p63RhoGEF Couples $G_{\alpha q/11}$-Mediated Signaling to Ca$^{2+}$ Sensitization of Vascular Smooth Muscle Contractility

Ko Momotani, Mykhaylo V. Artamonov, Darkhan Utepbergenov, Urszula Derewenda, Zygmunt S. Derewenda, Avril V. Somlyo

**Rationale:** In normal and diseased vascular smooth muscle (SM), the RhoA pathway, which is activated by multiple agonists through G protein-coupled receptors (GPCRs), plays a central role in regulating basal tone and peripheral resistance. This occurs through inhibition of myosin light chain phosphatase, leading to increased phosphorylation of the myosin regulatory light chain. Although it is thought that specific agonists and GPCRs may couple to distinct RhoA guanine nucleotide exchange factors (GEFs), thus raising the possibility of selective targeting of specific GEFs for therapeutic use, this notion is largely unexplored for SM contraction.

**Objective:** We examine whether p63RhoGEF, known to couple specifically to $G_{\alpha q/11}$ in vitro, is functional in blood vessels as a mediator of RhoA activation and if it is selectively activated by $G_{\alpha q/11}$-coupled agonists.

**Methods and Results:** We find that p63RhoGEF is present across SM tissues and demonstrate that silencing of the endogenous p63RhoGEF in mouse portal vein inhibits contractile force induced by endothelin-1 to a greater extent than the predominantly $G_{\alpha 12/13}$-mediated thromboxane analog U46619. This is because endothelin-1 acts on $G_{\alpha q/11}$ as well as $G_{\alpha 12/13}$. Introduction of the exogenous isolated pleckstrin-homology (PH) domain of p63RhoGEF (residues 331–580) into permeabilized rabbit portal vein inhibited Ca$^{2+}$ sensitized force and activation of RhoA, when phenylephrine was used as an agonist. This reinforces the results based on endothelin-1, because phenylephrine is thought to act exclusively through $G_{\alpha q/11}$.

**Conclusion:** We demonstrate that p63RhoGEF selectively couples $G_{\alpha q/11}$ but not $G_{\alpha 12/13}$ to RhoA activation in blood vessels and cultured cells and thus mediates the physiologically important Ca$^{2+}$ sensitization of force induced with $G_{\alpha q/11}$-coupled agonists. Our results suggest that signaling through p63RhoGEF provides a novel mechanism for selective regulation of blood pressure. (*Circ Res.* 2011;109:00-00.)

**Key Words:** Ca$^{2+}$ sensitization ■ RhoA ■ signal transduction ■ vascular smooth muscle ■ RhoGEF

The contractile response in smooth muscle (SM) to agonist stimulation is mediated through the phosphorylation of the myosin regulatory light chain (RLC$_{20}$). The level of this phosphorylation reflects the balance of the opposing activities of the calcium-calmodulin–dependent myosin light chain kinase (MLCK) and myosin light chain phosphatase.1–3 Although an increase in intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]) serves as a primary on-switch for the activation of MLCK, both MLCK and myosin light chain phosphatase are additionally regulated by diverse kinases, which in turn are regulated by diverse physiological agonists.4,3 Importantly, the activity of myosin light chain phosphatase is negatively regulated when Thr853, located in its regulatory, myosin targeting phosphatase subunit (MYPT1) is phosphorylated by the Rho-kinase (ROCK).1,3 This inhibition of myosin light chain phosphatase activity results in an increase in force generating phosphorylated myosin motors, without further increase in intracellular [Ca$^{2+}$]. This physiologically important phenomenon is known as Ca$^{2+}$ sensitization.3,5–7

The Ca$^{2+}$ sensitization pathway is initiated when ROCK is activated by the GTP-bound, biologically active form of the cytosolic GTPase, RhoA.8 Like other GTPases of the Ras-homology (Rho) family,9,10 RhoA functions as a molecular switch; it is biologically inert in the GDP-bound form, but on exchange of GDP to GTP, it undergoes a conformational change enabling it to interact with various target effectors.11–13 This results, for example, in the
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activation of kinases, such as ROCK. RhoA can serve as a switch, because it is a poor hydrolase, with $k_{cat}$ of $\approx 1$ min$^{-1}$, and can therefore exist for considerable time period as a substrate-enzyme complex (the on-state). To switch between the on- and off-states, RhoA is therefore assisted by other regulatory proteins in its catalytic cycle. The GTPase-activating proteins enhance the hydrolysis of GTP to GDP, thereby terminating the biological signal. The GDP-bound RhoA is then sequestered in the cytosolic pool by the Rho-nucleotide dissociation inhibitor, RhoGDI. Finally, a family of guanine nucleotide exchange factors (GEFs) catalyze the loading of GTP for GDP, reinitiating the signaling cycle. Both GTPase-activating proteins and GEFs are themselves tightly regulated upstream of RhoA.

Basal tone and the contractile state of SM are regulated by multiple agonists acting through G-protein-coupled receptors (GPCRs) leading to a rise in intracellular $[\text{Ca}^{2+}]$. However, the magnitude of force at a specific level of $[\text{Ca}^{2+}]$ is highly variable depending on the nature of the stimulus and on the vascular bed. In cases in which the force/$[\text{Ca}^{2+}]$ ratio is high, the force is enhanced due to the activation of a RhoA/ROCK signaling pathway, and consequently $\text{Ca}^{2+}$ sensitization. Thus, diverse agonists acting on heterotrimeric GPCRs can exert different magnitudes of RhoA-mediated $[\text{Ca}^{2+}]$-sensitized contractile force. This strongly suggests the presence of coupling in SM between agonist-activated GPCRs and RhoA, most likely through different GEFs. However, it is not clear how many RhoA-specific GEFs are functional in SM, whether specific GEFs are coupled to specific agonists and GPCRs, or if they regulate different functions in SM cells.

Consistent with the notion that specific agonists and GPCRs couple to specific RhoGEFs, a small family of GEFs was recently found to be activated directly by $\text{G}_q/11$-coupled receptors. These proteins contain a unique RGSL (regulator of G-protein signaling-like) domain, which recognizes and binds to $\text{G}_q/11$. It has been demonstrated that one of these GEFs, the leukemia-associated RhoGEF (LARG), plays a central role in the regulation of vascular SM tone in salt-dependent hypertension but not in the maintenance of basal blood pressure. The other 2 related RGSL-containing GEFs, PDZRhoGEF and p115-RhoGEF, were also found in SM tissues, but whether they couple to specific or multiple agonists and GPCRs is unknown.

Agonist coupling through GPCRs to $\text{G}_q/11$ is thought to be involved in the stimulation of phospholipase C ( PLC )-mediated, $\text{Ca}^{2+}$ -calmodulin activation of MLCK$^{12,33,35}$ and activation of RhoA. It has been shown that the RhoA-specific GEFs, p63RhoGEF$^{37,38}$ and p115RhoGEF in SM signaling in response to stimulation by angiotensin II. In this report, we present evidence for the involvement of p63RhoGEF specifically in mediation of $\text{G}_q/11$-coupled, agonist-induced $\text{Ca}^{2+}$-sensitized force in SM. We determined that p63RhoGEF mRNA and protein is ubiquitously present in SM cells and in vascular and gastrointestinal tissue across several species, including the human. We further show that after stimulation through $\text{G}_q/11$ with agonists such as endothelin-1 (ET-1) and phenylephrine, p63RhoGEF catalyzes GTP exchange on RhoA in SM and regulates $\text{Ca}^{2+}$ sensitization.

### Methods

All procedures using animals were carried out according to protocols approved by the Animal Care and Use Committee at the University of Virginia. Detailed experimental procedures are in Supplemental Material available at http://circres.ahajournals.org.

#### Anti-p63RhoGEF Antibodies

Two anti-p63RhoGEF antibodies are purchased from Proteintech, Illinois (51004–1-AP and 14839–1-AP).

#### Expression Plasmids and Recombinant Protein Production

Human p63RhoGEF cDNA was PCR-amplified and introduced into pGST-Parallel1, p3xFLAG-Myc-CMV-24 (SIGMA), and pcDNA3 (Invitrogen) plasmids. Glutathione S-transferase (GST)-fused p63RhoGEF recombinant protein was produced in Escherichia coli BL21 and purified using glutathione beads. GST was subsequently cleaved off using recombinant tobacco etch virus (TEV) protease.

#### SM Contraction Experiment

Force measurements on intact, $\alpha$-toxin, or $\beta$-escin permeabilized muscles were carried out as detailed in the Supplemental Material.

#### Rhotekin Assay

Rabbit portal vein strips were prepared and treated using the same protocols as in the contraction experiments and harvested at each critical time point. Mouse embryonic fibroblast (MEF) cells were transfected with mammalian expression plasmids to overexpress FLAG-p63RhoGEF. RhoA activity was assayed as detailed in the Supplemental Material.

#### RLC$_{20}$ and MYPT1 Phosphorylation

Rabbit portal vein strips were treated using the same protocols as in the contraction assays and processed as described previously. Phosphorylation measurements are detailed in the Supplemental Material.

#### Coimmunoprecipitation Assays

Coimmunoprecipitation assays on human embryonic kidney (HEK) 293 cell transfectants (expressing combinations of FLAG-

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### Non-standard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>DH</td>
<td>Dbl-homology</td>
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<tr>
<td>ET-1</td>
<td>endothelin-1</td>
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<tr>
<td>GEF</td>
<td>guanine nucleotide exchange factor</td>
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<td>GPCR</td>
<td>G-protein coupled receptor</td>
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<td>HEK</td>
<td>human embryonic kidney</td>
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<td>MEF</td>
<td>mouse embryonic fibroblast</td>
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<tr>
<td>MYPT1</td>
<td>myosin targeting phosphatase subunit</td>
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<tr>
<td>PH</td>
<td>pleckstrin-homology</td>
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<tr>
<td>RLC$_{20}$</td>
<td>myosin regulatory light chain</td>
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<tr>
<td>ROCK</td>
<td>Rho-kinase</td>
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<tr>
<td>shRNA</td>
<td>short hairpin RNA</td>
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<td>SM</td>
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p63RhoGEF-Full-Myc and/or FLAG-p63RhoGEF and/or Gα11 wild-type or Gα11 Q209T constitutively active mutant) are detailed in the Supplemental Material.

p63RhoGEF Knock-Down
An RNA interference sequence [GCCAAGCTGGATGAAGATGAG] was designed to target both mouse and human p63RhoGEF mRNAs that coincidentally match rat p63RhoGEF mRNA sequence. Short hairpin RNA (shRNA) was delivered and expressed either by pENTR/U6 plasmid (Invitrogen) or adenovirus including the sequence for the expression of shRNA in mammalian cells.

Quantitative Polymerase Chain Reaction
Total mRNA libraries prepared from unpassaged aortic, pulmonary artery and brain vascular SM primary human cell cultures were purchased from ScienCell Research Laboratories (Carlsbad, CA). RNA was also prepared from animal tissue samples. mRNA expression levels of p63RhoGEF and other GEFs were quantified by RT-PCR.

Statistical Analysis
All data are presented as mean±SEM. Differences were considered significant at a probability value <0.05 using 2-tailed Student t test.

Results
p63RhoGEF Transcription and Expression in SM
We chose the mouse as our principal model system. To quantify the level of p63RhoGEF transcription in comparison to those of other GEFs in mouse SM, we performed quantitative RT-PCR using mouse vascular SM tissues. To assess if the transcription patterns are representative of those observed in human, we also screened mRNA libraries from human aorta, pulmonary artery, and brain vascular primary, unpassaged SM cells. The p63RhoGEF mRNA was detected in all of the mouse tissues screened and showed particularly high transcription levels in portal vein (Figure 1)—which was subsequently used in our functional assays—as well as in aorta and pulmonary artery. Of significance is the presence of p63RhoGEF mRNA in mouse resistance arteries, such as the thoracodorsal and mesenteric arteries. In human cells, p63RhoGEF mRNA level was the highest in the aorta, followed by pulmonary artery and brain vascular SM cells (Figure 1, inset).

To assess the expression levels of p63RhoGEF we turned to rat tissues, because of the larger body size of the animal. As shown in Figure 2, p63RhoGEF was detected in diverse tissues, except for brain, liver, diaphragm, and heart. Similar results were obtained for select mouse and rabbit tissues indicating a consistent trend across species (data not shown). Importantly, we also screened rat tissues for the expression of Gα11, and we discovered that it

Figure 1. GEF mRNA transcription profile in mouse portal vein and p63RhoGEF mRNA expression levels in different human cell lines (inset 1) and mouse smooth muscle tissues (inset 2). Human cell lines (inset 1): HASMC indicates aorta SM; HPASMC, pulmonary artery SM; HBVSMC, brain vascular SM; and HEK293, human embryonic kidney cells.

Figure 2. p63RhoGEF, its upstream effector Gαq11, and downstream effector RhoA in a variety of rat tissues and cultured cell lines. p63RhoGEF, Gαq11, and RhoA proteins are expressed in a variety of rat blood vessels and ileum. R518 rat aortic cells and MEF cells used in this study express p63RhoGEF. Protein expression is quantified by Western blot analysis and levels normalized to total protein. Relative expression levels are presented arbitrarily.
follows a trend similar to p63RhoGEF. Similarly, RhoA expression was high in SM (Figure 2). Expression of p63RhoGEF was also detected in cultured rat aortic SM cells (R518) and mouse embryonic fibroblast (MEF) cells (Figure 2) but not in human embryonic kidney (HEK) 293 cells (Online Figure I, B). The anti-p63RhoGEF antibody typically gave triplet bands across species by Western blot and the lowest molecular weight band is predominant in mouse samples and is demonstrated to be nonspecific (Online Figure I, B). The top band (80 kDa) corresponds to the full-length p63RhoGEF consisting of 580 amino acid residues. Further details of experiments characterizing the p63RhoGEF antibodies and showing that the lowest band is nonspecific while the middle band probably is a truncated form of p63RhoGEF are described in the Supplemental Material. Although the presence of p63RhoGEF mRNA in brain and heart and HEK293 cells has been reported previously, we only observed the lowest protein molecular weight band in brain samples (Figure 2).

Suppression of Mouse Portal Vein Contractility by p63RhoGEF Knock-Down

Next, we asked if knock-down of p63RhoGEF might suppress contractility in intact blood vessels. Using LacZ-expressing adenovirus, we found that we can successfully infect mouse portal veins and cerebral vessels but not the aorta or mesenteric arteries. Therefore, we treated mouse portal veins with a viral construct to express short hairpin RNA (shRNA) targeting p63RhoGEF mRNA. This construct was effective as it knocked down the protein expression of p63RhoGEF by up to 79% in cultured cells (Online Figure I, A). In portal vein, after 5 days of treatment with the viral particle, the p63RhoGEF mRNA was reduced by 40%, based on quantitative RT-PCR analysis (Figure 3A). Reduction of p63RhoGEF at the protein level by ~35% was confirmed by Western blot analysis, using 2 pooled mouse portal veins (Figure 3A). Similar effects were also observed in 3 other Western blot analyses using the same portal vein SM strips used in contraction experiments, although the small sample size did not allow accurate quantification (data not shown).

To probe the impact of the p63RhoGEF knock-down on $G_{q11}$ and $G_{12/13}$-mediated SM contractility, we used, respectively, endothelin-1 (ET-1) and a thromboxane analog U46619 as agonists. The ET-1 receptor is coupled to $G_{q11}$, whereas U46619 acts primarily through $G_{12/13}$. Both agonists induce strong contractile response in intact SM through a combination of Ca$^{2+}$ influx, Ca$^{2+}$-dependent activation of PLC, Ca$^{2+}$ release from intracellular compartments, as well as Ca$^{2+}$ sensitization. Contraction in response to increasing concentrations of ET-1 was significantly lower in p63RhoGEF shRNA-treated vessel strips compared with control (nontargeting) treated vessels (Figure 3B, Online Figure II). Contraction induced by U46619 in shRNA-treated vessels was only slightly lower at the higher concentrations of U46619, than that observed in control treated vessels, although the difference did not reach statistical significance (Figure 3C and Online Figure II).

p63RhoGEF Knock-Down Disrupts the $G_{q11}$-Coupled Ca$^{2+}$ Sensitization in Mouse Portal Vein

Having established a link between p63RhoGEF and SM contractility in response to ET-1, we set out to examine the effect of reduced p63RhoGEF levels specifically on the RhoA-mediated, Ca$^{2+}$ sensitization pathway. To assess contractility in the absence of Ca$^{2+}$ influx, Ca$^{2+}$ release
from intracellular compartments, and other [Ca\(^{2+}\)]-dependent phenomena, we studied contractile activity of mouse portal vein permeabilized to ions by α-toxin at a constant buffered intracellular [Ca\(^{2+}\)], a protocol that retains receptor-coupling to RhoA.\(^3\,^6\) Contraction induced by stimulation with ET-1 at pCa 6.3 was significantly reduced in vessels where p63RhoGEF expression was knocked-down vessels. In contrast, the U46619 contractile response measured after stimulation with ET-1 at pCa 6.3 was significantly inhibited by partial silencing of p63RhoGEF. GTP\(_\gamma\)S-induced contraction is a measure of the residual component of Ca\(^{2+}\) sensitization. pCa 4.5: maximal force. C, Summary of changes in ET-1 (50 nmol/L and subsequent addition of 150 nmol/L final concentration) induced Ca\(^{2+}\) sensitized force as shown in A and B and in U46619-induced (150 nmol/L) Ca\(^{2+}\) sensitized force as in the same experimental scheme in A and B. The reduction in force, normalized to maximal force induced by pCa 4.5, for 50 nmol/L ET-1 (IC\(_{50}\)) was 29.0±3.4 versus 19.7±2.6, respectively, and at maximal ET-1 (150 nmol/L) was 38.1±4.0% versus 23.0±2.5%, respectively; no significant change in U46619-induced force between control and treated samples. Shown are mean values ±SEM, n=12 to 14, **P<0.01. D, Summary of changes in phosphorylation of the Rho kinase substrate MYPT1 at Thr853 after stimulation by ET-1 and U46619 in the Ca\(^{2+}\) sensitization protocol as detailed in Supplemental Material. Shown are mean values ±SEM, n=4, ***P<0.02.

Ca\(^{2+}\) Sensitized Force Is Relaxed by the Recombinant Extended PH Domain Fragment of p63RhoGEF

It has been established that in its nascent form, p63RhoGEF is autoinhibited. The molecule contains a DBI-homology (DH domain), a minimal unit to catalyze the GTP exchange reaction\(^{20\,\,44}\) and an adjacent C-terminal pleckstrin-homology (PH) domain, which exerts an autoinhibitory function through an interaction with the DH domain. This mechanism appears to be conserved in the related Dbl family members, Trio and Kalirin.\(^{45}\) Activation occurs when the \(G_{\alpha_q1}\) subunit binds to specific amino acids in the PH domain plus its adjacent conserved amino acid stretch (extended PH domain) of p63RhoGEF, thus causing a structural reorganization in p63RhoGEF and relief of autoinhibition. We hypothesized that if the Ca\(^{2+}\)-sensitization effect is mediated by the same mechanism, then the isolated, recombinant extended PH domain (p63RhoGEF\(^{331\,\,-580}\)) should interfere with the process, either by binding to the activated \(G_{\alpha_q1}\) and blocking downstream signaling, or by binding to the transiently open, that is, active conformation of endogenous p63RhoGEF. To test this hypothesis in intact blood vessels, we used a vessel that responds to phenylephrine and can be well permeabilized to proteins to allow for the introduction of the p63RhoGEF fragment into the SM cells, while retaining the receptor-coupling to RhoA. Because this was not possible using mouse portal vein, we used instead rabbit portal vein permeabilized with

Figure 4. Suppression of p63RhoGEF expression significantly inhibits ET-1-induced Ca\(^{2+}\) sensitized force and MYPT1 Thr853 phosphorylation, which is not observed in U46619 stimulation in permeabilized portal veins. A and B, ET-1-induced and GTP\(_\gamma\)S-induced force responses in permeabilized mouse portal veins after treatment with shRNA targeting p63RhoGEF (B) or a nontargeting control (A). CA\(^{2+}\) has been clamped to pCa 6.3. Note that ET-1 Ca\(^{2+}\) sensitized force (shaded component) is inhibited by partial silencing of p63RhoGEF. GTP\(_\gamma\)S-induced contraction is a measure of the residual component of Ca\(^{2+}\) sensitization. pCa 4.5: maximal force. C, Summary of changes in ET-1 (50 nmol/L and subsequent addition of 100 nmol/L; ie, 150 nmol/L final concentration) induced Ca\(^{2+}\) sensitized force as shown in A and B and in U46619-induced (150 nmol/L) Ca\(^{2+}\) sensitized force as in the same experimental scheme in A and B. The reduction in force, normalized to maximal force induced by pCa 4.5, for 50 nmol/L ET-1 (IC\(_{50}\)) was 29.0±3.4 versus 19.7±2.6, respectively, and at maximal ET-1 (150 nmol/L) was 38.1±4.0% versus 23.0±2.5%, respectively; no significant change in U46619-induced force between control and treated samples. Shown are mean values ±SEM, n=12 to 14, **P<0.01. D, Summary of changes in phosphorylation of the Rho kinase substrate MYPT1 at Thr853 after stimulation by ET-1 and U46619 in the Ca\(^{2+}\) sensitization protocol as detailed in Supplemental Material. Shown are mean values ±SEM, n=4, ***P<0.02.
Fortuitously, unlike the mouse vessel, rabbit portal vein responds to phenylephrine, reported to act purely through $\alpha_1$ and not $\alpha_2$. Phenylephrine/$\alpha_1$ coupling was established in mouse aorta. Phenylephrine is a specific $\alpha_1$-adrenoreceptor agonist shown to specifically mediate contractile responses through activation of $\alpha$-adrenoreceptors. $\alpha_1$-Adrenoreceptors have been shown to be present and functional in rabbit arteries including the carotid aortic, iliac, mesenteric, renal, ear, and resistance arteries. Therefore, we assume that they are also present in rabbit portal vein, additionally this vessel contracts in response to phenylephrine. When the contractile force induced by phenylephrine reached a plateau, a control filtrate (ie, buffer from which recombinant p63RhoGEF protein was removed) was added, followed by the p63RhoGEF protein. The filtrate produced no effect (Figure 5A), whereas the recombinant p63RhoGEF fragment induced relaxation of the SM strips (Figure 5A; Online Figure III). Both RhoA activity and RLC20 phosphorylation levels increased when MEF cells were stimulated by phenylephrine (5 mol/L) (Figure 6A), and dropped in the presence of overexpressed p63RhoGEF fragment. A similar trend was also observed on ET-1 stimulation (data not shown).

RhoA-GTP Turnover Is Suppressed in MEF Cells by Overexpression of p63RhoGEF Fragment

Having established an inhibitory role for p63RhoGEF in blood vessels, we investigated if it acts in the same way in cells. We could not use SM cells for that purpose because their transfection efficiency is generally only 2% to 3% by traditional methods and about 30% by electroporation. Instead, we resorted to mouse embryonic fibroblasts (MEF), in which we were able to achieve 80% transfection efficiency. Using MEF cells we investigated the changes in relative levels of RhoA and phospho-RLC20 on phenylephrine stimulation in the presence and absence of exogenous p63RhoGEF. RhoA and RLC20 levels were increased when MEF cells were stimulated by phenylephrine (5 μmol/L) (Figure 6A), and dropped in the presence of overexpressed p63RhoGEF fragment. A similar trend was also observed on ET-1 stimulation (data not shown).

p63RhoGEF Associates With Full-Length p63RhoGEF and the Constitutively Active $\alpha_1$-Adrenoreceptor

We then asked if p63RhoGEF interferes with the RhoA activation pathway by interacting with $\alpha_1$-adrenoreceptors. For this experiment, it was necessary to use cells that do not express endogenous p63RhoGEF, and we chose the HEK293 cells, which provide an additional benefit of easy transfection. Therefore, we overexpressed in HEK293 cells p63RhoGEF with and without full-length p63RhoGEF. Due to compet-
and constitutively active Gαq11 (Q209L) expressed with FLAG-tagged p63RhoGEF331–580 or FLAG-tagged full-length p63RhoGEF in HEK293 cells. Western blots of immunoprecipitation using anti-FLAG antibody show that Gαq11 (Q209L) interacts more strongly with both p63RhoGEF331–580 and full-length p63RhoGEF than wild-type.

**Discussion**

The RhoA/ROCK pathway serves as a final signaling hub in all SM tissues, accepting stimulatory signals initiated by diverse agonists, and activating Ca2+ sensitization through inhibition of myosin light chain phosphatase. This physiological phenomenon is of critical significance in control of blood pressure, airway resistance, erectile dysfunction, gut motility, bladder and ureter function, and so forth. The central role of the ROCK kinase prompted a number of investigations into its potential as a drug target. However, the ubiquitous distribution of ROCK reduces the clinical potential of this approach, as it lacks the desired tissue specificity. On the other hand, downstream of ROCK, RhoA appears to be activated through diverse pathways, each utilizing a specific combination of GPCRs, trimeric G-proteins and GEFs. This raises the exciting possibility of selective targeting of tissue-specific GEFs for therapeutic use.

To better understand the intricate nature of the Ca2+-independent component of contractile stimulation in SM, it is necessary to dissect the molecular pathways and identify the individual GPCRs as well as their partner G-proteins and coupled GEFs. Most vasoconstrictors, such as angiotensin II, ET-1, phenylephrine, and thromboxane A2 bind to GPCRs coupled either to Gα11 or Gα12/13 or both. In mice, knockout of Gαq11, but not Gα12/13, results in a fall in basal blood pressure while both are needed for the development of salt-induced hypertension. Studies of contractility of aorta from these mice showed that phenylephrine-induced force was completely abolished in the Gαq11 deficient mice but unaffected in the absence of Gα12/13. On the other hand, contractions in response to ET-1 or U46619 are mediated by both G-proteins, with Gαq11 dominating for ET-1 and Gα12/13 for U46619. The key question is what portion of Ca2+-sensitized contractile response is transmitted by each of these G-proteins, and if their signaling pathways are routed through distinct GEFs.

Although Gα12/13 was shown to activate RhoA through the three RGS-containing, RhoA-specific GEFs, for example, PDZRhoGEF, LARG, and p115RhoGEF, the pathways involving Gαq11 remained unclear. It has been established that the RhoA-specific exchange factor p63RhoGEF is selectively activated by Gαq11. Recently, mRNA transcripts for p63RhoGEF were identified in rat aorta. In an unrelated study, p63RhoGEF coupling through Gαq11 has been shown in cultured rat aortic SM cells to mediate angiotensin II signaling. It was found that angiotensin II first induces a rapid influx of intracellular [Ca2+]i and activation of p63RhoGEF, followed by a significantly slower, [Ca2+]i/Janus kinase 2–dependent activation of p115RhoGEF to sustain RhoA signaling. However, the role of this pathway in contractility was assessed only by a collagen contraction assay using cultured rat aortic SM cells known to dedifferentiate in culture. As expected, basal contraction increased on angiotensin II pathway stimulation but not in p63RhoGEF depleted cells.

The purpose of our investigation was to dissect the specific role of p63RhoGEF-mediated Ca2+ sensitization...
in blood vessels in response to stimulation through Gq/11, but through receptors other than that activated by angiotensin II. Our results confirm the presence of p63RhoGEF mRNA transcripts and protein in SM tissues and cells. Importantly, we show that expression profiles of upstream and downstream partners of p63RhoGEF (ie, Gq/11 and RhoA) correlate well with p63RhoGEF across the tissues screened. We demonstrate for the first time that p63RhoGEF plays an important part in vessel contractility induced by ET-1, coupled primarily to Gq/11 through the ETA receptors but not by U46619, a thromboxane A2 analog, known to couple predominantly by Goi2/13. The Goi2/11 coupling leads to PLCβ activation, production of IP3 (inositol 1,4,5-trisphosphate) and DAG (diacylglycerol) and consequently increased intracellular [Ca2+] as well as activation of the RhoA pathway. To evaluate if the Ca2+-sensitization pathway is affected by the knock-down of p63RhoGEF, we used α-toxin permeabilization, a method that retains agonist-GPCR coupling to RhoA under conditions where intracellular [Ca2+] is clamped. Ca2+ sensitization of contractile force induced by ET-1 but not by U46619 was significantly reduced when p63RhoGEF expression was suppressed (Figure 4). Furthermore, this was accompanied by a decrease in MYPT1 phosphorylation indicative of decreased RhoA/ROCK activity. These and other findings lead us to suggest that the Gq/11 contribution to the regulation of basal and stimulated blood pressure may signal through p63RhoGEF rather than solely through PLCβ-induced Ca2+ release. Consistent with this idea is the observation that ROCK inhibitor Y-27632 reduces hypertension45,46 and that the Rho/ROCK signaling pathway in SM cells is an important contributor to peripheral resistance.55 Significantly, p63RhoGEF mRNA is expressed in the resistance vessels, thoracodorsal and mesenteric arteries (Figure 1). P63RhoGEF protein was also detected in these vessels (data not shown). The contribution of this RhoGEF to the contractile behavior of resistance vessels will be explored in the future.

We also wondered if the in vivo mechanism by which p63RhoGEF operates in SM is consistent with a model derived from crystallographic studies of its complex with activated Goi2/11.40 We hypothesized, that if the Ca2+-sensitization effect is mediated by the same mechanism, then the isolated, recombinant extended PH domain (p63RhoGEF 331–580) should interfere with the process, either by binding to the activated Goi2/11, and blocking downstream signaling, or by binding to the transiently open, for example, active conformation of endogenous p63RhoGEF. To test this hypothesis in blood vessels, we performed contractility assays using β-escin permeabilized rabbit portal vein to allow introduction of the p63RhoGEF fragment into the SM cells. As expected, p63RhoGEF 331–580 induced relaxation after phenylephrine-induced contraction, which is known to be mediated by Goi2/11.30 This was accompanied by a decrease in RhoA-GTP and phospho-RLC20, suggesting suppression of Goi2/11-coupled RhoGEF activity. Reduction of RhoA activity on stimulation by phenylephrine in the presence of exogenous p63RhoGEF 331–580, acting as a dominant negative, was also observed in MEF cells. Overexpressed p63RhoGEF 331–580 associated with the full-length p63RhoGEF and with activated Goi2/11. Our findings are consistent with the DH domain of p63RhoGEF being auto-inhibited through its extended PH domain under basal conditions with agonist activated Goi2/11 relieving this auto-inhibition as shown in compelling in vitro experiments.45,49 A similar fragment of p63RhoGEF, comprising residues 295 to 580, was also shown to interfere with the angiotensin II–mediated RhoA activation pathway in SM cells.35 Taken together, these data strongly support the notion that the molecular mechanism inferred from the crystallographic studies is representative of the in vivo phenomenon.

In summary, the evidence presented in our report shows that activation of p63RhoGEF by agonists that stimulate receptors coupled to Goi2/11 leads to Ca2+ sensitization in blood vessels and cultured SM cells. We show that in addition to angiotensin II, both phenylephrine and ET-1 also signal through p63RhoGEF to active RhoA and Ca2+ sensitized force. We conclude that different Goi2/11-coupled receptors can signal through p63RhoGEF to contribute to vascular tone and that it is not unique to angiotensin II stimulation. Indeed, angiotensin II plays a major role in hypertension, whereas the tonic discharge of catecholamines by vasoconstricting sympathetic nerves maintains normotensive blood pressure. Further identification of specific RhoGEFs and characterization of their function in SM is a fertile ground for finding new targets that mediate specific functions of normal and diseased SM including the regulation of vascular tone.

Acknowledgments

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Disclosures

None.

References

6. Kitazawa T, Kobayashi S, Horii K, Somlyo AV, Somlyo AP. Receptor-coupled, permeabilized smooth muscle: role of the phosphatidylinositol...
Novelty and Significance

What Is Known?
- The small GTPase RhoA is activated by multiple agonists and significantly contributes to vascular contractility under physiological as well as pathophysiological conditions such as hypertension.
- Multiple GTP exchange factors (GEFs) are expressed in smooth muscle raising the possibility that specific agonists of specific G-protein coupled receptors (GPCRs) may be associated with distinct RhoGEFs.
- Angiotensin II has been shown to signal through $G_{q/11}$ in cultured smooth muscle cells but the role of other agonists in intact blood vessels is unknown, particularly agonists that regulate basal vascular tone.

What New Information Does This Article Contribute?
- p63RhoGEF is selectively activated by agonists such as $\alpha$-adrenergic and endothelin-1 that signal through $G_{q/11}$ in blood vessels and maintain normotensive blood pressure.
- Knock down of p63RhoGEF decreases RhoA activity leading to increased myosin phosphatase activity, decreased myosin phosphorylation and decreased force development in blood vessels.
- We demonstrate in vivo that the molecular mechanism of action of p63RhoGEF is consistent with a model derived from crystallographic studies.
- p63RhoGEF is a potential selective therapeutic target for decreasing peripheral resistance and blood pressure.

Multiple agonists signaling through GPCRs activate the RhoA pathway which plays a central role in the regulation of basal tone, peripheral resistance, hypertension and cerebral vasospasm. RhoGEFs function upstream of RhoA, but it is unclear whether the multiple RhoGEFs present in smooth muscle (SM) couple selectively to specific agonists through GPCRs. If so, this would allow for selective therapeutic targeting of specific RhoGEFs in vascular SM. p63RhoGEF selectively coupled $G_{q/11}$ but not $G_{12/13}$ to RhoA activation in blood vessels, mediating the physiologically important Ca$^{2+}$ sensitization of force induced with $G_{q/11}$-coupled agonists. Experiments using a recombinant extended PH domain fragment of p63RhoGEF show that this region serves as an autoinhibitory domain, interfering with the regulatory pathway by sequestering activated $G_{q/11}$. Our study extends the reported findings on angiotensin II to other $G_{q/11}$-coupled agonists such as $\alpha$-agonists and endothelin-1. Angiotensin II plays a major role in hypertension while catecholamines maintain normotensive blood pressure. We suggest that signaling through p63RhoGEF contributes to selective regulation of blood pressure. Further identification of specific RhoGEFs and characterization of their function in SM could identify new targets for regulating specific functions of normal and diseased SM.
p63RhoGEF Couples $G_{\alpha q/11}$-Mediated Signaling to $Ca^{2+}$ Sensitization of Vascular Smooth Muscle Contractility
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Supplemental Material

1. Characterization of anti-p63RhoGEF antibodies

An anti-p63RhoGEF antibody (Proteintech 51004-1-AP) typically gave triplet bands across species (i.e. mouse, rat and rabbit) (Figure 2; Online Figure I; rabbit data is not shown) with an 80 kDa upper band which we propose is a full-length p63RhoGEF based on the following reasons: 1) Exogenously expressed N-terminally FLAG-tagged and C-terminally Myc-tagged full-length p63RhoGEF (580 amino acids) detectable by anti-FLAG and anti-Myc antibodies ran as a single 80 kDa band (Online Figure I, C). Over-expressed p63RhoGEF was also detectable by anti-p63RhoGEF antibody and ran as a single 80 kDa band (Online Figure I, B); 2) The antibody (Proteintech 51004-1-AP) did not recognize an exogenously expressed 80 kDa protein when a single mutation (L580Q) was introduced in its epitope region (Online Figure I, C); 3) This 80 kDa band was diminished when anti-p63RhoGEF antibody (Proteintech 51004-1-AP) was absorbed with the p63RhoGEF<sup>331-580</sup> recombinant protein, which includes the epitope of anti-p63RhoGEF antibody (Proteintech 51004-1-AP) (Online Figure I, D); 4) Expression of shRNA oligos, designed to target p63RhoGEF mRNA sequence but not non-targeting shRNA, in MEF cells expressing p63RhoGEF, significantly decreased the 80 kDa band (Online Figure I, A). Treatment with shRNA also reduced the middle band but not the lower band. The middle band may correspond to an N-terminally truncated p63RhoGEF isoform but the smallest band is most likely a non-specific cross-reaction. This result was consistent over 3 independent shRNA sequences; 5) The 80kDa band was also recognized by another rabbit polyclonal antibody generated against p63RhoGEF (Protein tech 14839-1-AP). This antibody also labeled a second band corresponding to the second band detected with the first antibody (Proteintech 51004-1-AP) but not the lower band (Online Figure I, B). Therefore the lowest molecular weight band is a non-specific cross-reaction.

The slower electrophoretic mobility of p63RhoGEF running at 80kDa rather than 63kDa is likely a reflection of unique migration properties of residues 503-580 based on the following observations: N-terminally and C-terminally truncated p63RhoGEF<sup>331-502</sup> comprising mainly the PH domain gave an apparent size of approximately 22 kDa by electrophoresis in agreement with its predicted size of 22.1 kDa. However, N-terminally truncated p63RhoGEF<sup>331-580</sup> comprising the PH domain and all the residues to its C-terminal end gave an apparent size of approximately 40 kDa whereas its predicted size is 30.3 kDa (data not shown). In other words, addition of residues from 503 to 580 resulted in approximately a 10 kDa upward shift in apparent molecular weight partially accounting for the higher than predicted molecular
weight of full-length p63RhoGEF measured by electrophoresis. This slower migration of p63RhoGEF\textsubscript{331-580} was further verified by Western blot analysis using anti-HA and anti-Myc antibodies to detect over-expressed N-terminally HA-tagged and C-terminally Myc-tagged p63RhoGEF\textsubscript{331-580}, resulting in a band slightly higher than 40 kDa (data not shown). Taken together, we concluded that the 80 kDa band corresponds to full-length p63RhoGEF and that its slower than predicted electrophoretic mobility reflects unique migration properties of C-terminal residues 331-580.

2. Detailed Methods

Antibodies
Rabbit polyclonal anti-G\textsubscript{aq/11} antibody (Santa Cruz Biotechnology California sc-392 1:1000); mouse monoclonal anti-RhoA(26C4) antibody (Santa Cruz Biotechnology California sc-418 1:500); mouse monoclonal anti-RLC antibody (Sigma); rabbit polyclonal anti-phospho-RLC antibody (Cell Signaling, Danvers MA). Mouse monoclonal anti-MYPT1 antibody (BD Bioscience 1:1000); Rabbit polyclonal anti-phospho-MYPT 853 antibody (Millipore 1:1000). Rabbit polyclonal anti-p63RhoGEF antibody against the C-terminal 11 residues (ARLAKLDEDEL) conserved in human, mouse and rat (Proteintech Illinois 51004-1-AP 1:1000). Rabbit polyclonal anti-p63RhoGEF antibody (Proteintech Illinois 14839-1-AP 1:1000).

Tissue screen
Tissues were isolated from mice, rats and rabbits and outer connective tissue was carefully removed. Longitudinal SM was isolated from ileum. The cerebral vessels on the ventral surface of the brain were used. This included the posterior inferior cerebral arteries and the contiguous vessels up to and including the circle of Willis. The endothelium was removed from blood vessels, except cerebral vessels, by gently rubbing with a cotton tip. Tissues were homogenized in homogenization buffer (1% SDS, 300 mM NaCl, 50 mM Tris pH 7.5) and cleared by centrifugation at 14,000 x g for 10 min. Protein content of supernatants were measured by Lowry method and equalized for loading. 4X Sample buffer (250 mM Tris pH 6.8, 60% glycerol, 60 mM DTT, 0.016% Bromophenol Blue) was added to 25% of the final volume and samples were subjected to Western blot analysis and the bands were quantitated.

Production of p63RhoGEF\textsubscript{331-580} recombinant protein
E. coli BL21 was transformed with pGST-Parallel1-p63RhoGEF\textsubscript{331-580}, pre-cultured to OD\textsubscript{600} 2.0 at 37°C and further cultured at 16°C in Terrific Broth containing 100 mg/l ampicillin for 24 h. Bacteria were
harvested by centrifugation, re-suspended in ice-cold PBS containing protease inhibitors (complete protease inhibitor cocktail tablets; Roche) and lysed by French press. Cell lysate was cleared by a two-step centrifugation, first at 20,000 g for 15 min followed by a 65,000 g spin for 1.5 h (both at 4°C). Resulting supernatant was incubated with glutathione sepharose 4 fast flow beads (Amersham) overnight at 4°C. After thorough wash by PBS, the beads were packed in a column, and GST-p63RhoGEF331-580 was eluted by 5 mM reduced glutathione in 50 mM Tris (pH 8.0). Cleavage of GST by the Tobacco Etch Virus (TEV) protease was carried out while the eluate was dialyzed against relevant buffer for TEV proteolysis [0.5 mM EDTA, 100 mM NaCl, 5 mM 2-mercaptoethanol, 50 mM Tris (pH 8.0)] overnight at 4°C followed by dialysis against PBS on the next day. Removal of cleaved GST, uncleaved protein and the TEV protease (polyhistidine-tagged) was done by incubating the sample with the glutathione beads together with Ni-NTA agarose overnight at 4°C.

**Smooth muscle contractility measurements**

SM strips were cut from freshly isolated blood vessels or from mouse blood vessels following organ culture for silencing p63RhoGEF (described below). SM strips 2 mm long and 200 μm wide were cut with razor knives from blood vessels, attached with monofilament silk to a force transducer at one end and a rigid hook at the other and mounted on a bubble plate as described previously or on a specially designed bubble plate myograph (Danish Myo Technology, Aahus Denmark). Muscle strips were stretched to 1.2 times resting length and equilibrated at room temperature in Hepes buffered Krebs’ solution. The responsiveness of the muscle strip was first tested by transferring it to high K⁺ solution. shRNA-treated intact SM strips of mouse portal vein were incubated in Hepes Krebs’ solution at room temperature and used to plot cumulative force dose response curves to ET-1 and U46619. Force responses are expressed as mN. All strips were cut to have the same cross sectional area.

For evaluation of Ca²⁺ sensitization in mouse PAMV strips treated with shRNAs, strips were permeabilized with *Staphylococcus aureus* alpha toxin (60,000 units·ml⁻¹·h⁻¹) in G1 solution. Treatment with 10 μM A23187 for 10 min was used to deplete Ca²⁺ stores. Muscles were contracted by transferring from a G1 solution to pCa6.3. When contraction reached a plateau, the Ca²⁺ sensitization pathway was activated by ET-1 (50 and then 100 nM) or U46619 (100nM) plus GTP (1 μM). Once this tension reached a plateau, GTPγS (10 μM) was added to measure the residual component of Ca²⁺ sensitized force. Final maximal tension was elicited by pCa4.5 solution. ET-1-induced force was normalized to maximal pCa4.5 force.
For the experiments in which recombinant p63RhoGEF<sup>331-580</sup> was added to PAMV muscle strips from rabbit, the strips were permeabilized with 50 µM β-escin in relaxing 1 mM EGTA (G1) solution<sup>2,3</sup> for 30 min. β-escin treatment allows access of the recombinant protein to the cytoplasm. Treatment with 10 µM A23187 for 10 min was used to deplete Ca<sup>2+</sup> stores. Calmodulin (1 µM) was added to all solutions. Muscles were contracted by transferring from a G1 solution to pCa6.3. When contraction reached a plateau, the Ca<sup>2+</sup> sensitization pathway was activated by phenylephrine (2.5 µM) plus GTP (1 µM). When contraction reached the second plateau, recombinant p63RhoGEF<sup>331-580</sup> protein (40 µM) was added, which relaxed the muscle.

Organ cultured tissues compared to untreated freshly-isolated tissues respond to the same agonists and the relationship of the responses to different agonists is conserved. We have not directly compared maximal contractile activity of freshly isolated with organ cultured mouse vessels. However, the sensitivity settings used for recording tension are the same, so we do not think that contractile activity is depressed by more than 10-20% over 5 days in organ culture. Organ cultured mouse PAMV displayed normal spontaneous rhythmic activity similar to freshly isolated tissue.

**Rhotekin assay**

Rabbit portal vein strips were prepared and treated (i.e. equilibration, permeabilization, induction of Ca<sup>2+</sup> dependent pathway and Ca<sup>2+</sup> sensitization by phenylephrine and addition of recombinant p63RhoGEF<sup>331-580</sup> protein) as in the contraction experiments. The tissue samples were collected 1) prior to addition of phenylephrine, 2) at contraction plateau following addition of phenylephrine and 3) at relaxation after addition of recombinant p63RhoGEF<sup>331-580</sup> protein. MEF cells were transfected with mammalian expression plasmids to over-express FLAG- p63RhoGEF<sup>331-580</sup>. Transfection was performed using Lipofectamine 2000 reagent (Invitrogen). Transfection efficiency was improved by repeating transfection process three times. Following overnight serum starvation, the transfectants along with non-tranfected control cells were stimulated either by addition of phenylephrine (5 µM) or ET-1 (100 nM) for 10 min. Subsequent rhotekin assay was performed adopting procedures described previously<sup>5</sup>. The rabbit PAMV strips or MEF cells were immediately lysed in ice-cold rhotekin buffer [25 mM HEPES, pH 7.5, 150 mM NaCl, 1% NP-40, 10 mM MgCl2, 1 mM EDTA, 7% glycerol, 1% protease inhibitor cocktail (SIGMA)]. Following centrifugation at 14,000 x g for 10 min, protein contents of the supernatants were equalized based on protein concentrations measured by Lowry method, and the supernatants were subjected to rhotekin RBD agarose. Relative quantities of RhoA associated with Rhotekin RBD agarose (i.e. RhoA-GTP) were estimated by Western blot analysis and normalized to total RhoA.
RLC$_{20}$ and MYPT1 phosphorylation

RLC$_{20}$ phosphorylation in rabbit portal vein strips was measured following the same treatment using the same protocol as described above for the Rhotekin assay. Tissues were quick-frozen in 10% trichloroacetic acid in acetone chilled to -80°C, at the time points described above for the rhotekin assay. Quantitation of MYPT1 phosphorylation at Thr853 in mouse portal vein strips was carried out following permeabilization and identical treatment as shown in the SM contractility measurement protocol (Fig. 4A,B). Tissues samples were rapidly frozen in 10% trichloroacetic acid in acetone chilled to -80°C after a 7-minute stimulation by ET-1 (50 nM) or U46619 (150nM). Subsequently, tissue samples were processed as described previously 6. Briefly, frozen strips were subsequently washed with pure acetone, dried, homogenized in homogenization buffer (1% SDS, 150 mM NaCl, 50 mM Tris pH 7.5), boiled and cleared by centrifugation at 14,000 x g for 10 min. Protein content in each sample was equalized based on the protein concentration measurement by Lowry method. 4X sample buffer (250 mM Tris pH 6.8, 60% glycerol, 60 mM DTT, 0.016% Bromophenol Blue) was added to 25% of the final volume of the samples. The samples were subjected to Western blot analysis to detect total RLC$_{20}$, phosphorylated RLC$_{20}$ at Ser19, total MYPT1 and MYPT1 phosphorylated at Thr853 using antibodies detailed above. The relative phosphorylation levels were quantitated by normalizing to total RLC$_{20}$ or total MYPT1 respectively.

Co-immunoprecipitation assays

FLAG-p63RhoGEF-Full-Myc was expressed by itself or with FLAG-p63RhoGEF$^{331-580}$ in HEK293 cells. The cells were lysed in buffer [0.5% Triton X-100, 150 mM NaCl, 50 mM Tris (pH 7.5), 2 mM EDTA, 2 mM sodium orthovanadate, 50 mM NaF, 1% protease inhibitor cocktail (SIGMA)] and subjected to protein G agarose with anti-Myc antibody. The agarose was washed with lysis buffer and subjected to Western blot analysis using anti-FLAG antibody.

Full-length FLAG-p63RhoGEF-Myc or FLAG- p63RhoGEF$^{331-580}$ were co-expressed with $G_{\alpha11}$ wild-type or $G_{\alpha11}$ Q209T constitutively active mutant in HEK293 cells. The cells were lysed in buffer [0.5% Triton X-100, 150 mM NaCl, 50 mM Tris (pH 7.5), 2 mM EDTA, 2 mM sodium orthovanadate, 50 mM NaF, 1% protease inhibitor cocktail (SIGMA)] and immunoprecipitation was performed using EZview Red ANTI-FLAG M2 Affinity Gel (SIGMA). The gel was washed with lysis buffer and subjected to Western blot analysis.
p63RhoGEF knock-down in organ cultured PAMV

RNAi sequences designed to target both mouse and human p63RhoGEF mRNAs are as follows. No sequences matching all other known mouse and human mRNA sequences were detected. The third sequence coincidentally matches with rat p63RhoGEF mRNA sequence.

GCATATGTATGTGGTGTACTG
GCAAATCATCATCTTCAGTGA
GCCAAGCTGGATGAAGATGAG

Those sequences were introduced in pENTR/U6 plasmid (Invitrogen) for shRNA expression in mammalian cells. Knocking-down of p63RhoGEF has been confirmed by 1) co-transfecting HEK293 cells, which lack endogenous p63RhoGEF, with low-level expression plasmid for p63RhoGEF expression and BLOCK-iT U6 RNAi Entry Vector for shRNA expression resulting in significant reduction in exogenous p63RhoGEF expression (Online Figure I, A) and by 2) transfection of MEF cells with BLOCK-iT U6 RNAi Entry Vector for shRNA expression resulted in significant reduction in endogenous p63RhoGEF expression. The first and third sequences showed higher potency of knock-down in both over-expressed and endogenous p63RhoGEF and therefore further introduced in pAdloss-adenoviral U6 siRNA shuttle vector for the production of adenovirus consisting of the sequence to express shRNA in mammalian cells. pAdloss-adenoviral U6 siRNA shuttle vector and control virus consisting of a randomized sequence [AAGTGGCGCGCTAGGAAGAGA] showing no homology with known genes were kindly provided by Dr. Kasahara.

Portal vein SM strips were isolated from male C57B mice and incubated in 100 ml of OPTIMEM containing adenovirus constructs to express control non-targeting shRNA or the shRNA sequence targeting p63RhoGEF mRNA at 1x10^10 PFU. After 24 h, 100 ml of serum free media containing additional adenovirus at 1x10^10 PFU was added and this was repeated one more time. After the 72 h of incubation time in the presence of virus, muscles were incubated in serum free media without virus for another 48 h. Quantitative assessment of the knock-down efficiency was performed by quantitative RT-PCR and Western blot analysis.

Quantitation of p63RhoGEF transcriptome in different cell lines and in comparison with the expression levels of other GEF transcriptomes by quantitative polymerase chain reaction

Total mRNA extracted from primary, unpassaged human aortic, pulmonary artery and brain vascular SM cell cultures were purchased from ScienCell Research Laboratories (Carlsbad, California). Isolated mouse SM tissue samples were immediately submerged in RNAlater (Applied Biosystems) solution to prevent
RNA degradation. RNA extraction from mouse thoracic aorta, abdominal aorta, pulmonary artery, portal vein and bladder SM and mouse brain was carried out using TRIzol Reagent (Invitrogen) according to manufacture's protocol. In order to remove traces of genomic DNA, RNA samples were treated with DNaseI (Invitrogen) prior to cDNA syntheses. cDNA was synthesized from each RNA sample using Superscript III (Invitrogen) or iScript cDNA Synthesis Kit (BioRad). For quantitation of p63RhoGEF mRNA in mouse portal veins used in contractility measurements, quantitative RT-PCR amplification was performed using StepOne 7200 (Applied Bioscience) and expression levels of p63RhoGEF mRNA was normalized to those of beta-2 macroglobulin (B2M). Primer sets used: p63RhoGEF GCAGAAGAAGATGGCTCTGG and CCAAAACACAAATCCTGTACG; B2M GGTCTTTCTGGCTTGCTAC and CAATGTGAGGCGGGTGAACTG. For quantitation of p63RhoGEF mRNA in different cell lines or transcriptome of various RhoGEFs, primers specific for each RhoGEF gene were designed to have uniform length and GC% content using PrimerExpress software. Each set of primers was designed to amplify fragments between 100 to 150 bp with melting temperature between 78°C and 85°C. To minimize cross-reactivity, primers were designed outside the regions coding for the DH-PH tandems. Primers sets were synthesized by Invitrogen and tested for performance using standard curve method and mouse brain cDNA. Primer quality was judged by three criteria: a) primer efficiency to be between 90% and 110%; b) melting curve analysis for each PCR product to give single peak close to calculated melting temperature of the amplicon; c) 4% agarose gel for each amplicon to give a single band of a predicted size. Quantitative RT-PCR amplification was performed using StepOne Plus Thermocycler (Applied Biosystems) at 95°C for 10min, followed by 40 cycles of 95°C for 15s and 60°C for 60s. Reactions consisted of 5µl Power SYBR Green PCR Master Mix (Applied Biosystems), 2.5µl of primer mix at 0.3µM final concentration and 2.5µl of cDNA. Three measurements were averaged for each cDNA sample to minimize experimental error. Cт values were determined using fluorescence baselines and thresholds calculated for each target by StepOne Plus software. Gene expression was normalized to GAPDH expression and calculated as $2^{-ΔCт}$, were $ΔCт = (Cт of GAPDH - Cт of target gene)$. Cт values were not adjusted for amplification efficiency, since all primers used for comparative gene expression studies were already optimized to have efficiency close to100%. For each tissue $ΔCт$ values were averaged and errors calculated from at least three animals.
References


Online Figure I. Western blot analyses of p63RhoGEF. A. Knock-down of human, mouse and rat p63RhoGEF by shRNA (GCCAAGCTGGATGAAGATGAG). Top: HEK293 cells lacking endogenous p63RhoGEF are co-transfected by mammalian expression plasmid of FLAG-tagged human p63RhoGEF with an expression plasmid to express either shRNA targeting p63RhoGEF or non-targeting shRNA control. Western blot analysis was performed using anti-FLAG antibody. Middle and Bottom: MEF cells or rat aortic cells (R518) were transfected by viral construct to express either shRNA targeting p63RhoGEF or non-targeting shRNA control. Western blot analysis was performed using anti-p63RhoGEF antibody (Proteintech 51004-1-AP). B. HEK293 cells, HEK293 with over-expressed p63RhoGEF, mouse aorta tissue and MEF cells blotted by different anti-p63RhoGEF antibodies. C. HEK293 cells, HEK293 cells with over-expressed N-terminally FLAG-tagged and C-terminally Myc-tagged full-length p63RhoGEF (580 amino acids) with a single mutation (L580Q) in the epitope of anti-p63RhoGEF antibody (Proteintech 51004-1-AP) blotted by anti-p63RhoGEF (Proteintech 51004-1-AP), anti-FLAG and anti-Myc antibodies. The over-expressed protein was not detected by anti-p63RhoGEF antibody (Proteintech 51004-1-AP) indicating specificity of this antibody to the epitope. D. Mouse ileum homogenate blotted by anti-p63RhoGEF antibody (Proteintech 51004-1-AP) in the presence and absence of the p63RhoGEF<sup>331-580</sup>, which include the epitope of anti-p63RhoGEF antibody (Proteintech 51004-1-AP). (Small asterisks indicate that extra lanes between lanes shown were cropped; i.e. horizontally aligned boxes in the same group are on the same blot.)
Online Figure II. Suppression of p63RhoAGEF expression significantly inhibits ET-1 but not U46619-induced contractions in intact portal veins. Tension traces showing normal spontaneous contractile activity of mouse portal veins following treatment in organ culture with a viral construct to express shRNA targeting p63RhoGEF as compared to the ones treated with a non-targeting control. Data is summarized in Figure 3 in the manuscript. Muscles are incubated in Hepes buffered Krebs solution and contractile force recorded in response to cumulative doses of ET-1 (A and B) and U46619 (C and D); each increment represents 0.3, 1.0, 3.0, 10, 30, 100 and 300 nM for both ET-1 and U46619. The area under the curve was measured and plotted in Figure 3. Rho kinase inhibitor, Y-27632, added to the maximal ET-1 force response (A and B) relaxed ET-1-induced Ca\(^{2+}\)-sensitized force.

Online Figure III. Dose-dependent effect of p63RhoGEF\(^{331-580}\) PH domain fragment on phenylephrine-induced Ca\(^{2+}\) sensitized force in SM strips. Ca\(^{2+}\) sensitized force was induced by phenylephrine (PE; 2.5 µM) following β-escin-permeabilization to allow passage of p63RhoGEF recombinant protein across the cell membranes of rabbit portal vein SM strips. [Ca\(^{2+}\)] was buffered to pCa6.3. An incremental increase (each increment represents 10 µM) in p63RhoGEF\(^{331-580}\) PH domain fragment concentration in the system induced relaxation of phenylephrine-induced Ca\(^{2+}\) sensitized force accordingly.