Arhgap28 Is a RhoGAP that Inactivates RhoA and Downregulates Stress Fibers

Ching-Yan Chloé Yeung1,2#*, Susan H. Taylor1, Richa Garva1, David F. Holmes1, Leo A. Zeef2, Raija Soininen3, Raymond P. Boot-Handford1, Karl E. Kadler1*

1 Wellcome Trust Centre for Cell-Matrix Research, University of Manchester, Manchester, United Kingdom, 2 Faculty of Life Sciences, University of Manchester, Manchester, United Kingdom, 3 Department of Dermatology, Oulu Center for Cell-Matrix Research, University of Oulu, Oulu, Finland

Abstract

The small GTPase RhoA is a major regulator of actin reorganization during the formation of stress fibers; thus identifying molecules that regulate Rho activity is necessary for a complete understanding of the mechanisms that determine cell contractility. Here, we have identified Arhgap28 as a Rho GTPase activating protein (RhoGAP) that switches RhoA to its inactive form. We generated an Arhgap28-LacZ reporter mouse that revealed gene expression in soft tissues at E12.5, pre-bone structures of the limb at E15.5, and prominent expression restricted mostly to ribs and limb long bones at E18.5 days of development. Expression of recombinant Arhgap28-V5 in human osteosarcoma SaOS-2 cells caused a reduction in the basal level of RhoA activation and disruption of actin stress fibers. Extracellular matrix assembly studies using a 3-dimensional cell culture system showed that Arhgap28 was upregulated during Rho-dependent assembly of the ECM. Taken together, these observations led to the hypothesis that an Arhgap28 knockout mouse model would show a connective tissue phenotype, perhaps affecting bone. Arhgap28-null mice were viable and appeared normal, suggesting that there could be compensation from other RhoGAPs. Indeed, we showed that expression of Arhgap6 (a closely related RhoGAP) was upregulated in Arhgap28-null bone tissue. An upregulation in RhoA expression was also detected suggesting that Arhgap28 may be able to additionally regulate Rho signaling at a transcriptional level. Microarray analyses revealed that Col2a1, Col9a1, Matn3, and Comp that encode extracellular matrix proteins were downregulated in Arhgap28-null bone. Although mutations in these genes cause bone dysplasias no bone phenotype was detected in the Arhgap-28 null mice. Together, these data suggest that the regulation of Rho by RhoGAPs, including Arhgap28, during the assembly and development of mechanically strong tissues is complex and may involve multiple RhoGAPs.

Introduction

The actin cytoskeleton is fundamental to a wide range of cellular functions including cellular contractility, stiffness sensing, tissue formation, cell migration and cell polarity but the molecular mechanisms are complex and not fully understood. Members of the family of Rho guanosine triphosphatases (GTPases) are major regulators of the assembly of actin-based stress fibers along with mammalian diaphanous 1 (mDia) and Rho-associated kinase (ROCK) [1–3]. ROCK positively drives the assembly of contractile actin stress fibers by directly phosphorylating the myosin light chain (MLC), and also by inactivating MLC phosphatase [2,4]. Dynamic reorganization of the actin cytoskeleton into stress fibers is essential for fibroblast assembly and is regulated by signaling from Rho GTPases [5,6]. Actomyosin contractility is required for the translocation of fibronectin-bound integrins in specialized cell-matrix adhesions along actin stress fibers, a process that is believed to stretch folded fibronectin dimers to facilitate their assembly [7–9]. The mechanism for stress fiber-mediated ECM assembly is mechano-sensitive (via cell-matrix adhesions) and tightly regulated. For examples, disruption to actin polymerization or loss of tension caused the misalignment of collagen fibrils in newly synthesized tendon ECM [10,11], and targeted ROCK overexpression in the epidermis led to increased collagen deposition and ECM stiffness [12]. While it is well known that Rho GTPases regulate actin stress fiber assembly, how they are regulated during tissue morphogenesis is less well understood.

Although Rho is a GTPase its rate of GTP hydrolysis slow. Efficient hydrolysis of GTP requires a Rho GTPase activating protein (RhoGAP), which accelerates the hydrolytic activity up to 105-fold [13]. There are over 70 genes encoding proteins that contain a RhoGAP domain [14]. This multitude of RhoGAPs is thought to ensure signaling specificity, for example, via tissue-specific expression, specificity for a single GTPase or signaling pathway, or that some RhoGAPs act as scaffold proteins or effectors for crosstalk between Rho GTPases and other signaling pathways (reviewed by [14]).

We show here that Arhgap28 is differentially regulated during mouse embryonic development. The functions of Arhgap28 have not been reported but its differential expression has been listed in a
variety of cDNA microarray studies, as summarized in Table S1. Based on amino acid sequence similarities Arhgap20 is closely related to Arhgap6, Arhgap11a, Arhgap11b, Arhgap18, Arhgap40, DLC1 (Arhgap7), DLC2 (Arhgap27) and DLC3 (Arhgap30). Some of these RhoGAPs have been shown to regulate actin reorganization. Knockout of DLC1 is embryonic lethal at E10.5 of mouse development and examination of fibroblasts isolated from E9.5 mouse embryos revealed disrupted stress fibers and focal adhesions [15]. Mice lacking functional Arhgap6 protein are phenotypically normal, despite the fact that Arhgap6 is a RhoGAP for RhoA and causes the loss of actin stress fibers in cultured cells [16]. Arhgap18 has specificity for RhoA and disrupts actin stress fibers, where knockdown of Arhgap18 can enhance stress fiber formation [17]. On the basis of these studies, we hypothesized that Arhgap28 regulates actin stress fiber assembly.

Results

Sequence alignment predicts that Arhgap28 has a RhoGAP function similar to Arhgap6 and Arhgap18

RhoGAP function is mediated via the RhoGAP domain (represented schematically in Figure 1A), which enhances hydrolysis of GTP by the target Rho GTPase. Alignment of the RhoGAP domains of murine Arhgap6, Arhgap18 and Arhgap28 showed high homology and confirms the presence of a putative catalytic arginine residue (R125) in Arhgap28 (Figure 1B), suggesting that Arhgap28 has RhoGAP function. No other conserved domains were identified.

Expression of Arhgap28-V5 inhibits RhoA activation and actin stress fiber assembly

To examine if Arhgap28 can regulate RhoA signaling and actin stress fiber formation, we created a V5-tagged Arhgap28 expression clone (Arhgap28-V5; see Figure S1 for details) and transfected SaOS-2 cells. SaOS-2 cells were selected because they form prominent stress fibers when cultured on plastic or glass. Expression of Arhgap28-V5 was confirmed in transiently transfected cells by western blotting using an anti-V5 antibody (Figure 2A). Active RhoA was examined by a Rhotekin-GST pull-down assay to precipitate GTP-bound Rho. Expression of Arhgap28-V5 caused a reduction in active RhoA compared to cells treated with the transfection reagent only or when transfected with the empty vector (data not shown). In further experiments we performed quantitative ELISA assays for active RhoA, Rac1 and Cdc42 in the presence and absence of expressed Arhgap28-V5 and a mutant Arhgap28R125A-V5 (R125A-V5) with control samples transfected with empty vector only. Note that the R125A-V5 construct lacks the putative catalytic arginine residue in the GAP domain (see Figure 1). We consistently observed reduction in the amounts of active RhoA (but not Rac1 and Cdc42) in the presence of Arhgap28-V5. The amounts of active RhoA remaining ranged from 62–78% of basal levels of active RhoA observed in controls. The results of the experiment that showed reduction to 78% is shown in Figure 2B; the expression of Arhgap28-V5 but not the R125A-V5 resulted in a significant reduction in basal levels of active RhoA with no significant effect on basal Rac1 and Cdc42 activity. The variability in the results between experiments was most probably because of differences in transfection efficiency between experiments. In separate experiments we prepared stably transfected SaOS-2 cells, expressing Arhgap28-V5. However, for reasons that were unclear to us, the expression levels were very low. Overall, the experiments showed that Arhgap28-V5 was effective at reducing the levels of RhoA.

In further experiments, actin stress fibers were examined by staining with phalloidin and immunofluorescence using the anti-V5 antibody; cells without staining for V5 exhibited prominent stress fibers (Figure 2C). In all cells expressing Arhgap28-V5, as identified by staining with V5 antibodies, disrupted actin stress fibers were observed. Multiple actin microspikes and membrane ruffles were also observed on the edge of these cells (Figure 2C, arrows). To test if these morphological changes were attributed to the GTP hydrolysis activity mediated by Arhgap28, cells were transfected with R425A-V5. All cells that stained positively for V5 contained prominent stress fibers (Figure 2D). These expression studies indicated that Arhgap28 is a RhoGAP for RhoA and its expression negatively regulates stress fibers.

Arhgap28 has a restricted expression pattern through embryonic development

We studied the spatial and temporal activation of Arhgap28 during embryonic development using an Arhgap28 gene trap (Arhgap28b) mouse. The gene trap cassette does not disrupt the expression of the wild type Arhgap28 transcript and so the animals develop normally (see Figure S2 for details). In this mouse, the endogenous promoter of Arhgap28 drives the expression of β-galactosidase, which can be localized by X-gal staining. We examined the expression of β-galactosidase in E11.5 to E18.5 embryos. At all time points examined, β-galactosidase activity had a restricted spatial pattern (Figure 3A). At E11.5 and E12.5, staining was localized to the dorsal region in what appears to contain somatic cells (Figure 3A arrows in lower panels). At E13.5 to E15.5 the staining in the body of the embryos spread to the limbs and regions where the ribs are formed. At E18.5, staining could be clearly seen in limb bones and in the dorsal portions of the ribs. E18.5 embryos were sectioned and counter stained with Alizarin red. The results showed that cells staining positively for β-galactosidase activity were localized to the calcified portions of long bones and ribs (Figure 3B). These data suggested to us that Arhgap28 has a role in regulating Rho in the initial stages (from E12.5) of ECM assembly throughout the embryo and becomes restricted to boney tissues at late stages of embryonic development, in the mouse.

Arhgap28 and related RhoGAPs are upregulated during Rho-mediated tissue stiffening in vitro

To further explore the possibility that Arhgap28 is involved in the assembly of ECM, we examined the expression of Arhgap28 in a 3D cell culture model of tissue assembly in which embryonic fibroblasts deposit and tension a collagen fibril-rich ECM [11]. In this system, fibroblasts are moved from conventional 2D culture to medium containing fibrinogen and thrombin. The formation of a fibrin gel occurs within 5 minutes to produce a loose gel in which the fibroblasts find themselves suspended. During the next ~10 days in culture, the fibroblasts replace the fibrin with a collagen/ fibronectin-rich matrix [18] and subsequently tension this matrix using non-muscle myosin II-derived forces [19]. Quantitative PCR showed that the expression of Arhgap28 was low in cells cultured on plastic (Figure 4A). The expression increased when the cells were in fibrin gels but was notably upregulated (15-fold) once the tissue constructs had tensioned (day 13; $p<0.05$). The expression levels continued to increase and were 35-fold higher (compared to 2D culture) after 14 days of 3D culture under tension (25 days in total; $p<0.001$; Figure 4A). Arhgap6 and Arhgap18 were also upregulated during tissue construct formation but not to the same extent as Arhgap28 (Figure 4A).
To test if Rho signaling is involved in 3D tissue construct contraction, fully formed tissue constructs (10 days of culture) were treated with lysophosphatidic acid (LPA) to activate Rho and the formation of stress fibers [20,21] or the ROCK inhibitor, Y27632. As shown in Figure 4B, actin polymerization was dramatically affected by these treatments; LPA induced thicker, more prominent actin stress fibers whereas Y27632 resulted in stress fiber shortening and disassembly.

The 3D tissue constructs recoil when unpinned, which is a process driven by non-muscle myosin II-dependent actomyosin contraction [22]. We made use of this feature to develop an assay of Rho-myosin II-dependent contraction of the tissue constructs. Thus, constructs were incubated with either LPA or Y27632 and the length of the unpinned construct was measured during 30 minutes. Tissue constructs treated with LPA significantly contracted to 53%±0.02 of the original length in 30 minutes.
compared to 59.6% ± 0.04 in DMSO-treated tissue constructs \( (p<0.001; \text{Figure 4C}) \). Y27632 treatment significantly inhibited the contraction of the construct, which contracted to only 71.2% ± 0.6 of the original length after 30 minutes \( (p<0.001; \text{Figure 4C}) \). The \( T_{1/2} \) for recoil was 444 s ± 16 for DMSO-treated control tissue constructs, 323 s ± 7 for LPA treated constructs, and 670 s ± 3 for Y27632 treated constructs (Figure 4D). In addition, expression of \( \text{Arhgap28} \) and \( \text{Arhgap6} \) was dramatically downregulated at 6 and 24 hours after tissue constructs were unpinned (Figure 4E). Together, these data show that \( \text{Arhgap28} \) is upregulated and Rho signaling is active during contraction of newly synthesized ECM, and the expression of \( \text{Arhgap28} \) is inversely related to the stiffness of the mechanical environment.

Normal embryonic development of \( \text{Arhgap28} \)-null mice

To test further the possible role of \( \text{Arhgap28} \) in tissue formation, we generated a functional \( \text{Arhgap28} \)-null mouse by crossing \( \text{Arhgap28}^{gt} \) mice with \( \text{Cre} \) transgenic mice. LoxP sites flank exons 7, 8 and 9 of the \( \text{Arhgap28}^{gt} \) gene. Therefore, Cre recombinase would be predicted to produce mice harboring an \( \text{Arhgap28}^{del7-9} \) \( \text{Arhgap28}^{del} \) allele (Figure S3A). Using PCR readouts with genomic DNA we were able to identify wild type, heterozygous and null mice (Figure S3B). The absence of exons 7–9 causes a frame shift and a smaller transcript that can be detected by RT-PCR using primers that span exons 6 to 11 (Figure S3C). Thus, transcripts from the defective allele would produce a truncated protein lacking the RhoGAP domain. The results showed that heterozygous \( \text{Arhgap28}^{del} \) mice were viable and fertile. Litters produced by cross breeding heterozygotes had a close to normal Mendelian distribution of genotypes; 30% ± 9 wild type; 49% ± 5 heterozygotes; and 22% ± 8 homozygotes (±SEM; from a total of 37 pups, in 8 litters). \( \text{Arhgap28}^{del} \) embryos (E15.5) were examined histologically and no apparent differences were observed compared to wild type mice (Figure S3D).

Figure 2. \( \text{Arhgap28-V5} \) inhibits RhoA activation and stress fiber formation in SaOS-2 cells. SaOS-2 cells were transfected with empty vector or \( \text{Arhgap28-V5} \). A. The expression of \( \text{Arhgap28-V5} \) was confirmed by western blotting using an antibody to V5. B. Effect of \( \text{Arhgap28-V5} \) expression on the basal activity of RhoA \((n = 5)\), Rac1 \((n = 3)\) and Cdc42 \((n = 3)\). Bars show SEM, * indicates significant difference found, \( p<0.05 \). C–D. F-actin in cells expressing \( \text{Arhgap28-V5} \) (C) and \( \text{Arhgap28R425A-V5} \) (D) was visualized by fluorescence microscopy using anti-V5 antibodies and Atto 488-conjugated phalloidin (representative images from 3 independent transfections). Arrows point to membrane ruffling and F-actin protrusions. Bars = 25 μm.

doi:10.1371/journal.pone.0107036.g002
Arhgap6 and RhoA are upregulated in Arhgap28-null bone tissue

To confirm the loss of Arhgap28 in bone tissues, RNA was isolated from the tibia and fibula of P0 wild type and Arhgap28<sup>del</sup> mice, and overlapping PCRs were performed (Figure 5A). Full-length Arhgap28 was detected in wild type bone tissues and overlapping PCRs confirmed that exons 7–9 were absent in the Arhgap28 transcript of Arhgap28<sup>del</sup> bone tissues (Figure 5B). The products of these reactions were sequenced to confirm that the transcript expressed in Arhgap28<sup>del</sup> bone encoded a 367 amino acid-long protein lacking a functional RhoGAP domain (Figure S4).

Due to the lack of an obvious phenotype in heterozygous or null mice, we investigated the possibility that other RhoGAPs (e.g. Arhgap6 and Arhgap18) were compensating for the absence of Arhgap28. Quantitative PCR showed that the truncated Arhgap28<sup>del</sup> transcript was detectable at low levels in tissues from Arhgap28<sup>del</sup> mice, which suggests that this truncated transcript (lacking the sequences encoding the RhoGAP domain) is at least partially stable. However, it was clear that the mutant transcript was at significantly lower levels compared to the expression of the Arhgap28 transcript in wild type mice ($p<0.001$; Figure 6A) presumably due to nonsense mediated mRNA decay. We also detected a significant 2-fold upregulation of Arhgap6 expression in Arhgap28<sup>del</sup> tissues ($p<0.001$). Noteworthy, no significant differences were found in the expression of Arhgap18 in Arhgap28<sup>del</sup> tissues (Figure 6A). The data show that in tissues devoid of functional Arhgap28, there is compensatory upregulation of Arhgap6. We have previously shown that the two RhoGAPs were upregulated during ECM assembly and tensioning and were downregulated in response to lack of tension, which indicate that the Arhgap6-Arhgap28 pair might co-regulate the same Rho signaling pathway for actin reorganization. We also examined the expression of Rho GTPases. A small but significant upregulation in RhoA expression, but not in expression of Rac1, Cdc42 or RhoQ, was found in Arhgap28<sup>del</sup> bone tissues compared to wild type ($p<0.05$; Figure 6B). Again, these data were indicative of a role of Arhgap28 in RhoA regulation.

Microarray comparison of gene expression between bone tissues of wild type and Arhgap28<sup>del</sup> mice

While Arhgap6 expression was upregulated in Arhgap28<sup>del</sup> bone tissues there was also a significant increase in RhoA gene expression, which suggests that Rho signaling might still be altered in Arhgap28<sup>del</sup> tissues despite Arhgap6 compensation. To determine if global changes in gene expression occurred, microarray analyses were performed in which gene expression from bone tissues (tibia and fibula) of wild type and Arhgap28<sup>del</sup> mice were compared. The integrity of the RNA samples and the microarray readouts were analyzed (see Figure S5 for details). A total of 45037 probe sets were detected of which 363 of these showed significant differential expression of $\pm 2$-fold difference ($q<0.05$). A heat map generated by hierarchical clustering of all the probe sets, based on similarities in the expression level and the expression profile is shown in Figure S5D.

As a cautionary note, three probe sets detected Arhgap28<sup>del</sup> expression in bone from knockout animals (see Table 1). This was not unexpected because a 3′ microarray was used and the mutant Arhgap28<sup>del</sup> transcript contains the endogenous 3′-end of the Arhgap28 transcript (see Figure 5). In contrast to the qPCR analyses performed previously, readouts for Arhgap6 did not show a fold change of $\pm 2$ in Arhgap28<sup>del</sup> bone (Table 1). Expression of Arhgap18 and Rho GTPases (RhoA, Rac1, Cdc42 and RhoQ) also showed no differential expression in Arhgap28<sup>del</sup> bone. The discrepancy between the microarray and the qPCR analyses could be because qPCR is a more quantitative and sensitive method of
detecting gene expression. Interestingly, the actin genes detected by this microarray study, \textit{Acta1} and \textit{Actc1}, were both significantly downregulated (3-fold) in the bone tissue of \textit{Arhgap28del} mice (q<0.05; Table 1). The collagen genes expressed in bone tissues, \textit{Col1a1}, \textit{Col1a2} and \textit{Col10a1}, were not significantly different in \textit{Arhgap28-null} bones.

DAVID online tool was used to perform gene ontology analyses on probe sets showing a differential expression $6^2$-fold. The analysis showed that 57 probe sets detected genes that were downregulated and 306 probe sets detected genes that were upregulated, in the mutant samples. DAVID identified 10 functional annotation clusters in the genes that were downregulated. The top 3 annotation clusters and the genes that are over-represented in these clusters are listed in Table 2. All three clusters contained genes indicative of a cartilage ECM, for example, \textit{Col2a1}, \textit{Col9a1}, \textit{Hapln1} (hyaluronan and proteoglycan link protein 1), \textit{Matn1} (matrilin 1) and \textit{Matn3}, and some genes that are linked to negatively regulating bone mass, \textit{Agtr2} (angiotensin II receptor, type 2) and \textit{Dlk1}, which encodes a transmembrane

![Figure 4. \textit{Arhgap28} is upregulated during ECM assembly and downregulated during Rho-dependent tissue recoil.](image-url)

Primary embryonic chick fibroblasts were seeded into a fibrin gel for the formation of 3D cell cultures containing aligned ECM. \textbf{A.} The expression of \textit{Arhgap6}, \textit{Arhgap18}, \textit{Arhgap28}, \textit{Col1a1}, and \textit{Fn} were quantified by qPCR (n = 3). \textbf{B.} 3D tissue constructs were treated with 5 µM LPA or 10 µM Y27632 for 2 hours in serum-free conditions and changes to the actin cytoskeleton were observed by phalloidin staining. Bars = 50 µm. Arrows indicate the alignment of the tissue. \textbf{C.} The recoil baseline, which is the calculated length of the construct at the end of the exponential fit (see Materials and Methods for details). \textbf{D.} The half-time, which is the time required to achieve half of the recoil baseline. \textbf{E.} The expression of \textit{Arhgap6}, \textit{Arhgap18} and \textit{Arhgap28} were quantified by qPCR during tissue recoil (n = 3). Fold changes in gene expression were normalized to \textit{Gapdh} (2$^{-\Delta\Delta Ct}$ values). Bars show SEM. *** and * indicate significant differences found, p<0.001 and p<0.05, respectively, one way ANOVA.

doi:10.1371/journal.pone.0107036.g004
protein called delta-like 1 homolog. These data suggest that a bone phenotype could still be expected upon closer analysis.

For genes that were upregulated in Arhgap28-null bone tissue, 65 annotation clusters were identified and the top 3 clusters are listed in Table 3. The genes that were over-represented in the first cluster were genes involved in targeting proteins for ubiquitination. For example, this cluster contained 13 E3 ubiquitin protein ligases, including March3 (membrane-associated ring finger (C3HC4) 3), March5, Mybp2 (MYC-binding protein 2); 3 peptidases; and a gene, Psmd14, which encodes a regulatory subunit of 26S proteasome (see Table 3 for full list of genes). The second cluster contained genes that promote actin polymerization, including Pafah1b1 (platelet-activating factor acetyl-hydrolase 1b), Tmod1 (tropomodulin 1) and Diap3 (also known as mDia2); actin nucleation (Spire1); and genes involved in linking the actin cytoskeleton to the plasma membrane including Utrn (utrophin) and Spn1 (spectrin). The third cluster of over-represented genes that were upregulated in Arhgap28del bone tissue contained zinc finger proteins (Rbm5, Neil3 and Fus), which bind DNA or RNA.

To summarize, the GO analyses showed that loss of Arhgap28-mediated RhoA signaling causes: (i) down-regulation of cartilage ECM genes; (ii) up-regulation in genes involved in targeting proteins for ubiquitination; and (iii) up-regulation of genes involved in anchorage of the actin cytoskeleton to the plasma membrane. Whether or not Arhgap28 regulation of Rho signaling is involved functionally in cartilage homeostasis, protein degradation of anchorage of actin to the plasma membrane will require further investigation. However, the results presented in Figure 2 are indicative of a role for Arhgap28 in actin stress fiber polymerization at the plasma membrane.

Figure 5. Comparison of the full-length transcripts of wild type and Arhgap28del alleles. A. Overlapping products of PCR reactions 1, 4, 6–7, 9–14 by specific primers (numbers in gray) are sequenced. ATG and TAG indicates the start and stop codons, respectively. Diagram not drawn to scale. B. RNA was isolated from the tibia and fibula of P0 mice, and the 10 overlapping RT-PCRs were performed and visualized by gel electrophoresis. Products were subsequently purified and confirmed by DNA sequencing. Reactions in the green boxes will not produce a product with cDNA from Arhgap28del (see Figure S4).

doi:10.1371/journal.pone.0107036.g005
genes associated with bone dysplasia were downregulated in the differences between wild type, heterozygous and Arhgap28-null measured the intercanthal distance and found no significant differences between wild type, heterozygotes and Arhgap28-null mice. As a measure of intramembranous ossification, we examined the skeletons of mice by X-ray at 10 weeks of age, when the skeletal growth has reached maturity. Skeletal analyses revealed no significant differences in the lengths of femur and tibia between wild type, heterozygous and Arhgap28-null animals (Figure 7). Together, these data suggest that although genes associated with bone dysplasia were downregulated in the Arhgap28del mouse there is no bone length phenotype, presumably because of compensation from Arhgap6.

**Discussion**

The mechanical stiffness of musculoskeletal tissues is directly related to the organization of collagen fibrils in the ECM. For example, the strongest tensile tissues such as tendon and ligament have collagen fibrils arranged in parallel register, which presumably is the best organization to resist uniaxial force; in bone the collagen fibrils provide a template for mineralization. Furthermore, the collagen fibrils are pre-stressed by cells to ensure that tissues can respond directly to applied forces. It is poorly understood how the tissue-specific arrangement and pre-stressing of collagen fibrils is achieved. Tissue stiffening requires cellular contraction via actin stress fibers, which are regulated by Rho GTPases. How these Rho GTPases are regulated during tissue development to ensure that the ECM is at optimal stiffness is unknown.

Here, we show that Arhgap28 is activated in bone tissues before birth and during the assembly of a stiff ECM. Experiments expressing Arhgap28-V5 suggest that Arhgap28 is a negative regulator of RhoA and actin stress fiber formation. Arhgap28-deficiency does not appear to affect bone development, which is most likely due to functional redundancy between Arhgap28 and a closely related RhoGAP, Arhgap6. It will be important in future work to investigate the mechanisms of how Arhgap28-regulated actincontractility determines stiffening of the ECM and to understand how RhoGAPs crosstalk regulates Rho and actin remodeling within developing musculoskeletal tissues.

Cellular tension is generated by actin stress fibers [26]. In this context, the closely related Arhgap6 and Arhgap18 regulate the formation of actin stress fibers via RhoA [16,17] and in this study, expression of Arhgap28-V5 caused similar effects. Sustained RhoA activity has inhibitory effects on Rac1- and Cdc42-activated lamellipodia and filopodia formation [27,28], which helps explain the appearance of actin microspikes and membrane ruffles in Arhgap28-overexpressing cells although there was no detectable activity of Arhgap28 against Rac1 and CDC42 in the assays used here.

Cells respond to stiff extracellular matrices via Rho-activated actin stress fibers [29–31] and overactive RhoA signaling is linked to cancer [see 32 for review]. Here, loss of RhoGAPs, such as DLC1, is associated with cancer [33], which suggest that RhoA signaling affects cell fate. Overexpression of RhoA and Rho GTPases are linked to cancer and the stability of RhoA transcripts in cancer cells has been shown to be a result of altered polyadenylation signals [34], further suggesting why it would be biologically important to have more than one RhoGAP regulating Rho GTPases.

There are a few studies that show that the differentiation of mesenchymal stem cells into an osteogenic lineage can be influenced by Rho/ROCK signaling, for examples see [35–37]. Surprisingly, mice expressing a dominant-negative RhoA have a bone sclerotic phenotype, which suggests that lack of RhoA signaling enhances mineralization [38]. These disagreements linking Rho/ROCK and bone development could be due to the need for a balanced signal and crosstalk between Rho/ROCK and growth factors rather than an all-or-nothing response. RhoA expression was also significantly upregulated in bone tissues of Arhgap28-null mice. RhoA expression is activated by transcription factor Myc [39], but Myc was not differentially regulated in the microarray comparison between wild type and Arhgap28del bone tissues.
Due to the large family of mammalian RhoGAPs, it was predicted that Arhgap28-deficiency might be compensated for by functional redundancy between Arhgap28 and other RhoGAPs. Indeed, absence of Arhgap6 in mice does not cause an overt phenotype, presumably because of the observed compensatory mechanisms [16]. It was surprising in our studies that Arhgap18 was not also upregulated because both Arhgap6 and Arhgap18 have been shown to negatively regulate RhoA and actin stress fibers. These results reveal the potential of a novel co-regulatory mechanism for RhoA signaling and actin stress fibers by Arhgap6 and Arhgap28. How these RhoGAPs are activated during the patterning of ECM is unknown. Matrix assembly and detection of the ECM occurs at cell-matrix adhesion sites, therefore it is hypothesized that Arhgap28 is activated by signals downstream of cell-matrix adhesions in similar mechanisms described for other RhoGAPs. For examples, upon activation of integrin $\beta_1$, p190-RhoGAP is activated via tyrosine phosphorylation at the N-terminus by Src [40], and more recently, the identification of protein-protein interaction domains within DLC1 suggest direction interaction between DLC1 and cell-matrix adhesion proteins tensin, talin and focal adhesion kinase [41,42]. Importantly, although there was no overt phenotype in the Arhgap28del mice (perhaps due to compensation by Arhgap6) the microarray study revealed downregulation in Acta1 and Actc1 genes, which encode isoforms of actin found most abundantly in muscle. Downregulation in both these genes has also been observed in a microarray study of chondrocyte differentiation in vitro [43] and downregulation of Acta1 was observed in bone in response to mechanical loading [44], which suggest of change in the cells of Arhgap28del bone tissues. Gene ontology analyses also revealed that genes that encode ECM molecules associated with a cartilage tissue (including Col2a1, Col9a1, Matn3 and Comp) were downregulated in Arhgap28-null bone tissues. Mutations in these genes are associated with bone dysplasia [for reviews, see 23,24,25]. Bone tissues from Arhgap28del mice also showed an upregulation of genes involved in ubiquitination and actin

| Gene | Gene symbol | wild type | Arhgap28del | Fold change | q value |
|------|-------------|-----------|-------------|-------------|---------|
| RhoGAP genes | Arhgap28 | Arhgap28 | 271.7 | 110.3 | $-2.5$ | 0.037 |
| | Arhgap6 | Arhgap6 | 62.2 | 62.1 | $-1$ | 0.656 |
| | Arhgap18 | Arhgap18 | 488.6 | 397.5 | $-1.2$ | 0.106 |
| Deleted in liver cancer 1 | DLC1 | DLC1 | 48.6 | 52.3 | 1 | 0.413 |
| Rho GTPases genes | RhoA | RhoA | 5112.5 | 5060.3 | $-1$ | 0.627 |
| | Rac1 | Rac1 | 3672.1 | 3349.2 | $-1.1$ | 0.254 |
| | Cdc42 | Cdc42 | 239.8 | 190.0 | $-1.3$ | 0.197 |
| | RhoQ | RhoQ | 2126.4 | 2001 | $-1.1$ | 0.52 |
| actin genes | Acta1 | Acta1 | 334.1 | 94.0 | $-3.6$ | 0.015 |
| | Actc1 | Actc1 | 687.1 | 223.8 | $-3.1$ | 0.046 |
| bone collagen genes | Col1a1 | Col1a1 | 10217.8 | 10997.6 | 1.1 | 0.413 |
| | Col1a2 | Col1a2 | 321.6 | 767.0 | 2.4 | 0.163 |
| | Col10a1 | Col10a1 | 1917.6 | 1024.8 | $-1.9$ | 0.056 |

Comparison of gene expression in wild type and Arhgap28del bone tissues. Mean intensities from hybridization of triplicate samples to probe set(s) for the genes listed. doi:10.1371/journal.pone.0107036.t001
reorganization compared to wild type. Most of the genes enriched in the top cluster were E3 ubiquitin ligases, which mediate the specificity of the ubiquitination pathway [reviewed by 45]. The target for many of these E3 ligases is unknown. The genes that determine specificity of the ubiquitination pathway [reviewed by 45]. The differential regulation of these genes is indicative of a response to redundancy presumably underpins the cell’s ability to generate extracellular region part 1.57E–07 14 

| Annotation cluster 2, Enrichment score 3.78 | 
|------------------------------------------|
| GO: 0031012– extracellular matrix 2.07E–07 10 |
| GO: 0005578– extracellular region 6.35E–07 22 |
| GO: 0044211– extracellular region part 1.57E–07 14 |

| Annotation cluster 3, Enrichment score 2.24 | 
|------------------------------------------|
| GO: 0007155– cell adhesion 7.13E–03 6 |
| GO: 0022610– biological adhesion 7.19E–03 6 |

| Annotation cluster analysis of probe sets detecting a significant fold change greater than 2 from wild type to Arhgap28wild produced 10 clusters. The top 3 annotation clusters with the highest enrichment score are listed here. A. The top gene ontology components of the cluster. B. The statistical significance of this grouping where the lower the score the more unlikely this clustering is due to chance. C. The number of probe sets that recognize genes contributing to the GO term. D. List of gene names of the Affymetrix Mouse Genome 430 2.0 array probe IDs. doi:10.1371/journal.pone.0107036.t002

Materials and Methods

Ethics

The care and use of all mice in this study was carried out in accordance with UK Home Office regulations. UK Animals (Scientific Procedures) Act of 1986 under the UK Home Office licence (PPL 40/3485). No experimental procedures were performed on live animals. All animals were sacrificed by Schedule 1 cervical dislocation by trained personnel, and all efforts were made to minimize suffering.

V5-His-tagged murine Arhgap28 and Arhgap28R425A expression clones

For the generation of a C-terminus V5-His-fusion Arhgap28 clone, the sequence encoding V5-His (GKIPPNPLLGLDST-(His)6) was introduced immediately 5’ of the stop codon of the endogenous, larger Arhgap28 isoform cDNA. The DNA sequence of the short Mus musculus Arhgap28 isoform (BC066788.1; Source BioScience Geneservice) was digested with EcoRI and BamHI (New England Biolabs, Hertfordshire, UK) following the manufacturer’s instructions to produce a 1123 bp fragment of the 5’ portion of the Arhgap28 clone. For the preparation of the 3’ portion of endogenous Arhgap28 transcript, a 1209 bp fragment was amplified from mouse fibroblast cDNA (made with random hexamers) using a forward primer 5’ of a unique endogenous BamHI restriction recognition site 3’-AAG ATT TGG GTT GAC CGA GAC G-5’ and a reverse primer that created a new BamHI site 3’-AAT TCG AAG GGC TTC ATG ACC G-5’ immediately before the stop codon. iProof High-Fidelity DNA Polymerase (Bio Rad Laboratories) was used following the manufacturer’s protocol. The PCR fragment was sequenced and cut with BamHI and BstBI

| Cluster components\(^a\) | \(p\) value\(^b\) | Matched genes\(^c\) | Gene names (if annotated) of corresponding probe IDs in the GO list\(^d\) |
|------------------------------------------|
| Annotation cluster 1, Enrichment score 6.72 |
| SP_PIR_KEYWORDS secreted 4.13E–11 21 |
| GO: 0005576– extracellular region 6.53E–10 22 |
| GO: 0044211– extracellular region part 1.57E–07 14 |

Table 2. Top 3 annotation clusters of genes downregulated in Arhgap28wild bone.
restriction enzymes (New England Biolabs). pcDNA6/V5-His C vector was prepared by digestion with EcoRI and BstBI restriction enzymes (New England Biolabs). The three cut fragments were purified using QIAquick Gel Extraction kit (Qiagen) and ligated using T4 DNA Ligase (New England Biolabs).

Arhgap28R425A clone containing a codon change from CGA to GCA (Genscript) was subcloned into pcDNA6/V5-His C between EcoRI and BstBI sites. DNA was purified from each culture using QIAprep Spin Miniprep kit (Qiagen) following the manufacturer’s protocol.

Transient transfections and GTPase activation assays
NIH3T3 fibroblasts were cultured in DMEM, supplemented with 10% (v/v) FCS, 2 mM L-glutamine, 10 000 U/ml penicillin and 10 mg/ml streptomycin. SaOS-2 osteosarcoma cells were cultured in RPMI-1630, supplemented with 10% (v/v) FCS, 2 mM L-glutamine, 10 000 U/ml penicillin and 10 mg/ml streptomycin. Cells were cultured at 37°C, 5% CO2. DNA was transfected using Lipofectamine 2000 (Invitrogen) according to manufacturer’s protocol. Cells were analyzed 24 hours after transfection by indirect immunofluorescence, active GTPase G-LISA assays or western blotting. Expression of V5-tagged constructs was confirmed by western blotting using a mouse monoclonal anti-V5 epitope antibody (MCA1360 from AbD Serotec). The activities of RhoA, Rac1 or Cdc42 were measured.
24 hours after transfection using G-LISA Activation Assays (Cytoskeleton), according to manufacturer’s protocol.

**3D tissue constructs and recoil assays**

Primary embryonic chick fibroblasts were isolated from E14 chick metatarsal tendons. For the formation of 3D tissue constructs in vitro, cells were seeded into fibrin gels as described previously [11]. To modulate Rho signaling in vitro tissue constructs were washed with phosphate buffered saline (PBS) three times and then equilibrated for 30 minutes with serum-free medium and then treated for 30 minutes with final concentration of 5 μM lysophosphatidic acid (LPA; Sigma) in 0.1% (v/v) DMSO or 10 μM Y27632 (ROCK inhibitor; Sigma) in 0.1% (v/v) DMSO, diluted in serum-free medium, all at 37°C, 5% CO2 in a humidified environment. For the recoil assay, the tissue constructs were treated for 2 hours and was then cut from one suture to allow contraction to occur. The constructs were imaged using a digital single lens reflex camera at a fixed focal point for 30 minutes at 10-second intervals. The length of the constructs in each image was measured using ImageJ software. The length was converted to a percentage of the original and the means for each experimental group were calculated. The mean values were then fitted to a 3-parameter exponential decay function using SigmaPlot (Systat Software Inc.). One-way ANOVA and a Dunnett’s test were used to determine significance differences between the derived T1/2 and recoil baseline values compared to control constructs incubated in DMSO.

**RNA and PCRs**

RNA was isolated with TRIzol reagent (Invitrogen) and treated with DNase (Promega) following manufacturers’ protocols. cDNA was synthesized using TaqMan Reverse Transcriptase reagents (Applied Biosystems) and used for analyses by PCR and qPCR. A list of primers used can be found in Table S2. All PCR products produced from primers were validated and confirmed by DNA sequencing using the BLAST software against the NCBI nucleotide database and aligned with the expected sequence. For qPCR analyses, the 2^(-ΔΔCt) method [48] was used to analyze relative fold changes in gene expression compared to the control group. For older embryos the skin was removed before being fixed for 1 hour in 3.7% (w/v) PFA in PBS pH 7.4 at room temperature. Embryos were then washed with PBS containing 0.1% (v/v) Triton X-100 for 15 minutes twice. Each embryo was then stained with 20 ml of freshly prepared X-gal staining solution (1 mM X-gal (Qiagen), 5 mM potassium ferricyanide (K₃Fe(CN)₆), 5 mM potassium ferrocyanide (K₄Fe(CN)₉), 1 mM MgCl₂ in PBS containing 0.1% (v/v) Triton X-100) for 24 hours at 37°C. After staining, the embryos were rinsed with PBS and post-fixed in 4% (w/v) PFA in PBS overnight at 4°C. After post-fix, the embryos were either processed for paraffin embedding or dehydrated in 70% (v/v) ethanol for 6 hours and cleared with glycerol (10 ml of each 30, 50 and 80% (v/v) glycerol in 1% (w/v) potassium hydroxide and then 100% glycerol), incubating at 37°C for 2-3 days each.

**Immunofluorescence**

Cells fixed with freshly prepared 4% (w/v) paraformaldehyde in PBS for 20 minutes at RT, then washed with PBS containing 0.1% (v/v) Tween 20 three times and blocked with 1% (w/v) bovine serum albumin, 0.1% (v/v) Triton X-100 in PBS containing 0.1% Tween 20 for 1 hour. Cells were incubated with primary antibodies, mouse monoclonal anti-V5 epitope or the appropriate control IgGs, diluted in the blocking buffer overnight at 4°C. Cells were washed with PBS containing 0.1% (v/v) Tween 20 for 10 minutes three times and incubated with Alexa Fluor 594-conjugated antibodies (Invitrogen) and Atto 488-conjugated phallolidin (Sigma) diluted with the blocking buffer for 1 hour at room temperature, protected from light. Stained cells were washed with PBS containing 0.1% (v/v) Tween 20 for 10 minutes three times and mounted using Vector Shield containing DAPI (Vector Laboratories). Fluorescent images were taken using a digital camera attached to an Olympus BX51 and captured using MetaVue imaging software (Molecular Devices).

**Reporter and knockout mice**

Using homologous recombination in agouti C57BL/6N embryonic stem (ES) cell, the L112_Bact_P targeting gene trap cassette (including the genes encoding β-galactosidase and neomycin) was introduced into intron 6 of the Arhgap28 gene by the NIH Knockout Mouse Project (KOMP; CA, USA). In addition, exons 7–9 are flanked by loxP sites. ES cells containing the Arhgap28 gene trap allele (Arhgap28Δg) were injected into C57BL/6J blastocysts to produce germ line-transmitting chimeras (prepared at the University of Oulu, Finland). Chimeric males were assessed on coat color and mated with wild type C57BL/6J females to produce heterozygous progeny, which were then mated to obtain Arhgap28Δg mice. For the generation of Arhgap28 deleted exon 7–9 (Arhgap28Δex) mutants, male Arhgap28Δg mice were mated with females from a deleter Cre transgenic mice (a gift from M. Briggs, University of Newcastle, UK) to ablate the loxP-flanked exons 7–9 in all tissues. F1 offspring heterozygous for the knockout allele were then mated to generate knockout (Arhgap28Δex) mice.

**Genotyping**

Mice were genotyped using genomic DNA extracted from ear punches of adult mice or amniotic sacs of embryos using 200 μg/ml Proteinase K (Invitrogen) in buffer containing 17.6 mM N-lauroyl sarcosine, 100 mM NaCl and 5% (w/v) Chelex 100 resin (Bio Rad Laboratories). Genotypes were determined using specific primer pairs (see Table S2) using BioMix Red PCR reagents (Bioline). The annealing temperature for the wild type/mutant allele PCR was 53°C. The annealing temperature for the Arhgap28Δex allele was touch-down from 70-60°C for 10 cycles followed by 20 cycles at 60°C. The annealing temperature for the Cre transgene was 50°C.

**Whole mount X-gal stain**

For detection of beta-galactosidase expression, E10.5–E15.5 embryos were fixed for 1 hour in 3.7% (w/v) PFA in PBS pH 8 at room temperature. For older embryos the skin was removed before being fixed for 1 hour in 3.7% (w/v) PFA in PBS pH 7.4 at room temperature. Embryos were then washed with PBS containing 0.1% (v/v) Triton X-100 for 15 minutes twice. Each embryo was then stained with 20 ml of freshly prepared X-gal staining solution (1 mM X-gal (Qiagen), 5 mM potassium ferricyanide (K₃Fe(CN)₆), 5 mM potassium ferrocyanide (K₄Fe(CN)₉), 1 mM MgCl₂ in PBS containing 0.1% (v/v) Triton X-100) for 24 hours at 37°C. After staining, the embryos were rinsed with PBS and post-fixed in 4% (w/v) PFA in PBS overnight at 4°C. After post-fix, the embryos were either processed for paraffin embedding or dehydrated in 70% (v/v) ethanol for 6 hours and cleared with glycerol (10 ml of each 30, 50 and 80% (v/v) glycerol in 1% (w/v) potassium hydroxide and then 100% glycerol), incubating at 37°C for 2-3 days each.

**Histology**

Sagittal sections (6 μm thick) were cut from wax embedded embryos and mounted for haematoxylin and eosin staining. Slides were stained using an automated stainer and cleared into Histo-Clear (Thermo Fisher Scientific). For Alizarin red staining, sections were then washed with distilled water and stained with 2% (w/v) Alizarin red pH 4.2 for 10 minutes, washed with distilled water and dehydrated. Images were captured using a Carl Zeiss Axiocam Colour CCD camera with associated AxioVision software.
Comparative gene expression microarrays

For the comparison of bone tissues between wild type and Arhgap28del mice, tibia and fibula were dissected from three P0 neonatal litters each from defined breedings. Bones were incubated in 1000 U/ml bacterial collagenase type 4 (Worthington Biochemical Corporation) in 0.25% (w/v) trypsin (Invitrogen) for 25 minutes at 37°C with agitation every 10 minutes. The bones were then removed of any excess muscle and cartilage tissues and washed in PBS. RNA was isolated using a dismembrator as described previously [49]. Integrity and measurement of total RNA was performed using Agilent 2100 Bioanalyzer (Agilent Technologies). RNA was amplified by two-cycle cDNA synthesis, and then labelled cRNA was synthesized and hybridized to Mouse Genome 430 2.0 GeneChip arrays (Affymetrix). Microarray data are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-2296. Microarray data sets were analyzed by dChip (DNA-Chip) Analyzer to normalize the array readouts [50]. Normalized readouts were analyzed using the Robust Multichip Average method as described by [51]. Principal component analysis (PCA) was employed to confirm that different variables were present as a quality control for the arrays. p values for each probe set were generated by Limma t-test and q values were subsequently generated by applying false discovery rate correction. Gene ontology analysis was performed on probe sets that have detected fold changes greater than 2 using Database for Annotation, Visualization and Integrated Discovery (DAVID) online tool [52].

Bone length measurements

X-rays of mice were produced using a Flaxitron x-ray specimen radiograph system (Flaxitron Biopics) and x-ray film (GE Healthcare). Bone measurements were taken from scanned radiographic images using ImageJ software. One-way ANOVA followed by a Dunnett’s test were used to determine significance differences compared to wild type.

Supporting Information

Figure S1 Cloning of a V5-tagged Arhgap28 overexpression construct. A. Difference in C-terminal ends of the two major Arhgap28 isoforms from amino acid residue 679. PCR strategy to determine the sequence of endogenous Arhgap28 transcript expressed in primary mouse fibroblasts. Overlapping products of PCR reactions 1 to 12 by specific primers (numbers in grey) were sequenced. Reactions in the boxes are unique to either products of PCR reactions 1 to 12 by specific primers (numbers in grey). The predicted molecular weight of Arhgap28-V5 is 85 kDa. Control lysates were included – Talin-V5 (60 kDa), mock transfection (−) and myristoylated-FAK-V5 (+; 175 kDa). (TIFF)

Figure S2 Arhgap28del mice are normal and express Arhgap28 due to unsuccessful gene trapping. A. Schematic showing the genotyping strategy for identifying the presence of the gene trap cassette targeted to the Arhgap28 gene. B. DNA was isolated from wild type, Arhgap28del and Arhgap28gt/gt mutant neonatal tail tendons cells to confirm genotypes. DNA from wild type (+/+), animals will only produce a 493 bp product whereas DNA from homozygous (g/g) animals will only produce a 334 bp product and DNA from heterozygous (g/wt) animals will produce both bands. C. RNA was also isolated and RT-PCR was performed to detect the expression of Arhgap28 transcript spanning from exon 6 to 11, ColIα1 and Gapdh. D. Sagittal sections of wild type (+/+), heterozygous (g/wt) and homozygous Arhgap28gt/gt mutant (g/gt) embryos at gestation day E15.5 stained with H&E and close-up of the limbs. (TIFF)

Figure S3 Arhgap28del mice express an Arhgap28 transcript lacking exons 7 to 9. A. Schematic showing the genotyping strategy for identifying the presence of the gene trap cassette targeted to the Arhgap28 gene and for the detection of Arhgap28 del7-9 KO allele. B. Representative gel image of genotyping PCR products. In the first genotyping PCR which distinguishes between wild type (493 bp) or mutant Arhgap28 allele (either the Arhgap28del or Arhgap28del allele; 354 bp). The second genotyping PCR tests for the presence of the Arhgap28del allele, the product of the mutant Arhgap28 allele after Cre recombinase-mediated DNA excision (400 bp). The third genotyping PCR tests for the presence of the deleter Cre transgene (350 bp). C. RNA was isolated from wild type, Arhgap28wt and Arhgap28del/del pups and RT-PCR was used to detect expression of wild type Arhgap28 (634 bp) and Arhgap28del (338 bp) transcripts spanning from exons 6 to 11. RT-PCRs for ColIα1 and Gapdh was used as loading controls. D. Sagittal sections of wild type and homozygous Arhgap28del/del embryos at gestation day E15.5 stained with H&E. (TIFF)

Figure S4 Translated sequences of Arhgap28 transcripts. Amino acid sequences transcripts of Arhgap28 from wild type, predicted Arhgap28del, actual Arhgap28del bone tissue. Sequence in blue is a result of remainder sequence from Cre-mediated recombination. Sequence in green is the RhoGAP domain. (TIFF)

Figure S5 Quality control of microarray comparing wild type and Arhgap28del bone tissues. A. Agarose gel showing total RNA isolated from triplicate samples of P0 tibia and fibula tissues from wild type and Arhgap28del mice. Sequence in blue is a result of remainder sequence from Cre-mediated recombination. Sequence in green is the RhoGAP domain.

(TIFF)
Acknowledgments

We would like to thank the University of Manchester Biological Support Facility, especially Emma Owen and Vicky Taylor. We thank Mike Briggs (University of Newcastle) for the transgenic Cov mice. We also thank Helen Rajpar, Mark Morgan (University of Liverpool), Louise Kung (University of Manchester), the Genomic Technologies and the Bioimaging core facilities in the Faculty of Life Sciences (University of Manchester) for providing technical support/advice.

Author Contributions

Conceived and designed the experiments: C-YC Y KEK. Performed the experiments: C-YC RS DFH LAZ SHT RG. Analyzed the data: C-YC RS DFH LAZ RPB H KEK. Wrote the paper: C-YC Y KEK.

References

1. Watanabe N, Madaule P, Reid T, Ishizaki T, Watanabe G, et al. (1997) p140mDia, a mammalian homolog of Drosophila diaphanos, is a target protein for Rho small GTpase and is a ligand for profilin. EMBO J 16: 3044–3056.

2. Hirose M, Ishizaki T, Watanabe N, Urakata M, Kranenburg O, et al. (1998) Molecular dissection of the Rho-associated protein kinase (p160ROCK)-regulated neurite remodeling in neuroblastoma N1E-115 cells. J Cell Biol 141: 1625–1636.

3. Hall A (1998) Rho GTPases and the actin cytoskeleton. Science 279: 509–514.

4. Noda M, Yasuda-Fukazawa C, Morikawa K, Tato T, Okada T, et al. (1995) Involvement of rho in GTP gamma S-induced enhancement of phosphorylation of 20 kDa myosin light chain in vascular smooth muscle cells: inhibition of phosphatase activity. FEBS Lett 367: 246–250.

5. Youeda S, Usukhov D, Mahlaugt HA, Coachman JR. (2007) Fibronectin matrix assembly requires distinct contributions from Rho kinases I and II. Mol Biol Cell 18: 66–75.

6. Zhong C, Chrzanowska-Wodnicka M, Brown J, Shaub A, Belkin AM, et al. (1998) Rho-mediated contraction exposes a cryptic site in fibronectin and induces fibronectin matrix assembly. J Cell Biol 141: 539–551.

7. Pankov R, Endo Y, Even-Ram S, Araki M, Clark K, et al. (2005) A Rac switch regulates random versus directionally persistent cell migration. J Cell Biol 170: 795–802.

8. Zanin E, Katz M, Posen Y, Erez N, Yamada KM, et al. (2006) Dynamics and segregation of cell-matrix adhesions in cultured fibroblasts. Nat Cell Biol 221: 196–196.

9. Ohashi T, Kichart DP, Erickson HP (2002) Dual labelling of the fibronectin matrix and actin cytoskeleton with green fluorescent protein. J Cell Sci 115: 1221–1229.

10. Canton EG, Starborg T, Lu Y, Humphries SM, Holmes DF, et al. (2006) Actin filaments are required for fibroblast-mediated collagen fibre alignment in tendon. J Biol Chem 281: 38592–38598.

11. Kapacee Z, Richardson SH, Lu Y, Starborg T, Holmes DF, et al. (2008) Tension is required for fibroblast formation. Matrix Biol 27: 371–373.

12. Samuel MS, Lopez JL, McGhee EJ, Croll DR, Strachan D, et al. (2011) Actomyosin-mediated cellular tension drives increased tissue stiffness and beta-catenin activation to induce epidermal hyperplasia and tumor growth. Cancer Cell 19: 776–791.

13. Rittinger K, Walker PA, Eccleston JF, Smerdon SJ, Gamblin SJ (1997) Structure at 1.65 A of RhoA and its GTPase-activating protein in complex with a phosphatase. Actomyosin-mediated cellular tension drives increased tissue stiffness and beta-catenin activation to induce epidermal hyperplasia and tumor growth. Cancer Cell 19: 776–791.

14. Tcherkezian J, Lamarche-Vane N (2007) Current knowledge of the large RhoGAP family of proteins. Biochim Biophys Acta 1778: 1221–1229.

15. Durkin ME, Avner MR, Huh CG, Yuan RX, Thorgeirsson SS, et al. (2005) DLG-1, a Rho GTPase-activating protein with tumor suppressor function, is essential for embryonic development. FEBS Lett 579: 1191–1196.

16. Prakash SK, Paylor R, Jenna S, Lamarche-Vane N, Armstrong DL, et al. (2000) Functional analysis of ARHGAP6, a novel GTPase-activating protein for RhoA. Mol Biol Cell 11: 2360–2371.

17. Maeda M, Hasegawa H, Hyodo T, Oto S, Asano E, et al. (2011) ARHGAP18, a novel RhoA regulating protein, is a ligand for profilin. EMBO J 16: 3044–3056.

18. Yoneda A, Ushakov D, Couchman JR (2007) Fibronectin and expression levels. Hierarchical clustering was performed using 20 kDa myosin light chain in vascular smooth muscle cells: inhibition of phosphatase activity. FEBS Lett 367: 246–250.

19. Kalson NS, Holmes DF, Kapacee Z, Otermin I, Lu Y, et al. (2010) An overview of RhoGAPs. PLoS One 5: e107036.
42. Li G, Du X, Vass WC, Papageorge AG, Lowy DR, et al. (2011) Full activity of the deleted in liver cancer 1 (DLC1) tumor suppressor depends on an LD-like motif that binds talin and focal adhesion kinase (FAK). Proc Natl Acad Sci U S A 108: 17129–17134.
43. James CG, Appleton CT, Ulici V, Underhill TM, Beier F (2005) Microarray analyses of gene expression during chondrocyte differentiation identifies novel regulators of hypertrophy. Mol Biol Cell 16: 5316–5333.
44. Mantila Roosa SM, Liu Y, Turner CH (2011) Gene expression patterns in bone following mechanical loading. J Bone Miner Res 26: 100–112.
45. Deshaies RJ, Joazeiro CA (2009) RING domain E3 ubiquitin ligases. Ann Rev Biochem 78: 399–434.
46. Torrino S, Virokis O, Doye A, Boyer L, Stefani C, et al. (2011) The E3 ubiquitin ligase HACE1 catalyzes the ubiquitylation of active Rac1. Dev Cell 21: 959–965.
47. Guo Q, Xie J, Dang C, Liu ET, Bishop JM (1998) Identification of a large Myc-binding protein that contains RCC1-like repeats. Proc Natl Acad Sci U S A 95: 9172–9177.
48. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402–408.
49. Kapacee Z, Yeung CY, Liu Y, Crabtree D, Holmes DF, et al. (2010) Synthesis of embryonic tendon-like tissue by human marrow stromal/mesenchymal stem cells requires a three-dimensional environment and transforming growth factor beta3. Matrix Biol 29: 661–677.
50. Li C, Wong WH (2001) Model-based analysis of oligonucleotide arrays: expression index computation and outlier detection. Proc Natl Acad Sci U S A 98: 31–36.
51. Irizarry RA, Bolstad BM, Collin F, Cope LM, Hobbs B, et al. (2003) Summaries of Affymetrix GeneChip probe level data. Nucleic Acids Res 31: e15.
52. Huang da W, Sherman BT, Lempicki RA (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nature protocols 4: 44–57.