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The PIP_2-binding sequence of transient receptor potential channel canonical 4α is critical for its contribution to cardiomyocyte hypertrophy

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Running title:

TrpC4α promotes hypertrophy

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Abbreviations:

TrpC, transient receptor potential channel canonical
PLC, phospholipase C
IP_3, inositol(1,4,5)*tris*phosphate
DAG, *sn*-1,2-diacylglycerol
PIP_2, phosphatidylinositol(4,5)*bis*phosphate
PKC, protein kinase C
Shank3, SH3 and ankyrin repeat protein 3
MCIP modulatory calcineurin inhibitory protein;
qRT-PCR, quantitative reverse transcription polymerase chain reaction
DMEM, Dulbecco's Modified Eagle's Medium
ANP, atrial natriuretic peptide

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Abstract

Cardiomyocyte hypertrophy requires a source of Ca^{2+} distinct from the Ca^{2+} that regulates contraction. The canonical transient receptor potential channel family (TrpC), a family of cation channels regulated by activation of phospholipase C (PLC), has been implicated in this response. Cardiomyocyte hypertrophy downstream of Gq-coupled receptors is mediated specifically by PLCβ1b that is scaffolded onto a Shank3 complex at the sarcolemma. TrpC4 exists as two splice variants (TrpC4α and TrpC4β) that differ only in an 84 residue sequence that binds to phosphatidylinositol(4,5)*bis*phosphate (PIP_2), the substrate of PLCβ1b. In neonatal rat cardiomyocytes, TrpC4α, but not TrpC4β, co-immunoprecipitated with both PLCβ1b and Shank3. Heightened PLCβ1b expression caused TrpC4α, but not TrpC4β, translocation to the sarcolemma where it co-localized with PLCβ1b. When overexpressed in cardiomyocytes, TrpC4α but not TrpC4β, increased cell area (893±18 to 1497±29 mm^2, $p<0.01$) and marker gene expression (atrial natriuretic peptide increased by 409±32% and modulatory calcineurin inhibitory protein 1 by 315±28%, $p<0.01$). Dominant negative TrpC4 reduced hypertrophy initiated by PLCβ1b, or PLCβ1b-coupled receptor activation, by 72±8% and 39%±5, respectively. We conclude that TrpC4α is selectively involved in mechanisms downstream of PLCβ1b culminating in cardiomyocyte hypertrophy, and that the hypertrophic response is dependent on the TrpC4α splice variant-specific sequence that binds to PIP_2.

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Introduction

Hypertrophic growth of the myocardium is a complex response initiated by pathological stimuli including pressure or volume overload, myocardial infarction and congenital factors. Significantly, pathological hypertrophic responses require the participation of G proteins of the Gq family (Akhter et al., 1998; Dorn and Brown, 1999). Despite this, the immediate signaling responses to Gq activation in heart, and their relationships to cellular growth, remain poorly understood. In cardiomyocytes, Gq activates one splice variant of PLCβ1 (PLCβ1b) exclusively (Filtz et al., 2009; Grubb et al., 2008) and PLCβ1b is the only PLC that induces hypertrophy when overexpressed in cardiomyocytes *in vitro*. PLCβ1b differs from the other splice variant PLCβ1a by only a 32 residue C-terminal sequence and the splice variant specificity allowed us to identify factors critically involved in Gq/PLCβ1b-mediated responses. Important among these are the scaffolding proteins, Shank3 and Homer1c (Grubb et al., 2011; Grubb et al., 2012), both of which form complexes specifically incorporating PLCβ1b. Within central neurons, Shank3 and Homers are associated with transient receptor potential channels (TrpC) (Bertaso et al., 2010) (Kaznacheyeva et al., 2007). This is of interest because TrpC are implicated in Ca^{2+} responses specifically targeted to hypertrophic responses (Eder and Molkentin, 2011; Gomez et al., 2013; Wu et al., 2010).

TrpC are non-selective, low conductance channels that mediate entry of calcium and sodium, in response to activation of receptors coupled to PLC. The association between TrpC and PLC activation is complex and differs between the TrpC subtypes, as well as the cell type of origin (Hardie, 2003). PLC activation generates inositol(1,4,5)*tris*phosphate (IP_3) and *sn*-1,2-diacyclglycerol (DAG) at the expense of the substrate lipid, phosphatidylinositol(4,5)*bis*phosphate (PIP_2), and each of these, IP_3, DAG and PIP_2, can influence TrpC activity independently as well as in concert. TrpC form homo-tetramers as

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well as hetero-tetramers with other TrpC, on a subtype selective basis. TrpC1, -4 and -5 constitute one subfamily and TrpC3, -6 and -7 another. TrpC3, -6 and -7 are activated directly by DAG as well as indirectly following DAG-induced protein kinase C (PKC) activation (Hardie, 2003). In contrast, TrpC1, -4 and -5 do not respond to DAG, although TrpC1 can be activated by protein kinase C (PKC), and thus indirectly by DAG.

Cardiomyocytes express multiple subtypes of TrpC, including TrpC1, -3, -4, -5 and -6 (Eder and Molkentin, 2011; Watanabe et al., 2009) making evaluation of individual contributions difficult, especially when the functional channels can be heteromeric. TrpC3 and TrpC6 are elevated in hypertrophy and heart failure, and both have been suggested to supply the Ca^{2+} to activate responses downstream of calcineurin (Onohara et al., 2006). In addition to the DAG-regulated TrpC3/6 channels, there has also been evidence for an involvement of the DAG-insensitive TrpC family members, TrpC1 and TrpC4. TrpC1 expression is heightened in hypertrophy models (Kiso et al., 2013) and contributes to pressure overload hypertrophy (Seth et al., 2009). Dominant negative TrpC4 reduces hypertrophy following pressure overload or infusion of the Gq/PLC coupled agonist, angiotensin II (Wu et al., 2010).

The initiation of hypertrophic signaling downstream of Gq in cardiomyocytes depends on a protein complex incorporating Shank3 and PLCβ1b, and we considered the possibility that TrpC family members also associated with this complex. The DAG-insensitive TrpC family members, TrpC4 and TrpC5, have an identical C-terminal PDZ-interacting domain (TTRL) (Kiselyov et al., 2005) suitable for binding directly to the PDZ domain of Shank3. TrpC4 has previously been shown to co-localize with PLC subtypes based on associations with PDZ-containing scaffolding proteins via the TTRL sequence (Tang et al., 2000).

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Shank3 may fulfil a scaffolding role in organising signaling complexes incorporating PLCβ1b and TrpC4/5 in cardiomyocytes (Grubb et al., 2011).

In the current study we investigated whether TrpC4/5 associated with the Shank3/PLCβ1b complex in cardiomyocytes and whether any PLCβ1b/Shank3 associated TrpC contributed to hypertrophic responses. We found that only one of the splice variants of TrpC4, specifically TrpC4α, which incorporates a PIP_2-binding domain, associated with the complex. Furthermore, expression of TrpC4α, but not TrpC4β, was sufficient to cause cardiomyocyte hypertrophy, even though these two proteins differ by only an 84 amino acid, PIP_2-binding, sequence near to the C-terminus of the proteins.

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Materials and Methods

Preparation of neonatal rat ventricular myocytes (NRVM). Experiments were approved by the AMREP Animal Ethics Committee and all work was performed in accord with the NHMRC provision for the care and use of laboratory animals. Ventricular myocytes were prepared from 1-2 day old Sprague-Dawley rats of either gender, using repeated pancreatin/collagenase digestion, followed by separation from non-myocytes using discontinuous Percoll gradients as described previously (Sah et al., 1996). Cells were plated at 400/mm^2 and were maintained in defined medium comprising DMEM, insulin (50 μg/mL), transferrin (10 μg/mL), sodium selenate (30 nM), bromodeoxyuridine (BrdU; 0.1 mmol/L) and antibiotics. Cells were used within 3 days of preparation. BrdU was omitted after 3 days.

Measurement of NRVM hypertrophy. Cell area was quantified from manually-outlined cells in digitized microscopic images (recorded by an Olympus phase contrast microscope) of randomly-chosen cell fields using Image-Pro Plus 6.0 software. A minimum of 40 cells over 4 wells per treatment were measured for each independent experiment and the experiments were repeated at least three times. Protein was measured using the BCA method for protein (Pierce) and Burtons' diphenylamine procedure for measurement of DNA, as described previously (Filtz et al., 2009).

Constructs and adenoviruses. Adenoviruses expressing FLAG-PLCβ1b and PLCβ1a, have been described previously (Filtz et al., 2009). cDNAs for TrpC4α and TrpC4β were provided by Dr J Putney (Research Triangle Park, NC) (McKay et al., 2000). cDNA for dn-TrpC4 N-terminally myc-tagged (Schindl et al., 2008; Wu et al., 2010) was provided by DNA 2.0 (Menlo Park CA 94025.). Adenoviruses expressing TrpC were generated using the

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Gateway® methodology (Life Technologies) and were used at 20-50 plaque-forming units per cell.

Co-immunoprecipitation and western blotting. NRVM plated on 9 cm dishes were washed with Hanks buffered salt solution (HBSS) and then lysed and harvested in buffer containing in mmol/L; 50 HEPES pH 7.4, 130 NaCl, $MgCl_2$, 1 $CaCl_2$, 40 KH_2PO_4, as well as 1% (v:v) Triton-X100, 0.5% NP-40, 15% glycerol, 0.2% (w:v) BSA plus a protease inhibitor cocktail (Roche). The extract was pre-cleared and then anti-FLAG antibody (FLAG-M2, Sigma F1804), anti-TrpC4 (Milipore, #AB15302), anti-Shank3 (Millipore #AB15302) or anti-myc (abcam #ab9106) antibody was added at 5 μL/mL of extract, followed by Protein A or Protein G Sepharose at 50 μL/mL of extract. After overnight incubation at 4^o C, antibody-bound material was harvested by centrifugation and the pellets were washed 3 times with lysis buffer containing protease inhibitors. Proteins were separated by SDS-PAGE using gradient 7.5%-15% gels and were transferred to nitrocellulose membranes. Antibodies were used at the following dilutions; Shank3 1/2000, FLAG 1/1000, myc 1/5000, TrpC4 1/500, TrpC5 (Alomone #ACC-020, 1/500). All blots were developed using HRP-conjugated secondary antibodies and images were quantified using a GelDoc XRS+ system (BioRad) using ImageLab 2.0.1 software.

Membrane raft separation. Membrane fractions were separated on the basis of buoyant density using detergent-free methodology, as described previously (Morris et al., 2006). Two mL fractions were collected, diluted and pelleted at 75,000 x g prior to western blotting. Bands corresponding to TrpC4α and TrpC4β in the pelleted fractions were quantified relative to bands in the original lysate (prior to gradient centrifugation).

Confocal microscopy. NRVM were plated onto gelatinized glass bottom confocal dishes (MatTek, USA) and infected with adenovirus, as indicated. Treated NRVM were fixed with

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PFA (4% w/v paraformaldehyde, 0.1 M Pipes pH 6.8) before permeabilization with Saponin buffer (0.05% w/v, 0.1 M Pipes pH 6.8). The plates were washed in PBS, and incubated with antibodies diluted in PBA (PBS + BSA 1% w/v). Plates were then further washed in PBS, and incubated with AlexaFluor-488- or AlexaFluor-546-conjugated secondary antibodies (Life Technologies). The samples were then further washed in PBS, and mounted using Vectashield mounting media (Abacus ALS, Australia). Images were captured using a Zeiss Meta-510 LSM (excitation 488nm and 543nm with emission at 519nm and 573nm).

Measurement of mRNA expression. RNA was extracted using RNEasy kits from Qiagen according to the manufacturer's instructions, and reverse transcribed using Superscript III (Life Technologies). Quantitative real-time qRT-PCR with SybrGreen (Life technologies) reagent was performed on an Applied Biosciences 7500 Fast Real Time PCR System using primers for rat GAPDH (5' CAGTATGATTCTACCCACGG, 3' CAGATCCACAACGGATACAT) was used as a reference gene. ANP (5' AGCCTGCGAAGGTCAAGCT, 3' CGCTCCAATCCTGTCAATCC) MCIP (5' TGGGGAAGGAGATGAAACTG, 3' TGACTGGGGTAGCGTCTTC). Values are expressed as the $2^{-\Delta Ct}$ value relative to GAPDH in each sample (Pfaffl, 2001). All qRT-PCR experiments were performed in triplicate on triplicate or quadruplicate samples.

Statistics. Values are shown as mean ± SEM. Comparisons between treatment groups were carried out using 1 way ANOVA followed by Fisher's exact test (Sigma Stat). Paired studies were evaluated using a paired 't' test.

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Results

TrpC4α associates with PLCβ1b and Shank3.

We have previously reported that PLCβ1b associates with Shank3 in cardiomyocytes (Grubb et al., 2011), and we next examined whether either TrpC4 or TrpC5 also associates with this complex. NRVM were treated with Ad-FLAG-PLCβ1b for 24h, extracts were prepared and immunoprecipitated with anti-TrpC4 or anti-TrpC5 antibodies and subsequent western blots developed with anti-FLAG antibody (Fig. 1A&C). Anti-TrpC4 antibody precipitated FLAG-PLCβ1b and Shank3 (Fig. 1A&B), and similarly TrpC4 was precipitated by anti-FLAG or anti-Shank3 antibodies (Fig. 1B). In contrast, anti-TrpC5 antibody did not precipitate either FLAG-PLCβ1b or Shank3 (Fig. 1C&D) and anti-FLAG or anti-Shank3 antibodies did not precipitate TrpC5 (Fig. 1D).

TrpC4 exists as two different splice variants that differ only by an 84 amino acid sequence close to the C-terminal end of the protein (Fig. 2A) (Otsuguro et al., 2008). Western blots depicted in Fig. 1 A&B show that only a single band is associated with PLCβ1b or Shank3, and we next undertook studies to identify which of the two splice variants was bound. N-terminally myc-tagged TrpC4α and TrpC4β were expressed in NRVM, along with FLAG-PLCβ1b. Extracts were prepared and immunoprecipitated with anti-myc or anti-FLAG antibodies. Both myc-TrpC4α and myc-TrpC4β were precipitated with anti-myc antibodies, but only myc-TrpC4α was precipitated by anti-FLAG antibody (Fig. 2B) or by anti-Shank3 antibodies (Fig. 2C). Similarly anti-myc antibody only precipitated FLAG-PLCβ1b or Shank3 when myc-TrpC4α was expressed (Fig. 2D). Anti-myc antibody did not precipitate either FLAG-PLCβ1b or Shank3 when myc-TrpC4β was expressed. Thus TrpC4α, but not TrpC4β, associates with the PLCβ1b/ Shank3 complex in NRVM.

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PLCβ1b causes translocation of TrpC4α to sarcolemmal fractions.

In cardiomyocytes, PLCβ1b and its scaffold Shank3 are localized primarily in light lipid raft fractions of the sarcolemma along with the PLC substrate PIP_2 (Grubb et al., 2011) (Morris et al., 2006). We examined the distribution of TrpC4α and TrpC4β between membrane fractions of different buoyant densities, and whether this distribution was altered by expressing PLCβ1b. When extracts from control NRVM were subjected to sucrose gradient centrifugation to separate membranes of different buoyant densities, TrpC4α was found in both heavy and light membrane fractions, at the bottom (fractions 1&2) and the top (fractions 5&6) of the gradient, respectively (Fig. 3A). Expression of FLAG-PLCβ1b resulted in an increase in TrpC4α in membrane fractions of low buoyant density, without any change in total TrpC4α expression (Fig. 3A, B&C). TrpC4β was not detected in light membrane fractions in the absence or presence of FLAG-PLCβ1b. FLAG-PLCβ1b expression did not alter the distribution between heavy and light fractions of TrpC1, or TrpC5.

Using immunofluorescence and confocal microscopy, we examined the localization of TrpC4 in NRVM and whether PLCβ1b influenced the localization. In control NRVM, TrpC4 was present in intracellular vesicles distributed throughout the cytosol (anti-TrpC4 antibody, AlexaFluor-546 (red) (Fig 3D). Images were cross sectioned, as indicated by the white lines in Fig. 3D, and pixel density was quantified for FLAG-PLCβ1b and TrpC4 (Fig. 3E). Increasing the expression of PLCβ1b, by treatment with Ad-FLAG-PLCβ1b for 24 h, resulted in translocation of TrpC4 to the sarcolemma where it co-localized with FLAG-PLCβ1b (anti-FLAG antibody, AlexaFluor-488 (green)). Co-localization of TrpC4 and FLAG-PLCβ1b appears as yellow (Fig. 3D) and as co-localized bands (Fig. 3E).

TrpC4α, but not TrpC4β, is sufficient to cause hypertrophy of NRVM

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As noted above TrpC4 exists as two splice variants, of which only TrpC4α was shown to be associated with PLCβ1b signaling (Figs. 1-3). We next expressed either TrpC4α or TrpC4β (Ad-myc-TrpC4α or Ad-myc-TrpC4β) in NRVM for 24 h and examined the effect on hypertrophic markers. TrpC4α and TrpC4β, both myc-tagged, were expressed at similar levels in NRVM following treatment with adenovirus for 24 h (Fig. 4A). Increasing the expression of TrpC4α promoted cardiomyocyte hypertrophy, indicated by increased cell area and protein/DNA ratio (Fig. 4B&C) as well as hypertrophic marker gene expression, atrial natriuretic peptide (ANP), along with modulatory calcineurin inhibitory protein 1 (MCIP), which is an indicator of calcineurin activation (Fig. 4D). In contrast, expression of TrpC4β at similar levels had no effect on cell size or gene expression.

TrpC4 is downstream of PLCβ1b in hypertrophic signaling

We next examined whether TrpC4 was required for hypertrophic signaling downstream of PLCβ1b. NRVM were treated with adenovirus expressing FLAG-PLCβ1b for 24 h. This resulted in increases in cell size, as well as in the expression of the hypertrophic marker genes, ANP and MCIP. Co-expression of dn-TrpC4 (Ad-myc-dn-TrpC4 (Wu et al., 2010; Schindl et al., 2008)) inhibited these PLCβ1b-induced responses, pointing to a role for TrpC4 in hypertrophic responses downstream of PLCβ1b (Fig. 5 A-E). Our previous studies have demonstrated that hypertrophy initiated by activation of Gq-coupled α_1-adrenergic receptors (50 μmol/L phenylephrine (PE) plus 1 μmol/L propranolol, 24 h) can be partially inhibited by inhibiting PLCβ1b selectively (Filtz et al., 2009). In the current studies, α_1-adrenergic receptor mediated hypertrophy was also partially inhibited by dn-TrpC4 (Fig. 5F & G). Hypertrophy initiated by growth factors does not involve either Gq or PLCβ1b (Filtz et al., 2009). To ensure that the observed inhibitory effect of dn-TrpC4 on hypertrophic responses reflected a specific anti-hypertrophic action rather than general

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cytotoxicity, we examined the effect of dn-TrpC4 on hypertrophic responses caused by fetal bovine serum (FBS) FBS- mediated hypertrophy was not inhibited by dn-TrpC4 (Fig. 5H). Thus TrpC4 is involved in hypertrophic signaling pathways downstream of Gq, pathways that are thought to reflect ‘pathological hypertrophy’ specifically.

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Discussion

The current studies provide evidence that TrpC4α, but not the closely related TrpC4β, is involved in pathways downstream of PLCβ1b and contributes to cellular hypertrophic responses. PLCβ1b is an immediate effector of signaling responses downstream of Gq coupled receptors and is required for Gq-initiated cardiomyocyte hypertrophy (Filtz et al., 2009). PLCβ1b expression and activity are elevated in diseased myocardium from humans, rats, mice and sheep and furthermore activity increases with disease progression (Woodcock et al., 2009). Thus PLCβ1b and its downstream effectors, including TrpC4α, may contribute to disease. TrpC4α differs from the other splice variant, TrpC4β, only in the inclusion of an 84 amino acid sequence located close to, but not at, the extreme C-terminal end of the protein (Fig. 2A). Despite the apparently small sequence difference between the two splice variants, we found that TrpC4α, but not TrpC4β, associated with both PLCβ1b and Shank3 (Fig. 2) and translocated in response to PLCβ1b (Fig. 3). Furthermore, TrpC4α but not TrpC4β, was sufficient to cause cardiomyocyte hypertrophy (Fig. 4). The TrpC4α specific sequence binds PIP_2 (Otsuguro et al., 2008), the substrate of PLCβ1b. Many of the TrpC family members require PIP_2 for channel activity (Lemonnier et al., 2008; Rohacs, 2007), but TrpC4α has the unique property of being negatively regulated by PIP_2 (Otsuguro et al., 2008; Zhang C. G. et al., 2013)

TrpC4α, but not TrpC4β, moved to the light membrane fraction in the presence of heightened PLCβ1b (Fig. 3). PLCβ1b itself and its substrate PIP_2, are localized in this light membrane fraction (Morris et al., 2006). This co-positioning of PLCβ1b close to PIP_2 is facilitated by its association with the scaffolding protein, Shank3, also present in the light membrane fraction (Grubb et al., 2011). In addition to binding PLCβ1b, Shank3 has the capacity to bind to Homer family members, and in cardiomyocytes Homer1c has been shown

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to associate with the PLCβ1b/Shank3 complex (Grubb et al., 2012). Homer1c has the capacity to bind any of the TrpC family and potentially could crosslink any TrpC to Shank3 (Kreienkamp, 2008). This lack of subtype specificity means that Homer crosslinking cannot explain the specific interaction between TrpC4α and the PLCβ1b/Shank3 complex. Whilst the Homer-interaction domain is common to all TrpC, the C-terminal TTRL sequence, which is an appropriate ligand for the PDZ domain of Shank3, is exclusive to TrpC4 and TrpC5 (Beck et al., 2013). Thus these are the only TrpC family members likely to bind Shank3 directly, ie independently of Homer (Goel et al., 2005; Suh et al., 2001). TrpC4α, TrpC4β and TrpC5 all have the same C-terminal TTRL motif and therefore have identical capacities to bind to the PDZ domain of Shank3 similarly. Thus a PDZ interaction between the TrpC and the PDZ domain of Shank3 cannot readily explain the specific interaction with TrpC4α. On this basis, another mechanism is required to explain the observed splice variant specificity of the TrpC4α/ PLCβ1b/Shank3 association. Possibly, the binding to PIP_2 by the splice variant-specific sequence of TrpC4α (Putney, 2007; Zhang C. G. et al., 2013) is critical in localizing TrpC4α close to Shank3 and allowing binding to occur, either directly by PDZ binding or indirectly via interactions with Homer1c, or possibly by both mechanisms.

Localized Ca^{2+} responses are critical for cardiomyocyte hypertrophy, by providing the Ca^{2+} required for calcineurin activation and the initiation of downstream transcriptional responses (Goonasekera and Molkentin, 2012). Recent studies have demonstrated that these 'hypertrophic Ca^{2+} responses' involve TrpC, in addition to a subset of L-type Ca^{2+} channels that are localized to light membrane fractions (Gao et al., 2012). Our studies showed that TrpC4α alone is sufficient to cause hypertrophy of cardiomyocytes in isolation (Fig. 4) and that dn-TrpC4 prevented hypertrophy caused by PLCβ1b (Fig. 5A-E) and substantially reduced hypertrophy downstream of α_1-adrenergic receptor activation (Fig 5F&G).). It is generally accepted that inhibitors targeting early signaling intermediates should show

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specificity for individual pathways, whereas targeting downstream responses will target hypertrophy more broadly (Zhang L. et al., 2011). As TrpC4α is involved early in Gq/PLCβ1b initiated hypertrophic signaling pathways, inhibition would be expected to be exclusive to these pathways. In keeping with this, dn-TrpC4 did not inhibit FBS-mediated hypertrophy, which involves pathways downstream of growth factor receptors and is independent of Gq and PLCβ1b (Maillet et al., 2013; Simpson et al., 1982) (Filtz et al., 2009). TrpC4 is more highly expressed in adult myocardium than in the neonate (Jiang et al., 2014), and would also be expected to contribute to hypertrophic responses *in vivo* when these involve Gq. Hypertrophic responses caused by pressure overload or by infusion of angiotensin II *in vivo* require activation of Gq (Akhter et al., 1998; Wettschureck et al., 2001) (Dorn and Brown, 1999). In keeping with our findings, previous studies have reported that expression of dn-TrpC4 reduced hypertrophy in both of these *in vivo* models (Wu et al., 2010).

Ascribing cardiomyocyte responses to specific TrpC subtypes has been challenging for a number of reasons. The channels have considerable structural similarity (Eder and Molkentin, 2011; Toko et al., 2007), similar channel activity (Abramowitz and Birnbaumer, 2009) (Plant and Schaefer, 2005) and are expressed at relatively low level in cardiomyocytes (Jang et al., 2012). TrpC channels form complexes that often include more than one subtype (Abramowitz and Birnbaumer, 2009), which means that overexpression or inhibition of a particular subtype may influence responses of other family members. There may also be some redundancy between the family members, whereby deletion or overexpression of one subtype results in compensation by other members, especially members of the same TrpC subfamily. These specificity issues are of less concern in the current study because overexpressing TrpC4α, but not TrpC4β promoted hypertrophy, even though both interact similarly with TrpC1 and TrpC5 (Cioffi et al., 2012).

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In the current study we provide evidence that TrpC4 contributes to responses downstream of PLCβ1b by mechanisms that are entirely dependent on the 84 amino acid sequence specific to the TrpC4α splice variant (Fig. 2A, Fig. 4). PIP_2 binding is the essential feature of TrpC4α that distinguishes it from TrpC4β in relation to its involvement in cardiomyocyte hypertrophy. As demonstrated previously (Morris et al., 2006), PIP_2 is located in light lipid raft fractions along with PLCβ1b and the importance of the PIP_2 binding domain might be in optimising the localization of TrpC4α in close proximity to PLCβ1b. The other possibility is that the channel is activated directly by PIP_2 hydrolysis resulting from PLCβ1b activation, as has been suggested in other cell types (Zhang C. G. et al., 2013). This intriguing possibility requires further investigation. We conclude that cardiomyocyte hypertrophy downstream of Gq/PLCβ1b involves specifically the higher MW splice variant of TrpC4, TrpC4α.

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Authorship contributions

Conducted experiments: Cooley, Grubb, Luo.

Wrote or contributed to the writing of the manuscript: Woodcock.

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References

Abramowitz J and Birnbaumer L (2009). Physiology and pathophysiology of canonical transient receptor potential channels. *Faseb Journal* **23**: 297-328.

Akhter SA, Luttrell LM, Rockman HA, Iaccarino G, Lefkowitz RJ and Koch WJ (1998). Targeting the receptor-Gq interface to inhibit in vivo pressure overload myocardial hypertrophy. *Science* **280**: 574-577.

Beck A, Speicher T, Stoerger C, Sell T, Dettmer V, Jusoh SA, Abdulmughni A, Cavalie A, Philipp SE, Zhu MX, Helms V, Wissenbach U and Flockerzi V (2013). Conserved Gating Elements in TRPC4 and TRPC5 Channels. *J Biol Chem* **288**: 19471-19483.

Bertaso F, Roussignol G, Worley P, Bockaert J, Fagni L and Ango F (2010). Homer1a-dependent crosstalk between NMDA and metabotropic glutamate receptors in mouse neurons. *PLoS One* **5**: e9755.

Cioffi DL, Wu SW, Chen HR, Alexeyev M, St Croix CM, Pitt BR, Uhlig S and Stevens T (2012). Orai1 Determines Calcium Selectivity of an Endogenous TRPC Heterotetramer Channel. *Circulation Research* **110**: 1435-NIL_1170.

Dorn GW, 2nd and Brown JH (1999). Gq signaling in cardiac adaptation and maladaptation. *Trends Cardiovasc Med* **9**: 26-34.

Eder P and Molkentin JD (2011). TRPC channels as effectors of cardiac hypertrophy. *Circ Res* **108**: 265-272.

Filtz TM, Grubb DR, McLeod-Dryden TJ, Luo J and Woodcock EA (2009). Gq-initiated cardiomyocyte hypertrophy is mediated by phospholipase Cbeta1b. *Faseb J* **23**: 3564-3570.

Gao H, Wang F, Wang W, Makarewich CA, Zhang HY, Kubo H, Berretta RM, Barr LA, Molkentin JD and Houser SR (2012). Ca(2+) influx through L-type Ca(2+) channels

and transient receptor potential channels activates pathological hypertrophy signaling. *Journal of Molecular and Cellular Cardiology* **53**: 657-667.

Goel M, Sinkins W, Keightley A, Kinter M and Schilling WP (2005). Proteomic analysis of TRPC5- and TRPC6-binding partners reveals interaction with the plasmalemmal Na(+)/K(+)-ATPase. *Pflugers Arch* **451**: 87-98.

Gomez AM, Ruiz-Hurtado G, Benitah JP and Dominguez-Rodriguez A (2013). Ca2+ Fluxes Involvement in Gene Expression During Cardiac Hypertrophy. *Curr Vasc Pharmacol* **11**: 497-506.

Goonasekera SA and Molkentin JD (2012). Unraveling the secrets of a double life: contractile versus signaling Ca2+ in a cardiac myocyte. *J Mol Cell Cardiol* **52**: 317-322.

Grubb DR, Iliades P, Cooley N, Yu YL, Luo J, Filtz TM and Woodcock EA (2011). Phospholipase Cbeta1b associates with a Shank3 complex at the cardiac sarcolemma. *Faseb J* **25**: 1040-1047.

Grubb DR, Luo JT, Yu YL and Woodcock EA (2012). Scaffolding protein Homer 1c mediates hypertrophic responses downstream of Gq in cardiomyocytes. *Faseb Journal* **26**: 596-603.

Grubb DR, Vasilevski O, Huynh H and Woodcock EA (2008). The extreme C-terminal region of phospholipase Cbeta1 determines subcellular localization and function; the "b" splice variant mediates alpha1-adrenergic receptor responses in cardiomyocytes. *Faseb J* **22**: 2768-2774.

Hardie RC (2003). Regulation of TRP channels via lipid second messengers. *Annu Rev Physiol* **65**: 735-759.

Jang Y, Lee Y, Kim SM, Yang YD, Jung J and Oh U (2012). Quantitative analysis of TRP channel genes in mouse organs. *Archives of Pharmacal Research* **35**: 1823-1830.

Jiang Y, Huang H, Liu P, Wei H, Zhao H, Feng Y, Wang W and Niu W (2014). Expression and localization of TRPC proteins in rat ventricular myocytes at various developmental stages. *Cell Tissue Res* **355**: 201-212.

Kaznacheyeva E, Glushankova L, Bugaj V, Zimina O, Skopin A, Alexeenko V, Tsiokas L, Bezprozvanny I and Mozhayeva GN (2007). Suppression of TRPC3 leads to disappearance of store-operated channels and formation of a new type of store-independent channels in A431 cells. *Journal of Biological Chemistry* **282**: 23655-23662.

Kiselyov K, Kim JY, Zeng W and Muallem S (2005). Protein-protein interaction and functionTRPC channels. *Pflugers Arch* **451**: 116-124.

Kiso H, Ohba T, Lino K, Sato K, Terata Y, Murakami M, Ono K, Watanabe H and Ito H (2013). Sildenafil prevents the up-regulation of transient receptor potential canonical channels in the development of cardiomyocyte hypertrophy. *Biochemical and Biophysical Research Communications* **436**: 514-518.

Kreienkamp HJ (2008). Scaffolding proteins at the postsynaptic density: shank as the architectural framework. *Handb Exp Pharmacol*: 365-380.

Lemonnier L, Trebak M and Putney JW, Jr. (2008). Complex regulation of the TRPC3, 6 and 7 channel subfamily by diacylglycerol and phosphatidylinositol-4,5-bisphosphate. *Cell Calcium* **43**: 506-514.

Maillet M, van Berlo JH and Molkentin JD (2013). Molecular basis of physiological heart growth: fundamental concepts and new players. *Nature Reviews Molecular Cell Biology* **14**: 38-48.

McKay RR, Szymeczek-Seay CL, Lievremont JP, Bird GS, Zitt C, Jungling E, Luckhoff A and Putney JW, Jr. (2000). Cloning and expression of the human transient receptor

potential 4 (TRP4) gene: localization and functional expression of human TRP4 and TRP3. *Biochem J* **351 Pt 3**: 735-746.

Morris JB, Huynh H, Vasilevski O and Woodcock EA (2006). alpha(1)-Adrenergic receptor signaling is localized to caveolae in neonatal rat cardiomyocytes. *J Mol Cell Cardiol* **41**: 17-25.

Onohara N, Nishida M, Inoue R, Kobayashi H, Sumimoto H, Sato Y, Mori Y, Nagao T and Kurose H (2006). TRPC3 and TRPC6 are essential for angiotensin II-induced cardiac hypertrophy. *Embo J* **25**: 5305-5316.

Otsuguro K, Tang J, Tang Y, Xiao R, Freichel M, Tsvilovskyy V, Ito S, Flockerzi V, Zhu MX and Zholos AV (2008). Isoform-specific Inhibition of TRPC4 Channel by Phosphatidylinositol 4,5-Bisphosphate. *J Biol Chem* **283**: 10026-10036.

Pfaffl MW (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* **29**: e45.

Plant TD and Schaefer M (2005). Receptor-operated cation channels formed by TRPC4 and TRPC5. *Naunyn Schmiedebergs Arch Pharmacol* **371**: 266-276.

Putney JW, Jr. (2007). Inositol lipids and TRPC channel activation. *Biochem Soc Symp*: 37-45.

Rohacs T (2007). Regulation of TRP channels by PIP(2). *Pflugers Arch* **453**: 753-762.

Sah VP, Hoshijima M, Chien KR and Brown JH (1996). Rho is required for G alpha(q) and alpha(1)-adrenergic receptor signaling in cardiomyocytes - Dissociation of Ras and Rho pathways. *J Biol Chem* **271**: 31185-31190.

Schindl R, Frischauf I, Kahr H, Fritsch R, Krenn M, Derndl A, Vales E, Muik M, Derler I, Groschner K and Romanin C (2008). The first ankyrin-like repeat is the minimum indispensable key structure for functional assembly of homo- and heteromeric TRPC4/TRPC5 channels. *Cell Calcium* **43**: 260-269.

Seth M, Zhang ZS, Mao L, Graham V, Burch J, Stiber J, Tsiokas L, Winn M, Abramowitz J, Rockman HA, Birnbaumer L and Rosenberg P (2009). TRPC1 channels are critical for hypertrophic signaling in the heart. *Circ Res* **105**: 1023-1030.

Simpson P, McGrath A and Savion S (1982). Myocyte hypertrophy in neonatal rat heart cultures and its regulation by serum and by catecholamines. *Circ Res* **51**: 787-801.

Suh PG, Hwang JI, Ryu SH, Donowitz M and Kim JH (2001). The roles of PDZ-containing proteins in PLC-beta-mediated signaling. *Biochem Biophys Res Commun* **288**: 1-7.

Tang Y, Tang J, Chen Z, Trost C, Flockerzi V, Li M, Ramesh V and Zhu MX (2000). Association of mammalian trp4 and phospholipase C isozymes with a PDZ domain-containing protein, NHERF. *J Biol Chem* **275**: 37559-37564.

Toko H, Shiojima I and Komuro I (2007). Promotion of cardiac hypertrophy by TRPC-mediated calcium entry. *Journal of Molecular and Cellular Cardiology* **42**: 481-483.

Watanabe H, Murakami M, Ohba T, Ono K and Ito H (2009). The pathological role of transient receptor potential channels in heart disease. *Circ J* **73**: 419-427.

Wettschureck N, Rutten H, Zywietz A, Gehring D, Wilkie TM, Chen J, Chien KR and Offermanns S (2001). Absence of pressure overload induced myocardial hypertrophy after conditional inactivation of Galphaq/Galpha11 in cardiomyocytes. *Nat Med* **7**: 1236-1240.

Woodcock EA, Grubb DR, Filtz TM, Marasco S, Luo J, McLeod-Dryden TJ, Kaye DM, Sadoshima J, Du XJ, Wong C, McMullen JR and Dart AM (2009). Selective activation of the "b" splice variant of phospholipase Cbeta1 in chronically dilated human and mouse atria. *J Mol Cell Cardiol* **47**: 676-683.

Wu X, Eder P, Chang B and Molkentin JD (2010). TRPC channels are necessary mediators of pathologic cardiac hypertrophy. *Proc Natl Acad Sci U S A* **107**: 7000-7005.

Zhang CG, Bosch MA, Ronnekleiv OK and Kelly MJ (2013). Kisspeptin Activation of TRPC4 Channels in Female GnRH Neurons Requires PIP2 Depletion and cSrc Kinase Activation. *Endocrinology* **154**: 2772-2783.

Zhang L, Malik S, Kelley GG, Kapiloff MS and Smrcka AV (2011). Phospholipase C{epsilon} Scaffolds to Muscle-specific A Kinase Anchoring Protein (mAKAP{beta}) and Integrates Multiple Hypertrophic Stimuli in Cardiac Myocytes. *J Biol Chem* **286**: 23012-23021.

Footnotes

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Legends to Figures

Figure 1. ***Association of TrpC4 and TrpC5 with PLCβ1b and Shank3.*** **A. & B.** NRVM were treated with Ad-FLAG-PLCβ1b or control virus for 24 h, extracts were immunoprecipitated with anti-FLAG, anti-TrpC4- or anti-Shank3 antibodies, and the content of FLAG-PLCβ1b, TrpC4, TrpC5 or Shank3 measured by western blotting. **C. & D**. Extracts were immunoprecipitated with anti-FLAG, anti-TrpC5 or anti-Shank3 antibodies and the content of FLAG-PLCβ1b, TrpC5 or Shank3 measured. 'Lys'. is lysate prior to precipitation, '-Ab' no precipitating antibody control. All experiments were performed 4 times with similar findings.

Figure 2. ***Association of TrpC4 splice variants with PLCβ1b and Shank3.*** **A**. Diagram showing the relationship between the two splice variants of TrpC4. TrpC4α includes the 84 residue sequence that binds PIP_2 and TrpC4β does not. **B - D.** Extracts were prepared from NRVM expressing myc-TrpC4α or myc-TrpC4β along with FLAG-PLCβ1b and immunoprecipitated with anti-myc, anti-FLAG or anti-Shank3 antibodies. Content of myc-TrpC4α, myc-TrpC4β, FLAG-PLCβ1b or Shank3 was measured by western blot. All experiments were performed 4 times with similar findings.

Figure 3. ***Localization of TrpC4 and the effect of heightened PLCβ1b expression***. **A.** Expression of TrpC4 splice variants and other TrpC in membrane fractions from NRVM following treatment with Ad-FLAG-PLCβ1b. Membranes of different buoyant densities were separated by sucrose gradient centrifugation and TrpC expression measured in the different fractions by western blotting. Expression of caveolin-3 (Cav3) is shown to indicate the light membrane fractions. MW makers are indicated. **B**. Content of TrpC4α and TrpC4β

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in NRVM lysate prior to gradient separation. The value for relative content of TrpC4α in lysate from PLCβ1b-expressing cells relative to control cells was 1.028 ± 0.015, mean ± SEM, n=5. **C**. Quantification of the relative amount of TrpC4α in light membrane fractions (Fr. 5) (upper panel) and in heavy membrane fractions (Fr. 1) (lower panel), relative to the total TrpC4α measured in the lysate prior to gradient centrifugation. Filled circles represent values from individual experiments, (black are control and red are PLCβ1b treated) and open circles are mean ± SEM, n=5 separate experiments. * $p<0.01$ relative to Ad-control (paired 't' test). **D**. Co-localization of TrpC4 and FLAG-PLCβ1b. NRVM, treated with control adenovirus or adenovirus expressing FLAG-PLCβ1b were fixed and stained with anti-FLAG antibody followed by AlexaFluor-488 (green) and with anti-TrpC4 antibody (AlexaFluor-546 (red)). **E**. Images were cross-sectioned (500nm x 5nm x10μm) excluding the nucleus as shown by the white line, and pixel density quantified for FLAG-PLCβ1b (green) and Shank 3 (red) across the cell. A total of 20 different cells from 4 experiments were examined. All cells expressing FLAG-PLCβ1b showed sarcolemmal TrpC4.

Figure 4. ***TrpC4α, but not TrpC4β, causes hypertrophy of NRVM***. **A**. Expression of myc-tagged TrpC4α and TrpC4β, western blot using anti-myc antibody. MWs are indicated. **B**. *Upper panel*; Phalloidin (Alexa Fluor® 488 Phalloidin, Life Technologies) myofillament staining of NRVM expressing myc-TrpC4α or myc-TrpC4β for 24 h. *Lower panel*; Phase contrast images. **C**. NRVM were infected with Ad-myc-TrpC4α or Ad-myc-TrpC4β and hypertrophy was assessed by cell area and from protein/DNA ratios. Values shown are mean ± SEM, n=6. * $p<0.05$ relative to control. **D**. Expression of TrpC4α, but not TrpC4β, increased mRNA expression of ANP and MCIP. Values shown are ANP or MCIP $2^{-\Delta Ct}$

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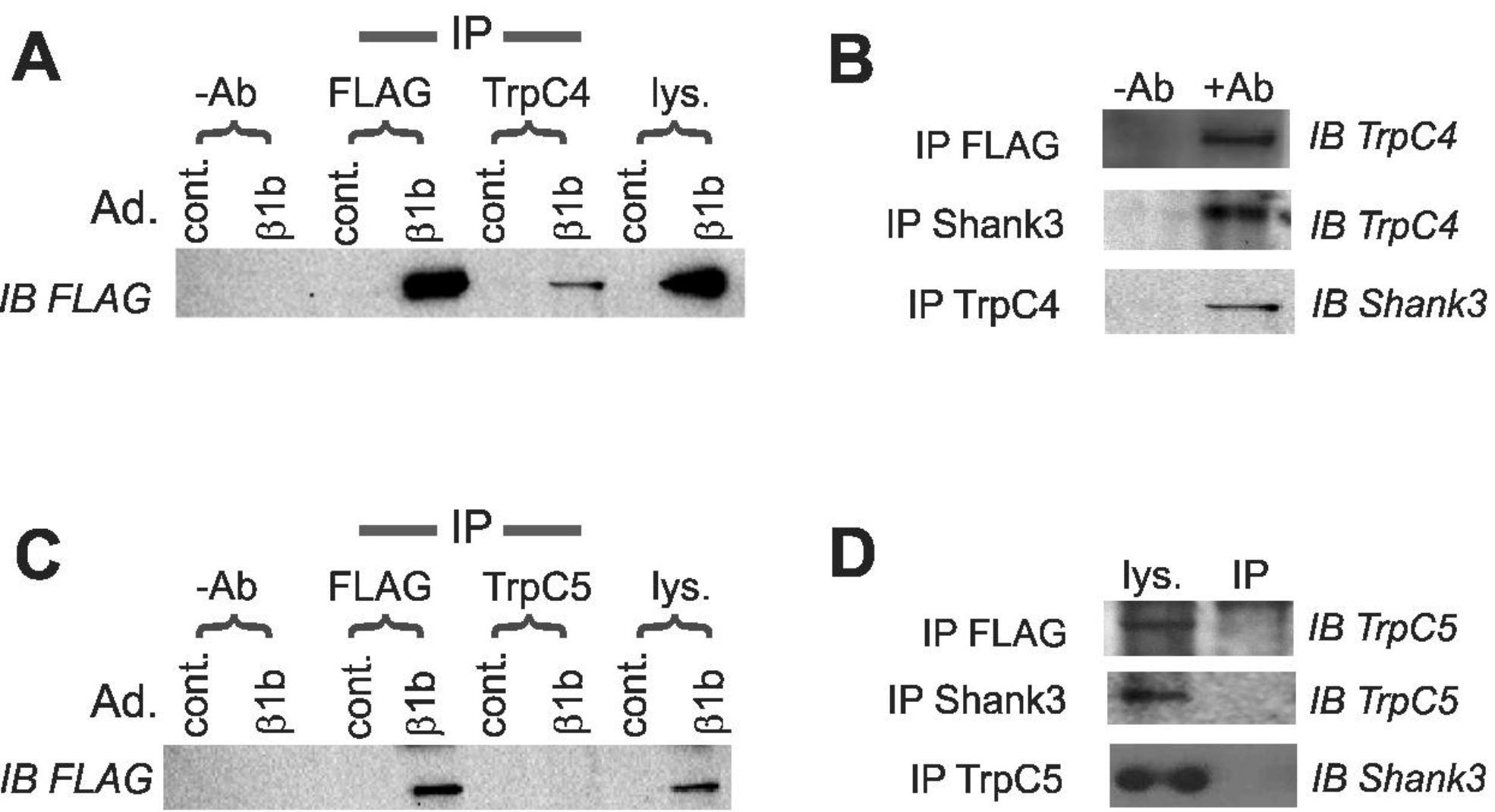
relative to GAPDH, mean ± SEM, n=4. *p<0.01 relative to control. *Open bars*, control; *Black bars*, TrpC4α; *Gray bars*, TrpC4β.

Figure 5. ***PLCβ1b-induced hypertrophy is inhibited by dn-TrpC4.*** **A-E**. NRVM were treated with Ad- FLAG-PLCβ1b together with Ad-myc-dn-TrpC4 (Ad-dn-C4) or control virus (Ad-con) for 24 h. **A**. Representative images, **B.** Cell area (μm^2), **C.** protein/DNA ratio, **D.** ANP expression ($2^{-\Delta Ct}$ relative to GAPDH) and **E**. MCIP expression ($2^{-\Delta Ct}$ relative to GAPDH) were measured. **F**. **& G**. NRVM were treated with phenylephrine (50 μmol/L) plus propranolol (1 μmol/L; PE) or together with Ad-myc-dn-TrpC (ad-dn-C4) for 24 h and cell area was measured. **H**. NRVM were treated with fetal bovine serum (FBS 5%) for 24 h and cell area quantified. Values shown are mean ± SEM, n=6. *p<0.01 relative to control. † p<0.01 relative to PLCβ1b or PE.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TrpC4
lys.
Ad.
cont.
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+Ab
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IP Shank3
IB TrpC4
IP TrpC4
IB Shank3
C
IP
-Ab
FLAG
TrpC5
lys.
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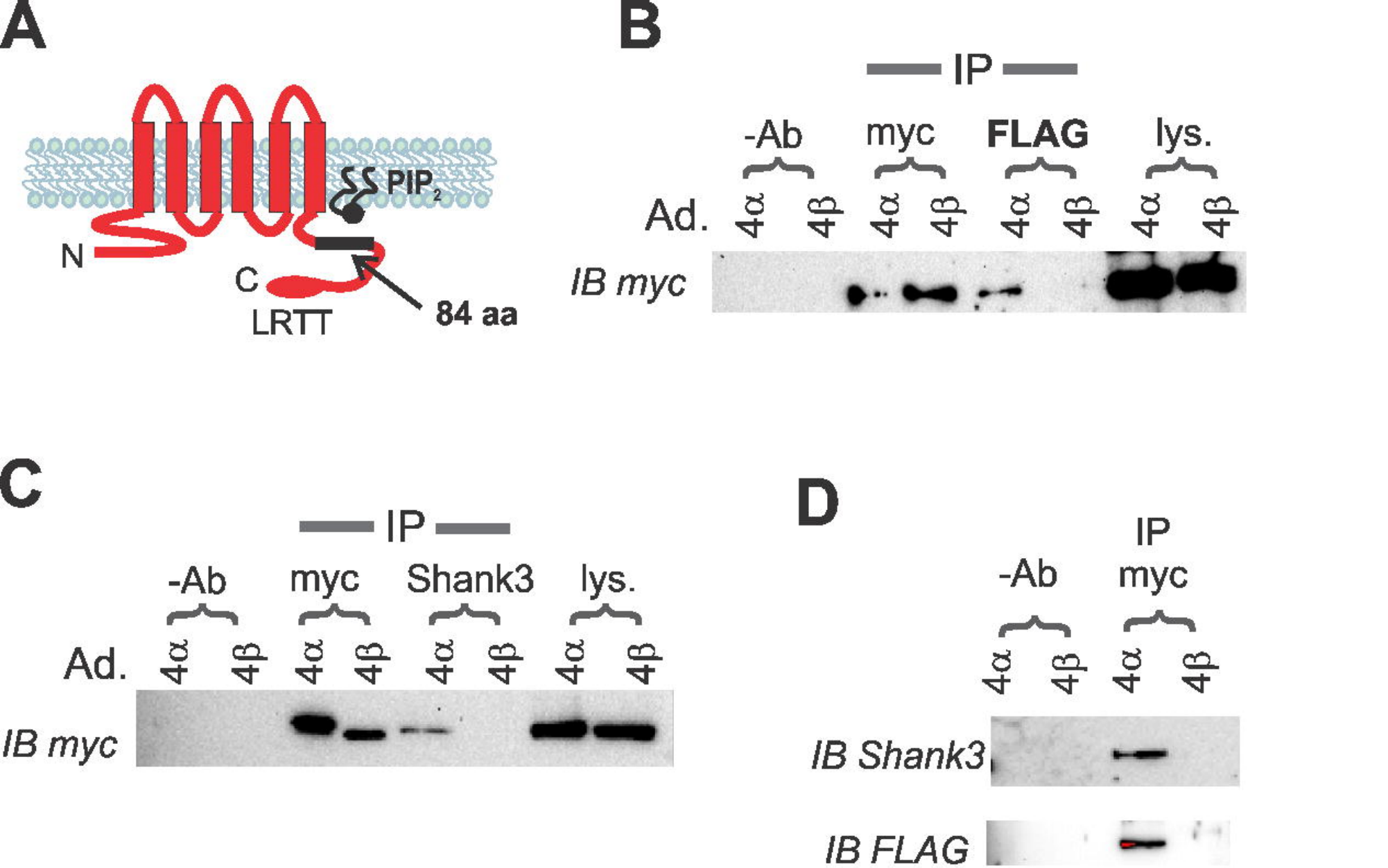
Fig. 1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IP
-Ab
myc
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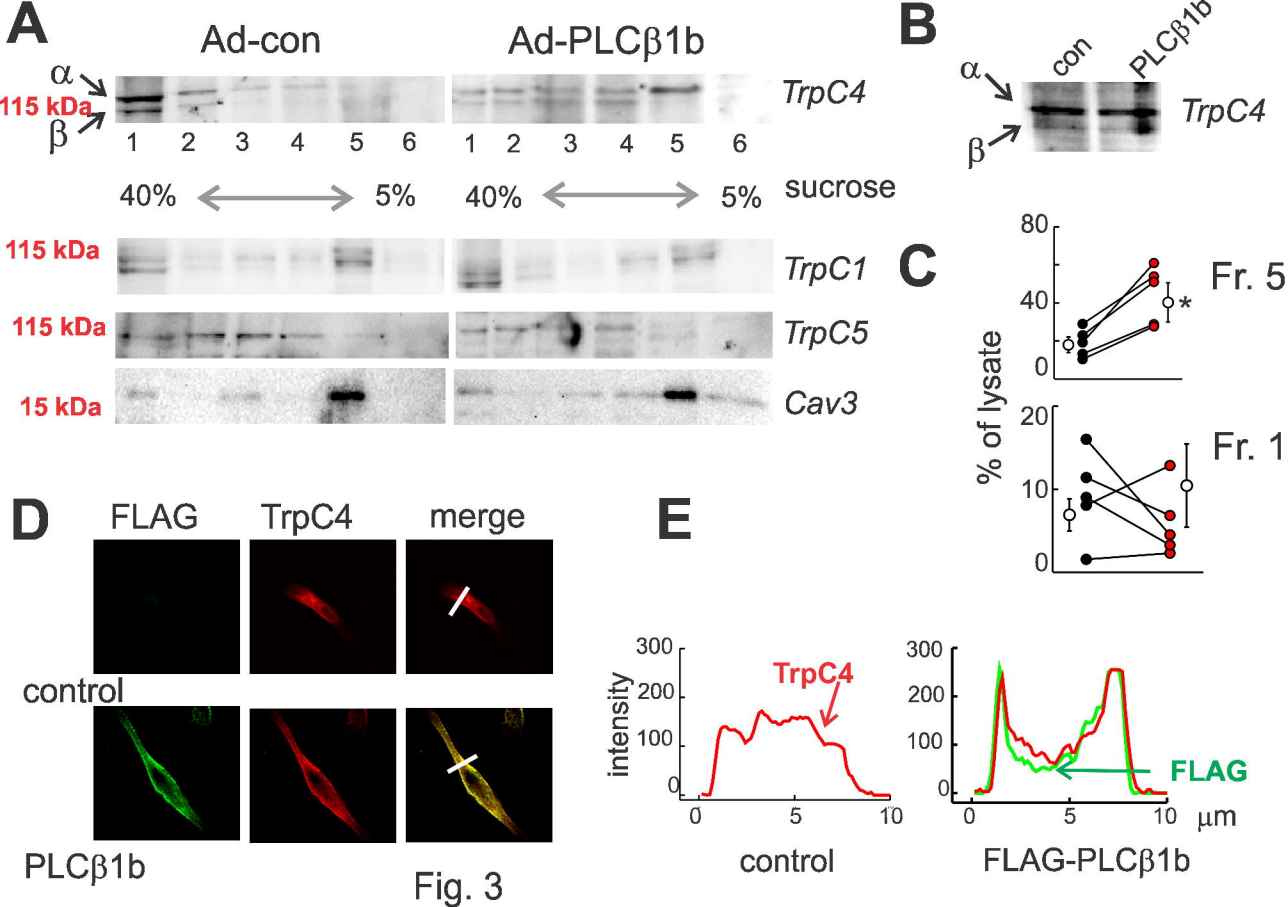
IB Shank3
IB FLAG

Fig. 2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Ad-con
Ad-PLCβ1b
α
115 kDa
β
TrpC4
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1 2 3 4 5 6
40%
5%
40%
5%
sucrose
115 kDa
TrpC1
115 kDa
TrpC5
15 kDa
Cav3
B
con
PLCβ1b
α
β
TrpC4
C
80
40
0
Fr. 5
*
% of lysate
20
10
0
Fr. 1
D
FLAG
TrpC4
merge
control
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300
200
100
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control
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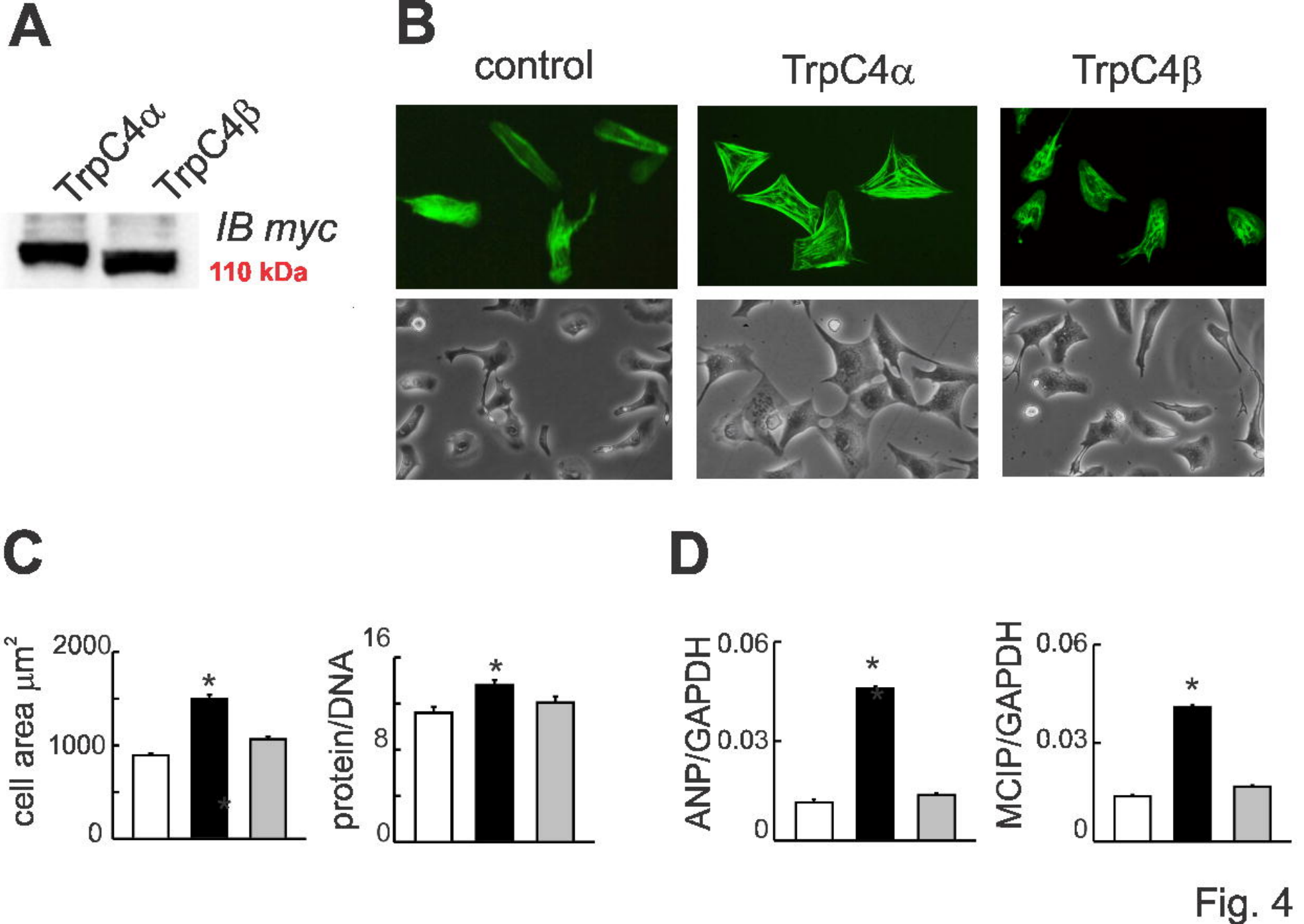
μm
FLAG-PLCβ1b

Fig. 3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110 kDa
B
control
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TrpC4β
C
cell area µm²
2000
1000
0
protein/DNA
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8
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D
ANP/GAPDH
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MCIP/GAPDH
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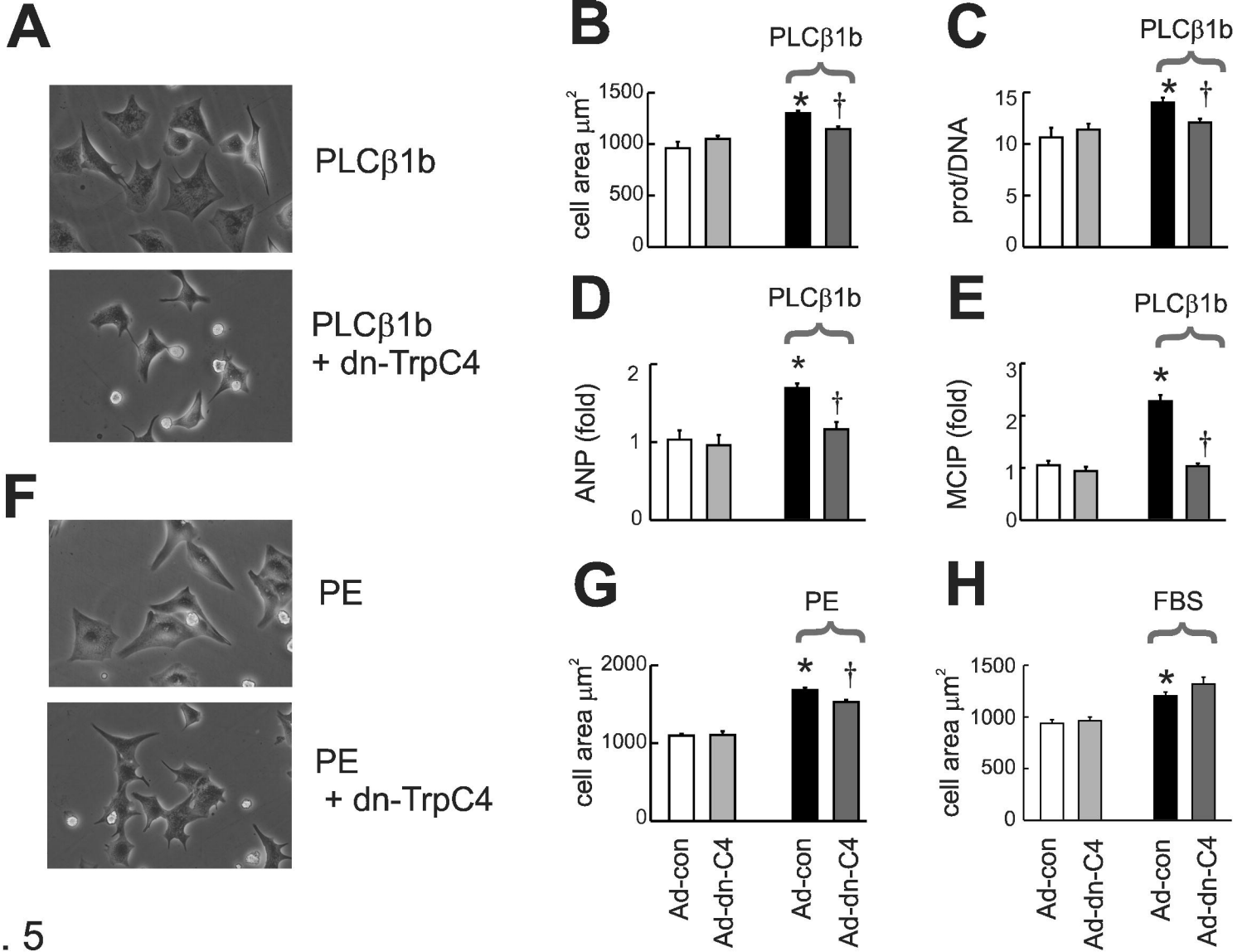
Fig. 4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PLCβ1b
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PE
PE
+ dn-TrpC4
B
PLCβ1b
cell area μm2
1500
1000
500
0
*
†
C
PLCβ1b
prot/DNA
15
10
5
0
D
PLCβ1b
ANP (fold)
2
1
0
E
PLCβ1b
MCIP (fold)
3
2
1
0
G
PE
cell area μm2
2000
1000
0
H
FBS
cell area μm2
1500
1000
500
0
Ad-con
Ad-dn-C4
Ad-con
Ad-dn-C4

Fig. 5