that hic-5 is also re-expressed in an adult model of decompensated hypertrophy provide the rationale for future in vivo studies in which the role of hic-5 in adult hypertrophic models can be directly tested.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Reference List

1. Pyle WG, Solaro RJ. At the crossroads of myocardial signaling: the role of Z-discs in intracellular signaling and cardiac function. Circ Res. Feb 20; 2004 94(3):296–305. [PubMed: 14976140]

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

2. Samarel AM. Costameres, focal adhesions, and cardiomyocyte mechanotransduction. Am J Physiol Heart Circ Physiol. Dec; 2005 289(6):H2291–H2301. [PubMed: 16284104]
3. Keller RS, Shai SY, Babbitt CJ, Pham CG, Solaro RJ, Valencik ML, et al. Disruption of integrin function in the murine myocardium leads to perinatal lethality, fibrosis, and abnormal cardiac performance. Am J Pathol. Mar; 2001 158(3):1079–90. [PubMed: 11238056]
4. White DE, Coutu P, Shi YF, Tardif JC, Nattel S, St AR, et al. Targeted ablation of ILK from the murine heart results in dilated cardiomyopathy and spontaneous heart failure. Genes Dev. Sep 1; 2006 20(17):2355–60. [PubMed: 16951252]
5. Shai SY, Harpf AE, Babbitt CJ, Jordan MC, Fishbein MC, Chen J, et al. Cardiac myocyte-specific excision of the beta1 integrin gene results in myocardial fibrosis and cardiac failure. Circ Res. Mar 8; 2002 90(4):458–64. [PubMed: 11884376]
6. Ross RS, Pham C, Shai SY, Goldhaber JI, Fenczik C, Glembotski CC, et al. Beta1 integrins participate in the hypertrophic response of rat ventricular myocytes. Circ Res. Jun 15; 1998 82(11): 1160–72. [PubMed: 9633916]
7. Ogawa E, Saito Y, Harada M, Kamitani S, Kuwahara K, Miyamoto Y, et al. Outside-in signalling of fibronectin stimulates cardiomyocyte hypertrophy in cultured neonatal rat ventricular myocytes. J Mol Cell Cardiol. May; 2000 32(5):765–76. [PubMed: 10775482]
8. Pham CG, Harpf AE, Keller RS, Vu HT, Shai SY, Loftus JC, et al. Striated muscle-specific beta(1D)-integrin and FAK are involved in cardiac myocyte hypertrophic response pathway. Am J Physiol Heart Circ Physiol. Dec; 2000 279(6):H2916–H2926. [PubMed: 11087248]
9. Taylor JM, Rovin JD, Parsons JT. A role for focal adhesion kinase in phenylephrine-induced hypertrophy of rat ventricular cardiomyocytes. J Biol Chem. Jun 23; 2000 275(25):19250–7. [PubMed: 10749882]
10. Krishnamurthy P, Subramanian V, Singh M, Singh K. Beta1 integrins modulate beta-adrenergic receptor-stimulated cardiac myocyte apoptosis and myocardial remodeling. Hypertension. Apr; 2007 49(4):865–72. [PubMed: 17283249]
11. Krishnamurthy P, Subramanian V, Singh M, Singh K. Deficiency of beta1 integrins results in increased myocardial dysfunction after myocardial infarction. Heart. Sep; 2006 92(9):1309–15. [PubMed: 16547211]
12. Melendez J, Welch S, Schaefer E, Moravec CS, Avraham S, Avraham H, et al. Activation of pyk2/related focal adhesion tyrosine kinase and focal adhesion kinase in cardiac remodeling. J Biol Chem. Nov 22; 2002 277(47):45203–10. [PubMed: 12228222]
13. Sussman MA, Welch S, Walker A, Klevitsky R, Hewett TE, Price RL, et al. Altered focal adhesion regulation correlates with cardiomyopathy in mice expressing constitutively active rac1. J Clin Invest. Apr; 2000 105(7):875–86. [PubMed: 10749567]
14. Brown MC, Turner CE. Paxillin: adapting to change. Physiol Rev. Oct; 2004 84(4):1315–39. [PubMed: 15383653]
15. Yuminamochi T, Yatomi Y, Osada M, Ohmori T, Ishii Y, Nakazawa K, et al. Expression of the LIM proteins paxillin and Hic-5 in human tissues. J Histochem Cytochem. Apr; 2003 51(4):513–21. [PubMed: 12642630]
16. Brunskill EW, Witte DP, Yutzey KE, Potter SS. Novel cell lines promote the discovery of genes involved in early heart development. Dev Biol. Jul 15; 2001 235(2):507–20. [PubMed: 11437454]
17. Thomas SM, Hagel M, Turner CE. Characterization of a focal adhesion protein, Hic-5, that shares extensive homology with paxillin. J Cell Sci. Jan; 1999 112(Pt 2):181–90. [PubMed: 9858471]
18. Rothermel BA, Berenji K, Tannous P, Kutschke W, Dey A, Nolan B, et al. Differential activation of stress-response signaling in load-induced cardiac hypertrophy and failure. Physiol Genomics. Sep 21; 2005 23(1):18–27. [PubMed: 16033866]
19. Osada M, Ohmori T, Yatomi Y, Satoh K, Hosogaya S, Ozaki Y. Involvement of Hic-5 in platelet activation: integrin alphaIIbbeta3-dependent tyrosine phosphorylation and association with proline-rich tyrosine kinase 2. Biochem J. May 1; 2001 355(Pt 3):691–7. [PubMed: 11311131]
20. Hagmann J, Grob M, Welman A, van WG, Burger MM. Recruitment of the LIM protein hic-5 to focal contacts of human platelets. J Cell Sci. Aug; 1998 111(Pt 15):2181–8. [PubMed: 9664039]

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

21. Rathore VB, Okada M, Newman PJ, Newman DK. Paxillin family members function as Csk-binding proteins that regulate Lyn activity in human and murine platelets. Biochem J. Apr 15; 2007 403(2):275–81. [PubMed: 17233630]
22. Yue TL, Gu JL, Wang C, Reith AD, Lee JC, Mirabile RC, et al. Extracellular signal-regulated kinase plays an essential role in hypertrophic agonists, endothelin-1 and phenylephrine-induced cardiomyocyte hypertrophy. J Biol Chem. Dec 1; 2000 275(48):37895–901. [PubMed: 10984495]
23. Glennon PE, Kaddoura S, Sale EM, Sale GJ, Fuller SJ, Sugden PH. Depletion of mitogen-activated protein kinase using an antisense oligodeoxynucleotide approach downregulates the phenylephrine-induced hypertrophic response in rat cardiac myocytes. Circ Res. Jun; 1996 78(6):954–61. [PubMed: 8635245]
24. Hagel M, George EL, Kim A, Tamimi R, Opitz SL, Turner CE, et al. The adaptor protein paxillin is essential for normal development in the mouse and is a critical transducer of fibronectin signaling. Mol Cell Biol. Feb; 2002 22(3):901–15. [PubMed: 11784865]
25. Ishibe S, Joly D, Zhu X, Cantley LG. Phosphorylation-dependent paxillin-ERK association mediates hepatocyte growth factor-stimulated epithelial morphogenesis. Mol Cell. Nov; 2003 12(5):1275–85. [PubMed: 14636584]
26. Avraamides C, Bromberg ME, Gaughan JP, Thomas SM, Tsygankov AY, Panetti TS. Hic-5 promotes endothelial cell migration to lysophosphatidic acid. Am J Physiol Heart Circ Physiol. Jul; 2007 293(1):H193–H203. [PubMed: 17337598]
27. Shibanuma M, Mashimo J, Kuroki T, Nose K. Characterization of the TGF beta 1-inducible hic-5 gene that encodes a putative novel zinc finger protein and its possible involvement in cellular senescence. J Biol Chem. Oct 28; 1994 269(43):26767–74. [PubMed: 7929412]
28. Tumbarello DA, Turner CE. Hic-5 contributes to epithelial-mesenchymal transformation through a RhoA/ROCK-dependent pathway. J Cell Physiol. Jun; 2007 211(3):736–47. [PubMed: 17299801]
29. Sun Q, Chen G, Streb JW, Long X, Yang Y, Stoeckert CJ Jr. et al. Defining the mammalian CArGome. Genome Res. Feb; 2006 16(2):197–207. [PubMed: 16365378]
30. Posern G, Treisman R. Actin' together: serum response factor, its cofactors and the link to signal transduction. Trends Cell Biol. Nov; 2006 16(11):588–96. [PubMed: 17035020]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

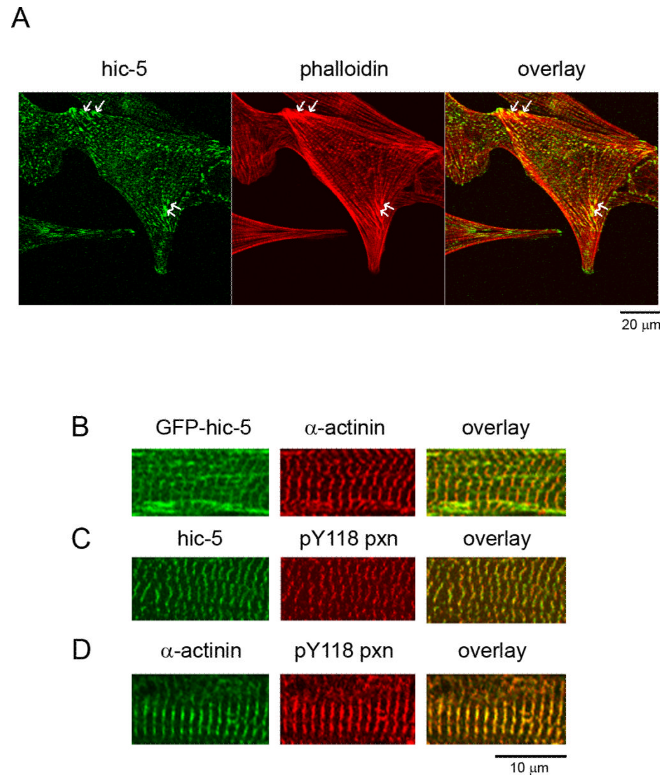

Figure 1. Hic-5 localization in NRVM

NRVM were fixed and immunostained with antibodies to (A) hic-5 (green) and phalloidin (red), (C) Hic-5 (green) and pY118 paxillin (red), (D) α-actinin (green) and pY118 pxn (red) (D). Arrows in (A) indicate colocalization of hic-5 and phalloidin. (B) NRVM were infected with GFP-hic-5 for 48 hours and fixed and immunostained with α-actinin antibody. All images were visualized using a Zeiss LSM 510 META confocal microscope.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

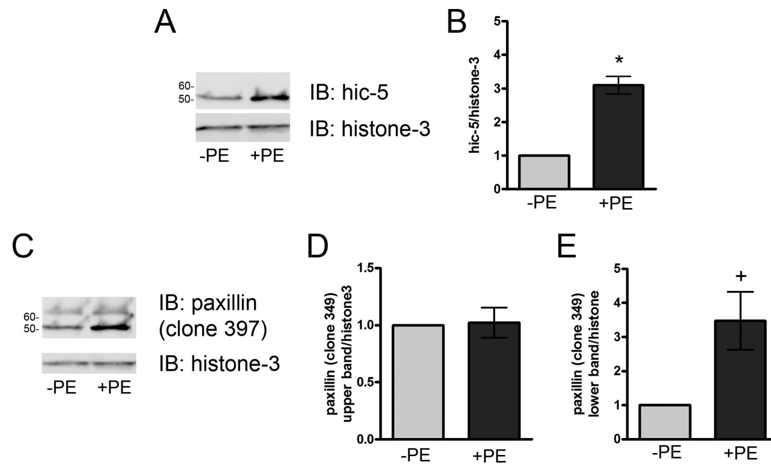

Figure 2. PE treatment (48 hour) increases hic-5, not paxillin expression in NRVM

NRVM were treated with PE (100 μM, 48 hours). Protein lysates were immunoblotted for hic-5, paxillin or histone-3 (loading control). Representative immunoblots for (A) hic-5 and histone-3 and (C) paxillin and histone-3. Quantification of (B) hic-5/histone-3 levels and (D) paxillin upper band/histone-3 and (E) paxillin lower band/histone-3 were graphed. Data are mean ± SEM, n=3. Data were analyzed by one-sample test "*" indicates $p<0.05$, "+" indicates $p=0.055$.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

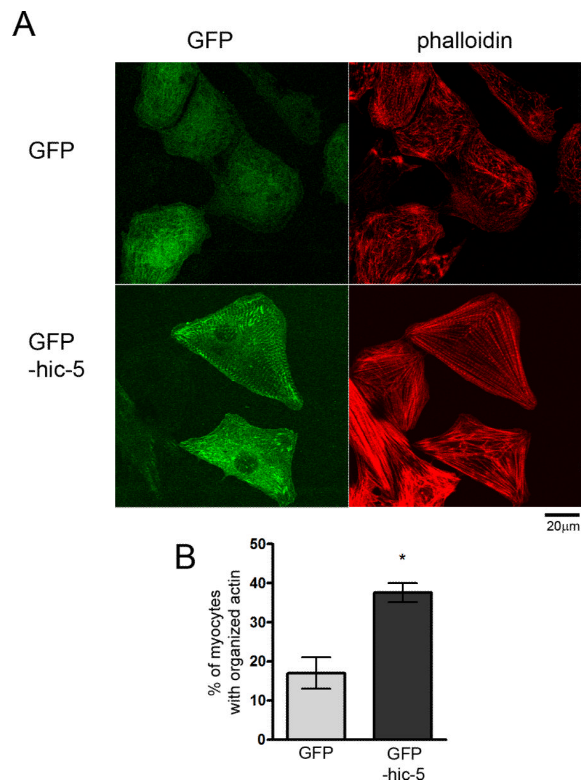

Figure 3. Hic-5 overexpression enhances cytoskeletal organization of NRVM

NRVM were infected with equivalent MOIs of GFP or GFP-tagged hic-5 for 48 hours in serum free culture media. Cells were labeled with antibodies to phalloidin. (A) Representative NRVM. (B) Percentage of cells with organized actin were quantified. Data are mean ± SEM, n=5. Actin organization of at least 50 myocytes were quantified for each group per "n". Data was analyzed by Mann-Whitney t-test, "*" indicates $p<0.05$.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

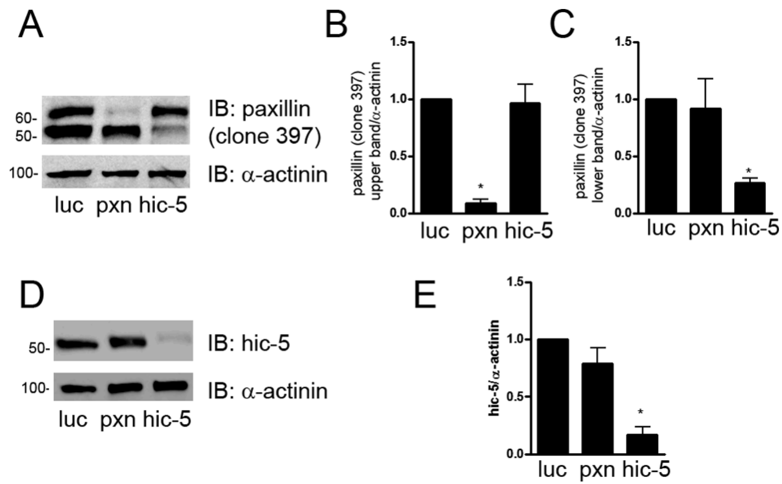

Figure 4. Validation of paxillin and hic-5 knockdown in NRVM

NRVM were transfected with 10 nM luc, pxn or hic-5 siRNA for 72 hours. Lysates were immunoblotted with antibodies to paxillin clone 349, hic-5, and α-actinin (load control). Representative western blots from (A) paxillin and α-actinin and (D) hic-5 and α-actinin are shown. Quantification of expression levels of (B) paxillin upper band, (C) lower band and (E) hic-5 relative to α-actinin levels are shown. Data are mean ± SEM, n=5. "*" indicates $p<0.05$ as compared to luc control, one-sample t-test.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

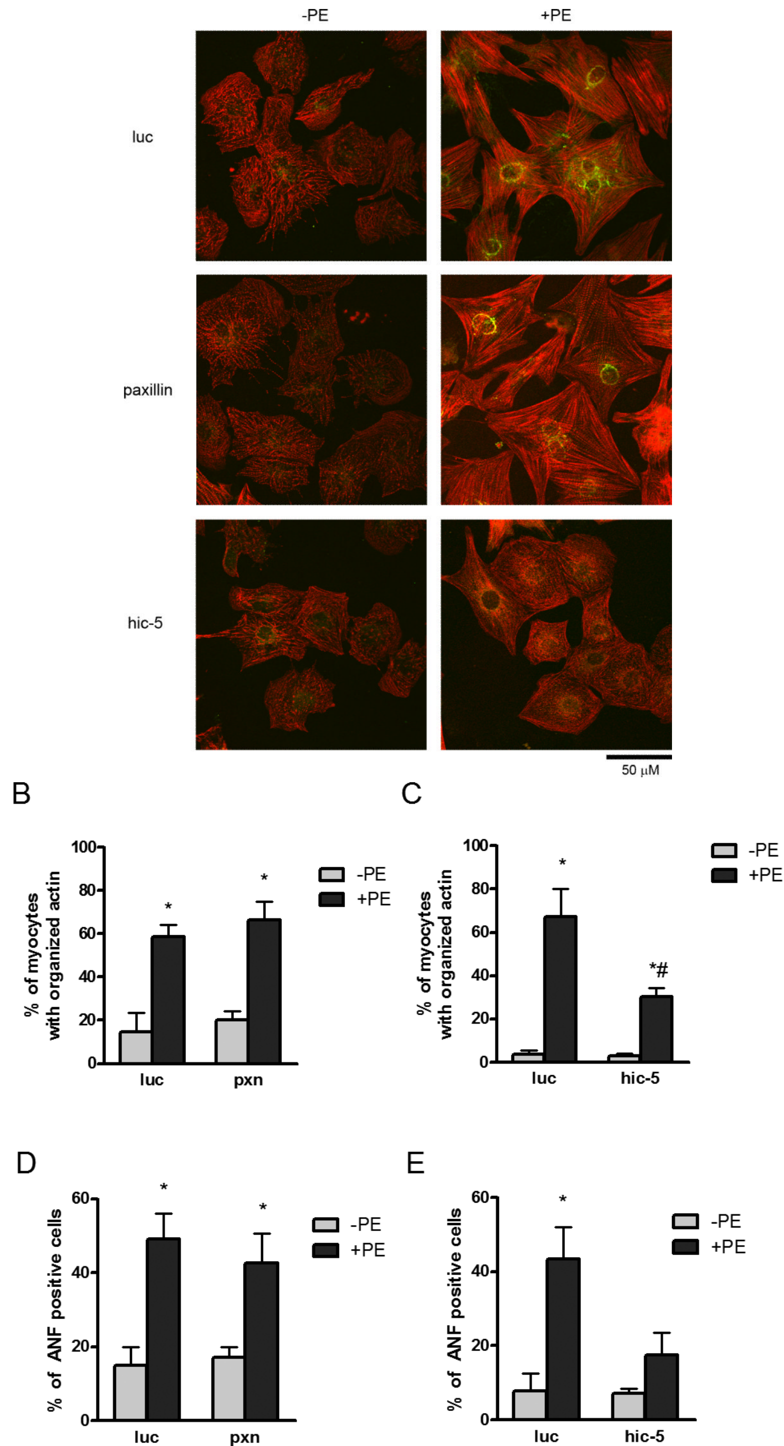

Figure 5. Hic-5, not paxillin, is required for PE induced cytoskeletal organization and ANF expression

NRVM were transfected with 10 nM of luc, pxn or hic-5 siRNA for 72 hours and subsequently treated with PE (100 µM, 48 hours). Cells were immunostained for ANF (green), DAPI (blue), and phallodin (red). (A) Representative cells for each condition. (B and C) Quantification of percent myocytes with organized actin. (D and E) Quantification of percent cells with ANF positive nuclei. Paxillin and hic-5 data are mean ± SEM, n= 5 and 3

respectively. 100-150 cells were randomly selected for analysis for each condition per n. Data was analyzed by 2-way ANOVA. “*” indicates $p<0.05$ effect of PE , “# “indicates $p<0.05$ effect of siRNA.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

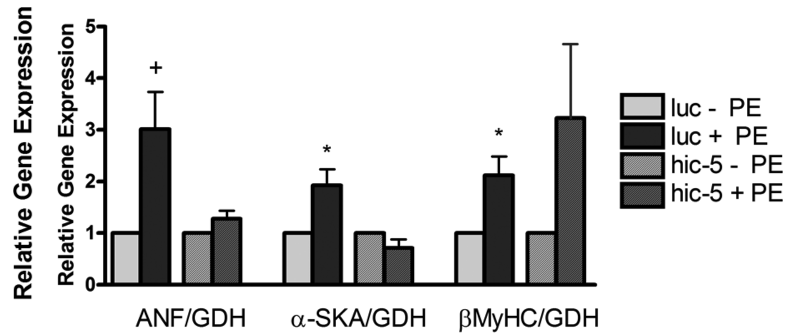

Figure 6. The effect of hic-5 knockdown on fetal gene expression

NRVM were transfected with 10 nM of luc or hic-5 siRNA for 72 hours and then treated with PE (100 μM, 48 hours). RNA was isolated and converted to cDNA. cDNA was amplified by qPCR using primers to ANF, α-skeletal actin, β-MyHC and GAPDH (GDH). GDH was used as a loading control for normalization of gene expression. qPCR data is representative of 4-6 independent myocyte preparations. "*" indicates $p<0.05$ as compared to minus PE control as analyzed by one sample t-test. "+" indicates data is approaching significance $p=0.06$.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

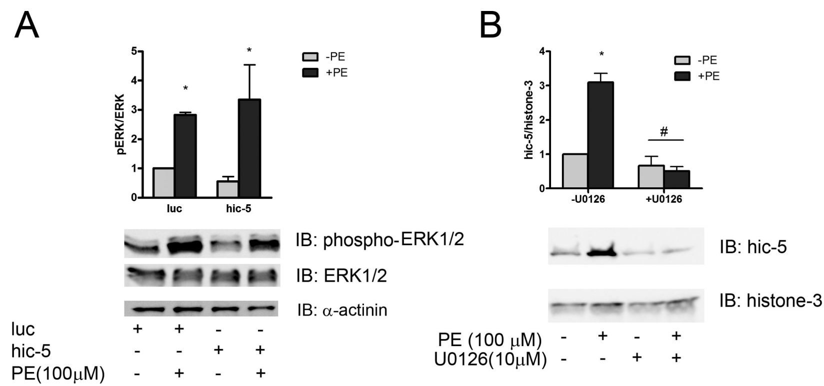

Figure 7. Hic-5 is downstream of ERK1/2 signaling

To assess the effect of hic-5 knockdown on ERK1/2 phosphorylation, PE (100 μM) was added for 10 minutes to NRVM transfected with luc or hic-5 siRNA for 72 hours. Lysates were immunoblotted for phospho-ERK1/2 and ERK1/2. (A) Quantification of phospho-ERK/ERK levels and representative western blot. To assess the effect of ERK1/2 phosphorylation on hic-5 expression, ERK phosphorylation was inhibited using MEK1/2 inhibitor U0126 (10μM, 30 minute pretreatment). Cells were then stimulated with PE (100μM, 48 hours) and hic-5 levels were assessed by western blot. Histone-3 was used as a loading control. (B) Representative western blot and quantification of hic-5/histone-3 levels. Data in A and B are mean ± SEM, n= 5 and 4, respectively. Data was analyzed by 2 way ANOVA where "*" indicates $p<0.05$ effect of PE, "#" indicates effect of siRNA.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

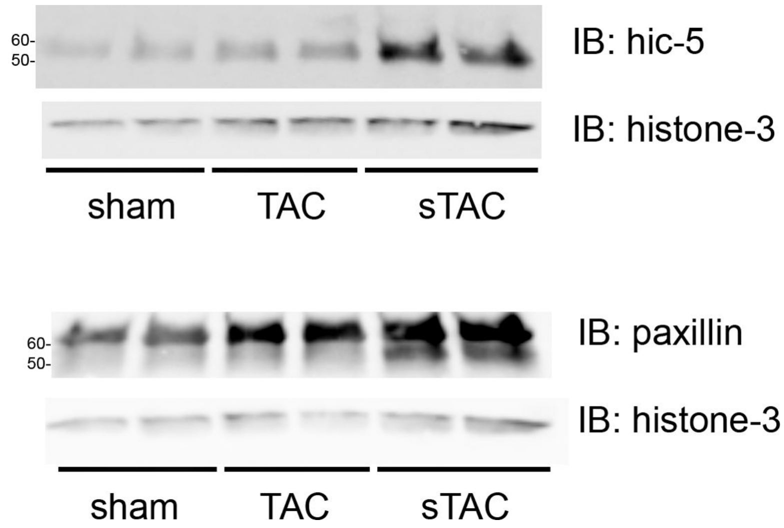

Figure 8. Expression of hic-5 and paxillin in two adult mouse models of cardiac hypertrophy

Mice were subject to sham, TAC or sTAC for 3 weeks as described in Materials and Methods. Protein was extracted from the left ventricle free wall. Protein lysates were loaded equally for protein and immunoblotted with antibodies to paxillin (clone 349), hic-5 and histone-3 (load control). Representative immunoblots of 2 mouse heart lysates per group are shown.