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## Desert Hedgehog-Primary Cilia Cross Talk Shapes Mitral Valve Tissue by Organizing Smooth Muscle Actin

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### Abstract

Non-syndromic mitral valve prolapse (MVP) is the most common heart valve disease affecting 2.4% of the population. Recent studies have identified genetic defects in primary cilia as causative to MVP, although the mechanism of their action is currently unknown. Using a series of gene inactivation approaches, we define a paracrine mechanism by which endocardially-expressed Desert Hedgehog (DHH) activates primary cilia signaling on neighboring valve interstitial cells. High-resolution imaging and functional assays show that DHH de-represses smoothed at the primary cilia, resulting in kinase activation of RAC1 through the RAC1-GEF, TIAM1. Activation of this non-canonical hedgehog pathway stimulates  $\alpha$ -smooth actin organization and ECM remodeling. Genetic or pharmacological perturbation of this pathway results in enlarged valves that progress to a myxomatous phenotype, similar to valves seen in MVP patients. These data identify a potential molecular origin for MVP as well as establish a paracrine DHH-primary cilium cross-talk mechanism that is likely applicable across developmental tissue types.

### Keywords

primary cilia; desert hedgehog signaling; valve development; myxomatous degeneration

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Disclosures

None.

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## Introduction

Mitral valve prolapse (MVP) is a progressive cardiac tissue disorder that affects 1 in 40 individuals worldwide (1–6). It is characterized by destruction of the extracellular matrix (ECM), which disrupts the valvular tissue architecture and renders the valves biomechanically incompetent. Patients with MVP often develop secondary complications including arrhythmias, heart failure and sudden cardiac death unless surgically corrected (7–9). Recent work has linked MVP to inborn errors of valve development and has identified variants in genes that are important for the formation and function of primary cilia (10–12). Primary cilia are singular, immotile projections of microtubules that extend from the surface of cells into the extracellular space. Although originally considered evolutionary remnants, primary cilia are now known to function as mechanosensors and signaling hubs to regulate many developmental processes. These structures possess complex repertoires of channel proteins and membrane-associated receptors that transduce chemical, electrical and mechanical cues from the surrounding microenvironment to control various cellular responses (13–26).

The hedgehog pathway is a well-established growth factor signaling pathway that requires the primary cilium for its function (17, 22, 23, 27–29). The initial input for this signaling cascade is at least one of three hedgehog ligands, Sonic Hedgehog (SHH), Indian Hedgehog (IHH), and/or Desert Hedgehog (DHH). These ligands interact with their cognate receptor, Patched (PTC) along the ciliary axoneme, which results in de-repression of the G-coupled protein receptor, Smoothened (SMO) at the cilia base. The canonical signaling pathway culminates with nuclear translocation of GLI transcriptional activators and/or repressors to modify expression of target genes primarily involved in cell cycle regulation. Due to the broad expression domains of hedgehog ligands and the presence of primary cilia on nearly every cell-type during development, it is not surprising that defects in ciliogenesis and its downstream signaling can cause a wide variety of congenital defects in humans (20, 22, 23, 30, 31).

In the heart, primary cilia are observed in the majority of cell types and have been associated with various congenital cardiac defects (11, 13, 30–38). Recent genetic and molecular studies have established primary cilia as important in forming both the mitral and aortic valves (11, 32, 34). However, how primary cilia function and whether hedgehog ligands are involved in cilia signaling during valve morphogenesis is currently unknown. Through a combination of *in vivo* and *in vitro* analyses, we show that among the three hedgehog ligands, only Desert Hedgehog (DHH) functions during fetal gestation to regulate cytoskeletal organization of the valve leaflets through a non-canonical, cilia-dependent pathway. The hedgehog signal emanates from the endocardium and is received by ciliated valve interstitial cells (VICs). This paracrine cross-talk is specific to developmental time points and is critical for shaping the valve into a thin, attenuated structure. Genetic perturbation of this pathway results in valvular defects during fetal gestation and leads to the generation of myxomatous valves later in life. Our studies establish a molecular and developmental origin for myxomatous mitral valves through a novel cross-talk mechanism and further implicate MVP as a congenital defect.

## Materials and Methods

The data that support the findings of this study are available from the corresponding author upon reasonable request.

### RT-PCR

Reverse transcribed RNA isolated from immortalized mouse mitral valve interstitial cells (mVICs) was used for RT-PCR analyses of *Gli1*, *Gli2*, *Gli3*, *Smoothened*, *Patched 1*, *Patched 2*, *Shh*, *Ihh*, *Dhh* and *Hprt* (Quantabio, Inc). The mVICs were a kind gift of Dr. Joey Barnett (Vanderbilt University) and previously published (11, 39). Primer sequences used: Patched 1: Forward: 5'-TAATGCTGCGACAACCTCAGG-3', Reverse: 5'-GGCTGGAGTCTGAGAACTGG-3', Patched 2: Forward: 5'-CTTGACTGCTTCTGGGAAGG-3'; Reverse: 5'-GCCAGCATAAGCAGATAGCC-3', Smoothened: Forward: 5'-CTGGGAGTCGGTTTTAATGG-3'; Reverse: 5'-ACACGTTGTAGCGCAAAGG-3', Gli1: Forward: 5'-CGGAGTTCAGTCAAATTAAC-3'; Reverse: 5'-CATCTGAGGTTGGGAATCC-3', Gli2: Forward: 5'-AGCCTCACCCACCTTCTTG-3'; Reverse: 5'-TGGGCGCAGGCCCTCAGC-3', Gli3: Forward: 5'-CCTTCTGAGTCCTCACAGAG-3'; Reverse: 5'-GACTAGGGTTGTTCCCTCCG-3', HPRT: Forward: 5'-GCGATGATGAACCAGGTTA-3'; Reverse: 5'-GTTGAGAGATCATCTCCACC-3', Shh: Forward: 5'-CAAAAAGCTGACCCCTTTAGC-3'; Reverse: 5'-CGTCTCGATCACGTAGAAGACC-3', Ihh: Forward: 5'-CTTGCCTACAAGCAGTTCAGC-3'; Reverse: 5'-TGCTGGTTCTGTATGATTGTCC-3', Dhh: Forward: 5'-CTTCAAGGATGAGGAGAACAGC-3'; Reverse: 5'-TGACTCTCTAGAACCGCGTAGC-3'.

### RNAseq

Mitral leaflets were dissected from P0 wildtype mice (n=3) and RNA was isolated using MicroRNeasy (Qiagen). Purity and quantification was determined by Bioanalyzer and the library preparation was done using the SMART-Seq v4 RNA-seq kit (Clontech Laboratories) per manufacturer instructions. The analysis was conducted on an OnRamp Bioinformatics Genomics research platform as previously described (11).

### In situ hybridizations

*In situ* hybridizations were performed as previously described (12, 40) and amplified with primers containing a SP6 RNA polymerase site engineered onto the 3' end of the reverse primer (5'-CGATCATTTAGGTGACACTATAGAAGAACCCCACTCACAGTTGGTATG-3', SP6 site underlined) or a T7 RNA polymerase site engineered onto the 3' end (Forward 5'-CGATCTAATACGACTCACTATAGGGAAGGACAGAAGTCCTCCAAGC-3', T7 site underlined.) n=3/timepoint.

### Mouse husbandry and genotyping

Animals were kept in a 12-hour light-dark cycle with food and water *ad libitum*. Genotyping was performed by Transnetyx, Inc or inhouse with the KAPA Mouse Genotyping Kit

(Kapabiosystems, #KK7301). The generation of conditional *Smo* mice has been previously described (41), and their genotyping was conducted with the following primers: the forward: 5'-GAAAGCTGGCCCCAGACTTTCG and reverse: 5'-AGTACCAGCAGCAGCAACTGC. Generation and genotyping of conditional *Ift88* and *Dzip1* have been previously described (11, 42). *WT1-Cre/ROSA-eGFP*, *Tie2-Cre*, *NfatC1-Cre*, and *NfatC1-enCre* lines were genotyped as previously described (11, 43). *Dhh* global null mice and conditional floxed alleles were previously described (44),(45). The forward primer for inhouse *Dhh* floxed allele genotyping was: 5'-TGGGTGTTTCCTTACAATCCGC-3'. The reverse primer for inhouse *Dhh* genotyping was: 5'-CAACCACTGGACCGAAGGAGGAA-3'. The forward delta primer was: 5'-TACGGTTCTCTCTGATTGTGATGAGGTC-3', and the reverse delta primer was: 5'-CAACCACTGGACCGAAGGAGGAA-3'.

### Immunohistochemistry and fluorescence imaging

Immunohistochemical and fluorescence stains were performed on 5  $\mu$ m, paraffin-embedded sections from E10.5, E13.5, E14.5, E15.5, E16.5, P0, and adult wildtype (6-month) and conditional *Dhh* mice as previously described (11, 12, 32, 34, 46, 47). Primary antibodies and their dilutions included: acetylated tubulin (Sigma, #T6793, 1:500), alpha-smooth muscle actin (Sigma, #A2547, 1:500), alpha-smooth muscle actin (Cell Signaling, #19245, 1:500), ARL13B (Protein Tech, #17711-1-AP, 1:500), Collagen Telo (a generous gift from Dr. Stanley Hoffman, 1:250), DHH (Santa Cruz, #sc-271168, 1:50), Elastin (Abcam, #ab77804, 1:250), Smoothed (Novus, #NSL2666, 1:100), Versican (a generous gift from Dr. Stanley Hoffman, 1:250). Secondary antibodies were all purchased from Invitrogen, used at a 1:100 dilution, and included fluorophores 488, 568, and Cy5. Nuclei were stained with Hoechst (Life Technologies, #H3569, 1:10,000). Slides were cover-slipped using Invitrogen SlowFade Gold Antifade Reagent (#S36936). Images were captured using: Leica TCS SP5 AOBS Confocal Microscope System and LAS AF v2.6.3 Build 8173 Acquisition and Analysis Software, Zeiss Axioscope M2, or Olympus BH-2 brightfield microscope. n>3/genotype.

### 3D reconstructions and morphometrics

3D reconstructions were generated from H&E images as previously described (11, 12, 32, 34) from P0 neonatal mice with the following genotypes: *NfatC1<sup>enCre(+)</sup>;Dhh<sup>f/f</sup>* (n=5), *NfatC1<sup>Cre(+)</sup>;Dhh<sup>f/f</sup>* (n=4), *Tie2<sup>Cre(+)</sup>;Dhh<sup>f/f</sup>* (n=5). Littermate controls were used as comparisons for phenotypic measurements (n=4 controls for each cKO group). Valve volumes were quantified with Imaris 9.2 software from the rendered 3D reconstruction. Length and thickness measurements were taken from the H&E images with Fiji (Image J) measuring tools. Length was measured along the entire vertical axis of each leaflet from the beginning, middle, and end of the valve. Thickness was measured in 3–4 evenly spaced intervals along the leaflet length to account for overall thickness from the leaflet hinge to tip. Each thickness measurement was taken across the horizontal axis on each 5  $\mu$ m section of each leaflet of the valve. 3D reconstruction of IHC's from valves were created from high-resolution confocal z-stacks using the Imaris 9.2 software.

### Movats pentachrome staining

Movats staining was performed on 5 $\mu$ m sections of wildtype, globally ablated *Dhh*, and *Dhh* conditional knockout (*NfatC1<sup>enCre(+)</sup>;Dhh<sup>f/f</sup>*) adult mitral valve tissue. Tissue sections were deparaffinized with xylenes and hydrated through graded ethanol to distilled water. Sections were incubated in Bouin's Fluid at 60°C for 1 hour. This was followed by a 5-minute wash in running water and a 20-minute incubation in 1% alcian blue at room temperature. Slides were then dipped 5 times in de-ionized water and incubated in alkaline alcohol for 10 minutes at 56°C. This was followed by a 2-minute wash in running water and a 15-minute room temperature incubation in Orcein-Verhoeff stain (0.2% Orcein, 5% alcoholic hematoxylin, 10% ferric chloride, 12% Lugol's iodine). Slides were washed for 3 minutes in running water and stained with woodstain scarlet-acid fuchsin for 2 minutes and 30 seconds. This was followed by incubations in 0.5% acetic acid (30 seconds), 5% phosphotungstic acid (9 minutes), 0.5% acetic acid (30 seconds), and 3 times in 100% EtOH (1 minute each). Slides were then incubated in alcoholic saffron for 20 minutes at room temperature. Finally, slides were dehydrated using two 5-minute incubations in 100% EtOH and three 5-minute incubations in xylenes and were coverslipped with Cytoseal. n=3/genotype.

### Western blotting

All Western blots were performed in a Bio-Rad mini blot electrophoresis chamber with semi-dry transfer in a Bio-Rad Trans-blot Turbo Transfer system. Protein samples were prepared using a 2x SDS Laemmli buffer (125mM Tris pH7, 10% Glycerol, 2% SDS, 5%  $\beta$ -mercaptoethanol, and .05% bromophenol blue) and boiled at 98°C for 5 minutes prior to loading. Proteins were separated in 4–20% Mini-PROTEAN TGX Stain-Free Protein Gels (Bio-rad, #456–8093) and transferred to Trans-Blot Turbo Mini Nitrocellulose Transfer Packs (Bio-rad, #170–4158). Membranes were blocked in 5% nonfat milk (Bio-rad, #170–6404) diluted in 1X Tris Buffered Saline, 0.1% Tween 20 (TBST) for 1 hour and incubated in primary antibodies at 4°C overnight with rotation. The next morning the membranes were rinsed 5 times with TBST and incubated at room temperature for 1 hour in secondary antibody. The membranes were then rinsed 5 times in TBST for 10 minutes each and imaged on a Bio-Rad ChemiDoc MP system with SuperSignal West Femto Maximum Sensitivity Substrate (ThermoFisher, #34096) or Pierce ECL Western Blotting Substrate (ThermoFisher, #32209) as appropriate. Primary antibodies and their dilutions included: GLI3 (Origene, #TA337186, 1:1000), RAC1 monoclonal antibody (Cytoskeleton Inc., #ARC03, 1:500) and Smoothed (GeneTex, #GTX60154, 1:1000). All secondary antibodies were purchased from Sigma and were used at 1:7500. These included: Anti-mouse IgG HRP antibody (#A9044) and Anti-rabbit IgG HRP antibody (#A9169).

### Cell density studies

Cell density was quantified as previously described (11, 32). Total cell numbers were counted using the open-source software, Cellprofiler. A pipeline was created to generate binary images from edited H&E images containing only the valve leaflets to count nuclei per section.

### Chicken valve dissection/cell culture/treatment groups

Eggs were purchased from Charles River Labs (#10100326) and incubated in a rotating egg incubator at 37°C with 55% humidity until Hamburger Hamilton Stage 30–31 (HH30–31), a stage of active valve remodeling. Embryos were then removed from the shell, and the mitral valves were dissected from the heart and stored in sterile PBS until all eggs were dissected. Mitral valves were pooled, and cells were dissociated with Trypsin containing EDTA (Corning, #25–053-CL). Cells were cultured in 100 mm dishes from Sigma (#93100), and maintained in M199 medium (Hyclone, #SH30253.01) supplemented with 5% heat-inactivated chicken serum (Bioworld, #30611183–1), 1% Pen-Strep (Life Technologies, 15070–063), and 0.1% Insulin-Transferrin-Selenium (ITS) (Gibco, #41400–045). Treatment groups for cell experiments included: Untreated, DHH 1µg/ml of recombinant mouse DHH C23II N-Terminus ligand (Novus Biologicals, #NBP2–35175), Antibodies (0.5µg/ml) against SHH (Santa Cruz, #sc-365112), IHH (Santa Cruz, #sc-166685), and DHH (Santa Cruz, #sc-271168), Cyclopamine 25µM (Tocris, #1623), and Cytochalasin D 1µM (Sigma, #C8273). All treatments, unless otherwise specified, were performed in low serum containing media: M199, 0.1% chicken serum, 0.1% ITS, 1% Pen-Strep.

### Hydrogel Remodeling assay

Collagen hydrogels were made with serum-free M199 media (Hyclone, #SH30253.01) and 1.5mg/ml Rat Tail Collagen I (Corning, #354236). Fetal chick mitral valve interstitial cells (cVICs) (stage HH30–31) were added to the M199/Collagen mixture after it was neutralized with 1N NaOH at a concentration of  $3 \times 10^5$  cells per hydrogel. 400 µl of cell/hydrogel mixture was added to each well of a 4-well plate (Thermo Scientific, #176740) and allowed to polymerize at 37°C for approximately 1 hour. After hydrogels solidified, 500µl of chick valve culture media was added to the top of the gels without detaching them and then incubated overnight at 37°C. The next morning, the culture media was aspirated and the gels were rinsed twice with sterile 1X Phosphate Buffered Saline (PBS). Treatments were then added in low serum containing media and hydrogels were detached from the well walls with a sterile pipette tip. Gels were imaged every hour for 8 hours and again at 24 hours (timepoints: 1, 2, 3, 4, 5, 6, 7, 8 and 24 hours). Following compaction, hydrogels were collected and embedded in paraffin for sectioning and immunostaining. The hydrogel assay for Supplementary Figure 8 was conducted in the same manner with the exception of using serum free media containing 1% Pen-Strep and 0.1% ITS during treatment. n=4 hydrogels/treatment group.

### Immunocytochemistry

cVICs were seeded into 4-well, collagen-coated chamber slides and cultured with normal media at 37°C for 16 hours. Cells were serum starved for 5 hours and treated in serum free media for 2 hours. Media was aspirated and cells were fixed in 4% paraformaldehyde (PFA) for 15 minutes and permeabilized in 0.1% Triton-X for 5 minutes. Fixed cells were washed twice in PBS and blocked with 1% bovine serum albumin (BSA) in PBS for 1 hour at room temperature. Cells were incubated in primary antibody diluted in 1% BSA for 16 hours at 4°C or 1.5 hours at room temperature as necessary, washed twice with PBS and incubated in secondary antibody for 1 hour at room temperature. Slides were washed three times for 5

minutes in PBS, including a wash step with Hoechst (Life Technologies, #H3569, 1:10,000). Slides were coverslipped with Invitrogen SlowFade Gold Antifade Reagent (#S36936). Primary antibodies and their dilutions included: acetylated tubulin (Sigma, #T6793, 1:500), alpha-smooth muscle actin (Sigma, #A2547, 1:500), Collagen Telo (a generous gift from Dr. Stanley Hoffman, 1:250). Secondary antibodies were purchased from Invitrogen, used at a 1:100 dilution, and included fluorophores 488, 568, and Cy5. n=4 slide chambers/treatment group.

### Co-immunoprecipitation

cVICs were cultured as described above, serum starved for 5 hours and treated for 1 hour in the respective treatment groups. Cell lysates were collected with non-ionic lysis buffer (25mM Tris pH7.4, 150mM NaCl, 1% Igepal, 5% Glycerol) containing 1X protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, #1861281), snap frozen with liquid nitrogen, and then stored at  $-80^{\circ}\text{C}$  until ready for use. Equal amounts of protein (300 $\mu\text{g}$ /sample) were then loaded into 1.5ml tubes with 20 $\mu\text{l}$  of A/G agarose beads (50% agarose, Pierce, #20421) and volumes were equalized to 1ml with lysis buffer. Samples were rotated at  $4^{\circ}\text{C}$  for 30 minutes to pre-clear the lysates. After centrifugation to pellet the beads, the supernatant was collected and split between two tubes for the co-IP. One tube contained 10 $\mu\text{l}$  A/G agarose beads with 3 $\mu\text{l}$  normal mouse IgG (400 $\mu\text{g}/\text{ml}$ , Santa Cruz, #sc-2025) and the other contained 10 $\mu\text{l}$  of agarose beads conjugated to TIAM1 antibody (500 $\mu\text{g}/\text{ml}$ , 25% agarose, Santa Cruz, #sc-393315 AC). Samples were rotated for 16 hours at  $4^{\circ}\text{C}$ . Supernatant was removed and beads were washed 6 times with 500 $\mu\text{l}$  of lysis buffer containing protease inhibitor. Each wash step included centrifuging to pellet the beads for 2 minutes at 14,000 X g and rotating the beads for 5 minutes at  $4^{\circ}\text{C}$ . All supernatant was removed to leave dry beads. Protein was then eluted at  $98^{\circ}\text{C}$  for 5 minutes in 15 $\mu\text{l}$  2x SDS laemmli buffer (125mM Tris pH7, 10% Glycerol, 2% SDS, 5%  $\beta$ -mercaptoethanol, and .05% bromophenol blue diluted in  $\text{H}_2\text{O}$ ), and used for Western blot analysis. n=4 samples/treatment group.

### RAC1 Activation Assay

RAC1 activation was performed on HH 30 chicken VICs per manufacturers recommendations (Cytoskeleton, Inc., #BK035). Serum starved VICs were treated with 1 $\mu\text{g}/\text{ml}$  of recombinant mouse DHH C23II N-Terminus ligand (Novus Biologicals, NBP2-35175) for 5, 10, 15, and 30 minutes. Proteins were isolated from treated and untreated control cells with kit buffers as indicated by instructions. n=4 replicates.

### Statistical analysis

Unless otherwise stated, all error bars are shown as means  $\pm$  SD. To detect statistically significant differences between groups/genotypes, a Student's t-test was used. P-values are listed in the figure legend for each image. Graphs were generated by SPSS v24 or Excel. Boxplots depict data quartiles and the error bars on these graphs represent the 95% confidence interval.

## Animal approval

All experiments on mice were approved by the Institutional Animal Care and Use Committee (IACUC) of the Medical University of South Carolina.

## RESULTS

### DHH ligand is developmentally expressed by valve endocardial cells and correlates with translocation of Smoothened protein on valve interstitial cell axonemes

RT-PCR was performed to assay the presence for hedgehog (HH) ligands and signaling responders in mouse VICs. HH ligands, Sonic (SHH), Indian (IHH), or Desert (DHH) were below the level of detection indicating very low to no expression within this valve interstitial cell line. HH responders: Smoothened (SMO), Patched1 (P1), and the downstream GLI transcription factors (GLI1, GLI2, GLI3) were, however, expressed by this cell population (Figure 1A). Three independent RNAseq analyses from isolated, individual P0 neonatal mitral valves identified significant levels of mRNA reads for *Dhh*, but no transcripts were detected for *Shh* or *Ihh* (Figure 1B). These data suggest that *Dhh* is the only hedgehog ligand present within the valves, but is not expressed in valve mesenchyme. To test whether *Dhh* is expressed by valve endocardial cells, section *in situ* hybridization was performed. As shown in Figure 1C, *Dhh* is expressed primarily within the valve endocardium at E15.5 with lower levels of expression within the trabeculated endothelium, primary atrial septum and epicardium. By the P0 neonatal timepoint, valvular *Dhh* mRNA expression shows regionalization within the mitral leaflet and is primarily restricted to the atrialis endocardium. Immunohistochemistry (IHC) confirmed the presence of DHH protein within the valve endocardium at embryonic, fetal and neonatal timepoints with evidence of protein diffusion into the interstitium (Figure 1D). Similar to the mRNA expression, DHH protein is primarily evident within the atrialis aspect of the anterior and posterior mitral leaflets with the highest signal present at the valve tip (Figure 1D and Supplemental Figure 1A). Expression of DHH protein was not detected in the adult mitral leaflets (Figure 1D), indicating a likely developmental role for this growth factor. To test whether hedgehog signaling was active in the valve interstitium, IHC was performed for axonemes and the G-protein coupled receptor (GPCR), Smoothened. In the presence of HH ligand, Smoothened is released from the membrane and travels into the primary cilium (48, 49). Thus, localization of Smoothened serves as a surrogate for hedgehog signaling. Using high resolution confocal microscopy, Smoothened was found both at the ciliary base and within the axoneme, strongly supporting active HH signaling with the valve. In addition, Smoothened staining was also observed in cytosolic regions and in cell types (valve endocardium) that are not associated with the primary cilia, suggesting a cilia-independent role consistent with previous reports (Figure 1E, Supplemental Figure 2.) Taken together, our RNA and protein expression studies support a paracrine cross-talk hypothesis in which valve endocardial-produced DHH communicates with valve interstitial cells (VICs) to cause smoothened translocation into the primary cilia to activate hedgehog signaling.



## Genetic depletion of *Dhh* in the valve endocardium results in abnormal valve morphogenesis and adult myxomatous mitral valves

DHH conditional mice (*Dhh*<sup>ff</sup>) were used (45) to assay the cell-specific function of DHH during valve development and to validate the DHH antibody (Supplemental Figure 1). We initially examined *Dhh*<sup>ff</sup> mice maintained on either the *Tie2*<sup>Cre</sup> or *NfatC1*<sup>Cre</sup> backgrounds. Both of these Cre lines genetically remove *Dhh* in endothelial and endothelial-derived cells. As the mitral valves are initially built through an endothelial-to-mesenchymal transformation (EndoMT), these Cre lines will remove *Dhh* from a majority of cells that make up the mitral valves in its entirety. Although subtle phenotypic differences were observed between each of these Cre-lines, the mitral leaflets from both lines displayed quantifiably altered valve morphology in both length and thickness (Supplemental Figure 3). In all animals examined, the valves exhibited regional thickness, width and length alterations, resulting in enlarged valve leaflets at P0. To test whether the DHH signal emanating from the endocardium is responsible for regulating valve morphogenesis as suggested by its expression pattern (Figure 1A–D), *Dhh* floxed mice were bred onto the *NfatC1*<sup>enCre(+)</sup>. This Cre is specifically expressed in valve endocardial cells that do not undergo EMT and thus, enables deletion of *Dhh* only in the valve endocardium (50). Depletion of *Dhh* in valve endocardial cells resulted in a mitral valve phenotype similar to the *Tie2*<sup>Cre</sup>;*Dhh*<sup>ff</sup> and *NfatC1*<sup>Cre</sup>;*Dhh*<sup>ff</sup> mice. Morphometric analyses of 3D reconstructions and measurements obtained from H&E sections demonstrated that mitral valve leaflets in the *NfatC1*<sup>enCre(+)</sup>;*Dhh*<sup>ff</sup> mice had abnormal valve morphology with significant increases in volume, length and thickness measurements at the neonatal P0 timepoint (Figure 2A–D). Consistent with our previous reports, we also observed a 50% (3 out of 6) penetrant right-noncoronary BAV phenotype in these mice (Supplemental Figure 4). We observed no significant changes in cilia length on valve interstitial cells (Supplemental Figure 5), demonstrating that loss of *Dhh* does not impair ciliogenesis.

To define whether loss of *Dhh* could result in a myxomatous valve as seen in patients with MVP, adult *NfatC1*<sup>enCre(+)</sup>;*Dhh*<sup>ff</sup> adult mice were examined histologically by Movats Pentachrome stains. As shown in Figure 2E, developmental depletion of *Dhh* in the valve endocardium resulted in adult myxomatous anterior and posterior leaflets in 100% of animals examined. This phenotype was consistent with the myxomatous valves observed in global *Dhh* deletion adult animals (Supplemental Figure 6). Both global and conditional knockout models for *Dhh* exhibit excess leaflet tissue and have disrupted ECM boundaries including loss of fibrillar collagen and increased proteoglycans and elastin.

## Cilia do not regulate canonical hedgehog signaling in the mitral valves

To begin exploring the mechanisms by which DHH signaling through primary cilia can regulate valve development and lead to myxomatous valves later in life, we initially examined whether canonical hedgehog signaling is impacted by primary cilia. Following a hedgehog signal through the primary cilium, GLI3 is cleaved into activator and/or repressor transcription factors (51–53). Thus, the ratios of GLI3 activator vs repressor are well established readouts for active hedgehog signaling through the primary cilium. In our study, isolated mitral valves from two independent models of cilia deficiency (*Ift88* and *Dzip1*), were assayed by Western analyses for differences in these cleavage products (Supplemental

Figure 7). Western analyses of isolated mitral leaflets from wildtype, conditional heterozygote, and knockout mice revealed no statistically significant change in the ratio's between full length (GLI3 transcriptional activator) or GLI3 repressor (Supplemental Figure 7 A, B). As transcription of GLI factors and Patched1 is under the control of GLI transcription regulators through an autocrine feedback loop, we examined whether mRNA of *Gli1*, *Gli2*, *Gli3* and *Patched 1* was changed as a global readout for altered canonical hedgehog signaling in the valves. In mitral leaflets isolated from cilia-deficient *Dzip1* and *Ift88* conditional knockout mice, no statistically significant change in mRNA levels for these *Gli* transcriptional targets were observed (Supplemental Figure 7C). Although *Gli* transcription factor mRNAs are expressed by mitral VICs, our data suggest that canonical hedgehog signaling does not impact GLI cleavage or transcription and thus, are unlikely to contribute to disease phenotype.

### Desert Hedgehog functions through a non-canonical pathway to remodel the cytoskeleton

There are currently two known non-canonical signaling pathways in which hedgehog can function. The first (type I) pathway results in increased proliferation. However, previous reports have shown that loss of cilia either through *Ift88* or *Dzip1* deletion in the valves does not result in a significant change in proliferation (11, 34). Consistent with these prior reports, we do not see a change in total cell number in the *Dhh* conditional knockout mitral leaflets (Supplemental Figure 8A–C) compared to control littermates. These data suggest that DHH signaling and/or primary cilia have little impact on cell proliferation during valve morphogenesis and type I noncanonical signaling is not likely impacting valve development. The second (type II) non-canonical hedgehog pathway involves cytoskeletal remodeling (54). During mitral valve development, VICs reorganize/activate their cytoskeleton and become aligned within the tissue (12, 55–57). As a consequence, the ECM, which is attached to the cells also becomes aligned in a process called “ECM compaction or ECM remodeling”. Thus, we hypothesized that DHH-signaling, through a type II non-canonical mechanism, activates the VIC cytoskeleton and results in ECM compaction, ultimately leading to leaflet attenuation and thinning. We initially tested this by assaying cellular density within the *Dhh* conditional knockout valves. Consistent with our previous findings in the *Ift88* and *Dzip1* conditional knockout mice (11, 32, 34), there is a reduction in cell density in the mitral leaflets at P0 (Supplemental Figure 8A, B) and an increase in extracellular space between cells compared to control littermates. This indicates a potential failure for the cells to actively remodel and condense the surrounding ECM.

To directly test the effect of DHH signaling and the role of primary cilia in ECM remodeling through activation of the cytoskeleton, we employed 3D collagen gel compaction assays. In these assays, cells are embedded within free-floating collagen gels and upon stimulation, will compact the gel over time. These assays are commonly used as an efficient and reproducible method for quantifying cytoskeletal dynamics and ECM compaction *in vitro* (57). Embryonic chicken valvular interstitial cells (cVICs) during stages of active valve remodeling (HH30–31) were dissected, cultured, and entombed with collagen hydrogels (1.5mg/ml), released from the side and bottom of the wells and subsequently treated with DHH ligand (mouse C23II, 1µg/ml). While the results showed that DHH treated cells had significantly smaller hydrogel diameters at 2–6 hours post treatment, the results were modest

and there was no difference in hydrogel compaction after 7 hours of treatment (Figure 3A & 3B). This prompted us to investigate whether hedgehog ligands were present within the media, potentially masking the effect of exogenously added ligand. Western blotting of unconditioned media confirmed the presence of each of the three hedgehog ligands present within the media (Figure 3C). To test whether hedgehog ligands were responsible for promoting collagen remodeling in this gel system, antibodies directed against each of the hedgehog ligands were added to the culture media. Addition of these blocking antibodies significantly impacted the rate of collagen remodeling in the gels at every timepoint investigated (Figure 3D–F) demonstrating a crucial role for hedgehog signaling in cell-driven ECM compaction. As DHH is the only hedgehog ligand expressed during cardiac valve development, the data strongly support that this growth factor is driving valve ECM compaction. This was further confirmed in serum free media stimulated with DHH ligand. As shown in Supplemental Figure 9, DHH was sufficient to stimulate robust ECM remodeling by cVICs in our collagen gel cultures.

To test whether DHH signaling is dependent on primary cilia, chicken VICs were treated with cyclopamine (Figure 4A,B). Cyclopamine is a Hedgehog-specific inhibitor that binds to Smoothened to prevent its signaling through the primary cilium (58). VICs stimulated with cyclopamine (25 $\mu$ m) displayed a pronounced delay in their ability to remodel the collagen ECM compared to control and DHH treated gels. Results from combinatorial treatment of DHH and cyclopamine were similar to those obtained from cyclopamine-only treated gels, suggesting a DHH-dependency on Smoothened at the primary cilium. Cytochalasin D (CytoD), an actin depolymerizing agent was used as a control to validate that gel compaction is due to actin organization. Chicken VICs, that were stimulated with CytoD failed to compact collagen gels over a 24-hour timepoint. The addition of DHH to CytoD treated cultures had no impact on the cVICs ability to remodel the ECM as anticipated (Figure 4C,D). Brightfield images of the hydrogels taken at the 24-hour timepoint confirmed that cell viability was likely not a factor in the differences in hydrogel remodeling (Supplementary Figure 10A). 3D reconstructions of cVICs from the hydrogels also showed that cells treated with DHH exhibited a different pattern of  $\alpha$ -SMA staining compared to cyclopamine and CytoD treated cells (Supplementary Figure 10B). In the cyclopamine treated cultures, the cells exhibited a rounded morphology and perturbed  $\alpha$ -SMA organization with no evidence of linearly organized actin filaments. The  $\alpha$ -SMA staining in the cytochalasin D treated cells appears jagged and consistent with depolymerized actin cables. Conversely, the DHH treated cells have elongated filopodia with organized actin and appear to make syncytial networks with neighboring cells. These data strongly support DHH as regulating  $\alpha$ -SMA organization and ultimately, ECM remodeling, through a Type II (Smoothened-dependent) non-canonical cilia signaling mechanism. Western analyses of these gels revealed no changes in total  $\alpha$ -SMA protein amounts, further indicating that cellular distribution and/or organization of  $\alpha$ -SMA is driven by DHH signaling (Supplemental Figure 11).

## Smooth muscle actin organization is regulated by DHH and primary cilia during development *in vivo*

Our data showing that DHH can regulate  $\alpha$ -SMA organization *in vitro*, combined with the observation that DHH and cilia are only present during developmental timepoints *in vivo* indicates that  $\alpha$ -SMA activation may represent a unique DHH-driven cell phenotype within the mitral valves during development. Immunohistochemical stains revealed that  $\alpha$ -SMA is expressed by VICs within specific regions of the valve during timepoints of active valve remodeling (Figure 5A). During early stages of valve morphogenesis,  $\alpha$ -SMA is not observed in newly transformed mesenchymal cells at E10.5, but is robustly expressed by developing myocytes (Figure 5A and (59)). Within the valves at E13.5, expression of  $\alpha$ -SMA is confined to a small group of cells at the junction where superior and inferior AV cushions have fused as well as in a few interstitial cells within the distal regions of the anterior and posterior leaflets (Figure 5A). By E15.5, expression becomes more pronounced within VICs localized to the distal tips of the mitral leaflets. The expression of  $\alpha$ -SMA continues to increase in this region of the leaflet at P0 and correlates with the timepoints of active valve compaction/remodeling, the expression of primary cilia and the presence of DHH protein (Figure 5B, C and Supplemental Figure 1). To test whether expression of  $\alpha$ -SMA is affected by DHH, we performed IHC and 3D-reconstructions of immunostains throughout the entire valve of control and *NfatC1<sup>Cre(+)</sup>* mice (Figure 5C and Supplemental Movies 1 and 2). In control mitral leaflets at P0, expression of  $\alpha$ -SMA is expressed primarily in regions of apposition between the anterior and posterior mitral leaflets. This pattern of expression was significantly attenuated in the *NfatC1<sup>Cre(+)</sup>;Dhh<sup>fl/f</sup>* valves (Figure 5C, Supplemental Movies 1, 2). Loss of cilia resulted in a similar change in  $\alpha$ -SMA expression and its spatial distribution (Figure 5D).

In an attempt to identify the tissue layer in which these  $\alpha$ -SMA cells reside, IHC co-stains with markers of the fibrosa (collagen 1 $\alpha$ 1) and spongiosa (versican) were performed and revealed an imperfect overlap between  $\alpha$ -SMA expressing cells and either of these markers (Supplemental Figure 12, 13). The altered distribution of  $\alpha$ -SMA in the conditional heterozygote and knockout correlates with areas of lower versican expression in the valve tips (Supplemental Figure 12). Although some overlap is evident between collagen and  $\alpha$ -SMA expressing cells in control mitral leaflets, this expression is limited to the middle of the mitral tip. The DHH heterozygote and knockout mice fail to show this pattern and the cells that express  $\alpha$ -SMA in this region do not appear to appreciably overlap (Supplemental Figure 13). Loss of one allele of *Dhh* was sufficient to generate these molecular changes indicating that gene dosage of this growth factor is critical. These data suggest that  $\alpha$ -SMA positive VICs likely mark a unique sub-population of endothelial-derived cells within the tips of the mitral valves. This hypothesis was further tested by performing IHC on WT-1 lineage traced mice, which labels epicardial-derived cells (EPDCs) (59). Consistent with  $\alpha$ -SMA marking a novel population of endothelial-derived cells within the mitral leaflet tips, expression is not observed within EPDCs of the anterior or posterior leaflets at P0 (Supplemental Figure 14).

## DHH promotes filamentous $\alpha$ -SMA organization through TIAM1 mediated RAC1 activation

Our data demonstrate a critical role for DHH signaling through the primary cilium in the regulation of collagen gel compaction (Figures 3 and 4) and  $\alpha$ -SMA organization (Figure 5, Supplemental Figure 10). Anisotropic filamentous actin is required for force generation and remodeling of ECM (60–67). Thus, we tested whether DHH could induce organized, anisotropic  $\alpha$ -SMA filaments. Immunocytochemistry of unstimulated cVICs in serum-starved cultures revealed prominent  $\alpha$ -SMA expression. This expression was primarily restricted to the cell periphery with a notable lack of mature  $\alpha$ -SMA filaments (Figure 6A). Under DHH stimulated conditions, however, we observed a profound increase in anisotropic,  $\alpha$ -SMA filaments that were well organized within the VICs (Figure 6A'). To test if this organization was dependent on active hedgehog signaling, the Smoothened inhibitor, Cyclopamine was used. ICC of cyclopamine-treated cultures revealed a complete loss of filamentous  $\alpha$ -SMA staining with no discernible difference in organization between DHH-treated and controls (Figure 6B, B'). The actin depolymerizing agent, Cytochalasin D gave a similar response with no evidence of mature filamentous  $\alpha$ -SMA between DHH-treated and controls (Figure 6C, C'). These data further demonstrate a role of DHH signaling through the primary cilium in organizing anisotropic  $\alpha$ -SMA filaments.

To gain mechanistic insight into how DHH might regulate smooth muscle actin polymerization through the primary cilium, we investigated whether RAC1 activation might be involved. RAC1 is a member of the p21 Rho-family of small GTPases that are master regulators of actin cytoskeletal rearrangements (54, 57, 68). Recent data has demonstrated that RAC1 is activated by the small GEF, TIAM1 (T cell lymphoma Invasion and Metastasis) (69). As TIAM1 has been shown to interact with Smoothened and upon hedgehog stimulation (69), function as a GEF for RAC1 to polymerize actin, we initially tested by Co-immunoprecipitation (Co-IP) whether this interaction was functioning in our culture models (Figure 6D). In full serum containing media (containing each hedgehog ligand and/or spiked with additional DHH protein), we detected no appreciable interaction between TIAM1 and Smoothened. However, following treatment of the cultures with antibodies against each of the hedgehog ligands to block receptor-ligand interactions, TIAM1 was observed as interacting with Smoothened (Figure 6D). This indicated that presence of hedgehog ligand resulted in a disruption of the TIAM1-Smoothened interaction. Similarly, cyclopamine, which binds to Smoothened and induces an inactive confirmation of Smoothened, resulted in retention of a Smoothened-TIAM1 interaction by Co-IP (Figure 6D). Treatment with the actin depolymerizing agent, cytochalasin D also led to an interaction between TIAM1 and Smoothened (Figure 6D). These data suggested that DHH is required for the release of a TIAM1-Smoothened interaction, coincident with smooth muscle actin polymerization. As free TIAM1 is a GEF for RAC1, we tested whether DHH treatment of serum starved cultures could result in an increase in RAC1 activation as assayed by RAC1-GTP levels. Consistent with this hypothesis, stimulation of serum-starved cVICs with DHH resulted in a dose-dependent increase in activated RAC1-GTP (Figure 6E, F). This dependency of RAC1 activation of DHH was further evaluated in the *NfatC1<sup>Cre(+)</sup>; Dhh<sup>f/f</sup>* mitral valves compared to littermate controls. Loss of DHH *in vivo* resulted in a profound loss of activated Rac1-GTP in the valve interstitial cells (Supplemental Figure 15).

## DISCUSSION

Mitral valve prolapse is a common disease affecting 2.5% of the general public with a poorly understood etiology. Until recently, studies on MVP have been relegated to end-stage phenotyping. These studies have led to the understanding of the molecular and structural phenotypes of a myxomatous valve (70–72). In these abnormal, biomechanically inferior tissues, there is very little phenotypic similarity with unaffected valves in terms of their structural organization. The beautifully organized ECM makeup of elastin, proteoglycans and collagen that is observed in normal mitral valves is lost in MVP patients. Blurring of these ECM boundaries in diseased tissues are only one difference. Cell phenotypes are also different between a normal and MVP disease valve with evidence of increased inflammatory cells (47, 73), myofibroblasts, robust cellular activities and overall lack of cellular quiescence (71). How the valve tissue progresses to this stage of disease in a patient with MVP is, however, unknown. Recent genetic discoveries have pointed to aberrant primary cilia as causative to MVP in affected families as well as in the population (10–12, 74). Uncovering how primary cilia function will likely provide key insight into mechanisms that go awry in MVP patients.

Although it is now clear that primary cilia are required for valve development and can cause myxomatous valve disease and MVP in mouse models and in patients with disease, the function of these cellular antennae in the valves have remained elusive. The classical signaling of hedgehog through the primary cilium to regulate GLI transcription factors is by far the most common mechanism of function. However, it is becoming clear that this signaling cascade, which ends in GLI transcriptional target gene regulation, is far more complex and can function through different mechanisms depending on cell-context. In this study, for example, we found no evidence to support a Hedgehog-cilia-GLI pathway in two independent cilia-deficient genetic models. This contradicts previous reports of *Ift88* (75) and *Dzip1* deletion (76–80), which showed a profound effect on GLI repressor versus activator forms with consequences on modulation of GLI transcriptional targets. The differences between our study and other previous reports likely reside in the context of the interrogated tissue-type, cell-type and cell-ECM microenvironment.

Another main difference may reside in the ligand itself. Whereas most reports focus on sonic hedgehog (SHH) as the major driver of cilia-dependent signaling in most cells within the body, our data do not support a role for SHH in the valves. RNAseq and RT-PCR revealed the presence of *Dhh* mRNA within the developing mitral valves and not *Shh* or *Ihh*. Previous reports have shown that, although the affinity between the hedgehog ligands is similar with Patched, their potencies to stimulate the canonical pathway are very different with a potency of  $Shh \gg Ihh \gg Dhh$  (54). Thus, the decision to function through a canonical (GLI-dependent) or non-canonical pathway likely resides in the type of hedgehog ligand present, concentration gradient within the tissue and diffusivity of the ligand. Our data showing activation of ciliated VIC's in close proximity to the highest degree of DHH expressing endocardial cells lend support to this hypothesis of a limited concentration gradient and/or restricted diffusion rate (Supplemental Figure 1).

For Hedgehog to function through the primary cilia, it must bind to its cognate receptor, Patched at the ciliary base. This binding induces a conformation change that de-represses Patched inhibition of the GPCR, Smoothened, which then traverses into the ciliary axoneme. Theoretically, knocking out Smoothened should show a similar phenotype in the valves compared to valves in *Ifi88*, *Dhh*, and *Dzip1* mouse models. Although previous reports have stated no valve phenotype in the *Tie2<sup>Cre</sup>;Smoothened* floxed mouse (81), our studies using the *NfatC1<sup>Cre</sup>* resulted in a robust phenotype (Supplemental Figure 16). The reason for these differences are unclear but may reside either in the type of Cre used, the degree of persistent smoothened expression in the valves, or technical differences between our imaging modalities.

Within the valves, primary cilia and desert hedgehog are primarily observed during development timepoints. It is becoming recognized that during fetal morphogenesis, cardiac valves need to be reshaped and reorganized to conform to the growing heart and increasing hemodynamic demands. Although still poorly understood, various groups have proposed a need for actin-driven mechanics to reshape the valve (57, 82–84). Consistent with this theory, we show that DHH expression is capable of regulating collagen gel remodeling *in vitro*, which is primarily dependent on actin kinetics since dismantling the actin cytoskeleton completely abrogates compaction. Our previous reports on cilia-deficient valves (11) and our current findings *in vivo* also highlight a function for primary cilia and DHH signaling in the regulation of fetal valve remodeling. Mechanistically, we identified a pathway in which DHH ligand, through the primary cilium could regulate the organization of  $\alpha$ -smooth muscle actin, typically viewed as a contractile myofibroblast marker. While, the presence of this gene is commonly associated with end-stage disease phenotypes, it is now clear that this is only a small part of  $\alpha$ -SMA's function. In fact, we detect  $\alpha$ -SMA protein expression in unique temporal-spatial patterns during valve development. Thus, categorizing  $\alpha$ -SMA solely as a myofibroblast marker is not necessarily correct as it is expressed within valve interstitial cells that appear phenotypically identical to their neighboring cells. As  $\alpha$ -SMA positive cells do not label exclusively with collagen or proteoglycan markers, respectively, we are unable to characterize them as a specific cell type. As such, our *in vivo* and *in vitro* data suggest that they likely represent a novel DHH-responsive cell type within the tips of the valves. Upon stimulation by DHH, these cells undergo a robust re-organization of the contractile  $\alpha$ -SMA cytoskeleton. Genetic perturbation of primary cilia or *Dhh* as well as pharmacological disruption of Smoothened function do not result in a change in  $\alpha$ -SMA expression levels (Supplemental Figure 11), but rather a change in the distribution and organization of the actin architecture. As we show, this change in  $\alpha$ -smooth muscle actin organization can be driven, at least in part, by a complex kinase cascade involving TIAM1 and RAC1.

How organizing the actin cytoskeleton into anisotropic arrays of  $\alpha$ -SMA leads to ECM remodeling is not well understood. It is logical to propose that  $\alpha$ -SMA organization results in shape changes that establish long, thinned fibroblasts. As these fibroblasts are connected to the ECM via integrins and other receptors, the ECM passively adopts a stratification similar to the cells. In this way, the cellular-ECM syncytium acquire an aligned shape within the valve tissue. With this perspective, stating that  $\alpha$ -SMA functions to promote “contraction” of VICs is incorrect and care should be placed on the description of such.

Additionally, the presence of bonafide acto-myosin contractile cables within the valve interstitial cells has not yet been fully described, and further questions the contractibility of these cells. Rather, it is more likely that  $\alpha$ -SMA induces cell shape changes and the ECM attached to the VICs through integrins becomes passively remodeled or re-organized. Nonetheless, the organization of  $\alpha$ -SMA as well as F-actin cables can induce tissue remodeling, potentially through the process described above. Previous TEM studies have shown that the neonatal valve and mature adult valve do indeed have longitudinal arrays of cell-ECM along the tension points of the valve ranging from annulus fibrosae to chordae tendineae (55). Thus, blocking VIC shape changes, integrin-mediated cell-ECM interactions, and/or cell-cell adhesions would likely disrupt cell-ECM alignment and result in a phenotypically enlarged, or uncompact tissue during development. In addition to our current data, recent findings from various *in vivo* mouse models, including perturbations of other genes that cause MVP (such as *FLNA* and *DCHS1*) and *in vitro* 3D models that incorporate tension (85–89), support this hypothesis.

## CONCLUSIONS

The concept that MVP, which is usually diagnosed in aged adults can be rooted in developmental defects may seem paradoxical. However, based on this report, in addition to papers by us and others, we now recognize that MVP has at least two major disease phases that represent cause and effect: 1) *disease inception* that occurs during development and (12, 46, 90–92) and 2) *disease pathogenesis* that occurs over the lifespan of the affected individual (47, 73). *This is akin to a house being built with a faulty structure that will take time to fail.* As such, myxomatous valve disease and MVP must then, by definition, be congenital in nature and influenced by secondary defects (e.g. inflammation, etc.) in valve homeostasis after birth. We posit that valves in MVP patients are not biomechanically inferior because they “degenerate” (as is current dogma), as this would imply destruction of a properly built structure. Rather, valves in MVP patients are built improperly during development and hence “generate” a myxomatous phenotype over time. This distinction is crucial as it places a critical need in understanding how valves are constructed during development with the goal of being able to rectify or correct a tissue that is being built improperly. Herein we have identified a novel mechanism by which Desert Hedgehog signals through the primary cilium in a paracrine, non-cell autonomous manner to activate and stabilize the cytoskeleton (Figure 7). When perturbed, the actin cytoskeleton is organized improperly resulting in developmental valve defects and *generation* of a myxomatous valve phenotype later in life. As cilia gene defects are common in MVP patients, this study sheds new light on how these structures are functioning in normal valves and the damaging consequences that ciliogenic signaling defects have on actin organization. Finding new methods for organizing and/or stabilizing the actin cytoskeleton may prove beneficial in blunting myxomatous valve progression and functional MVP.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.



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Dr. Norris and D. Fulmer designed the research studies and wrote the manuscript. D. Fulmer, Dr. Toomer, J. Glover, Dr. Guo, K. Moore, R. Moore, R. Stairley, C. Gensemer, M.K. Rumph, F. Emetu, C. McDowell, and J. Bian, C. Wang, T. Beck conducted experiments and acquired data. Drs. Norris, Lipschutz, Wessels, Renault and D. Fulmer analyzed and interpreted data and reviewed the manuscript.

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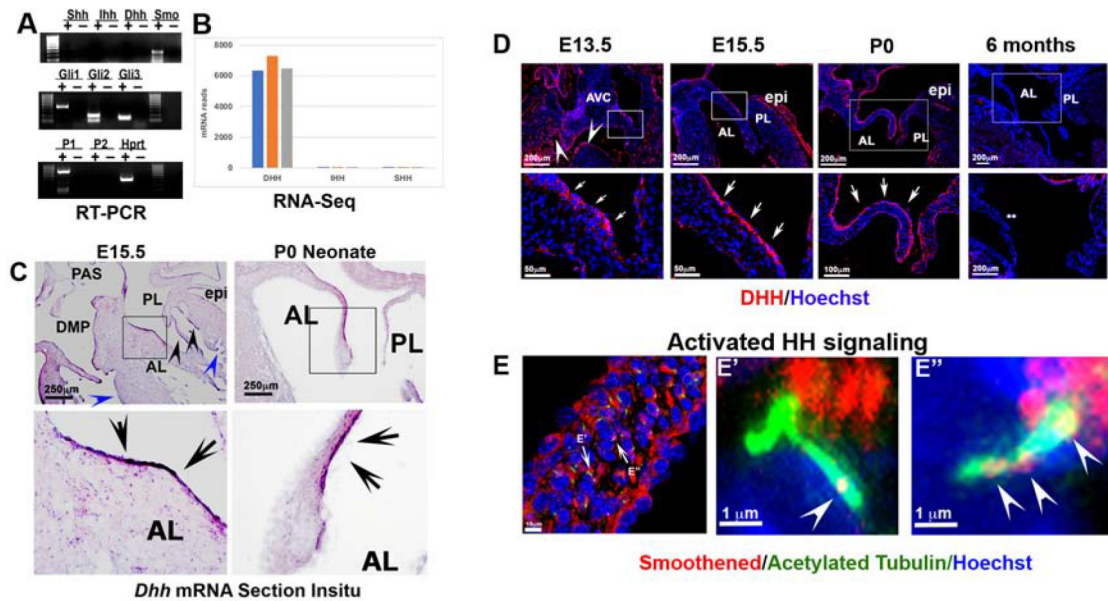
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**HIGHLIGHTS**

- Genetic variants in cilia genes cause Mitral Valve Prolapse
- Desert Hedgehog (DHH), and not other hedgehog ligands signal through the primary cilia during valve development
- Endocardial DHH activates primary cilium on valve interstitial cells through a paracrine cross-talk mechanism
- Non-Canonical hedgehog signaling, independent of GLI transcription factors function through the primary cilium during valve development
- Desert Hedgehog activates TIAM1-RAC1 kinase activity through the primary cilium
- Alpha-Smooth Muscle Actin is activated through a DHH-TIAM1-RAC1 signaling event to stimulate cell and tissue remodeling
- Myxomatous valves and MVP can be based on developmental defects in cytoskeletal remodeling dependent on DHH signaling through the primary cilium



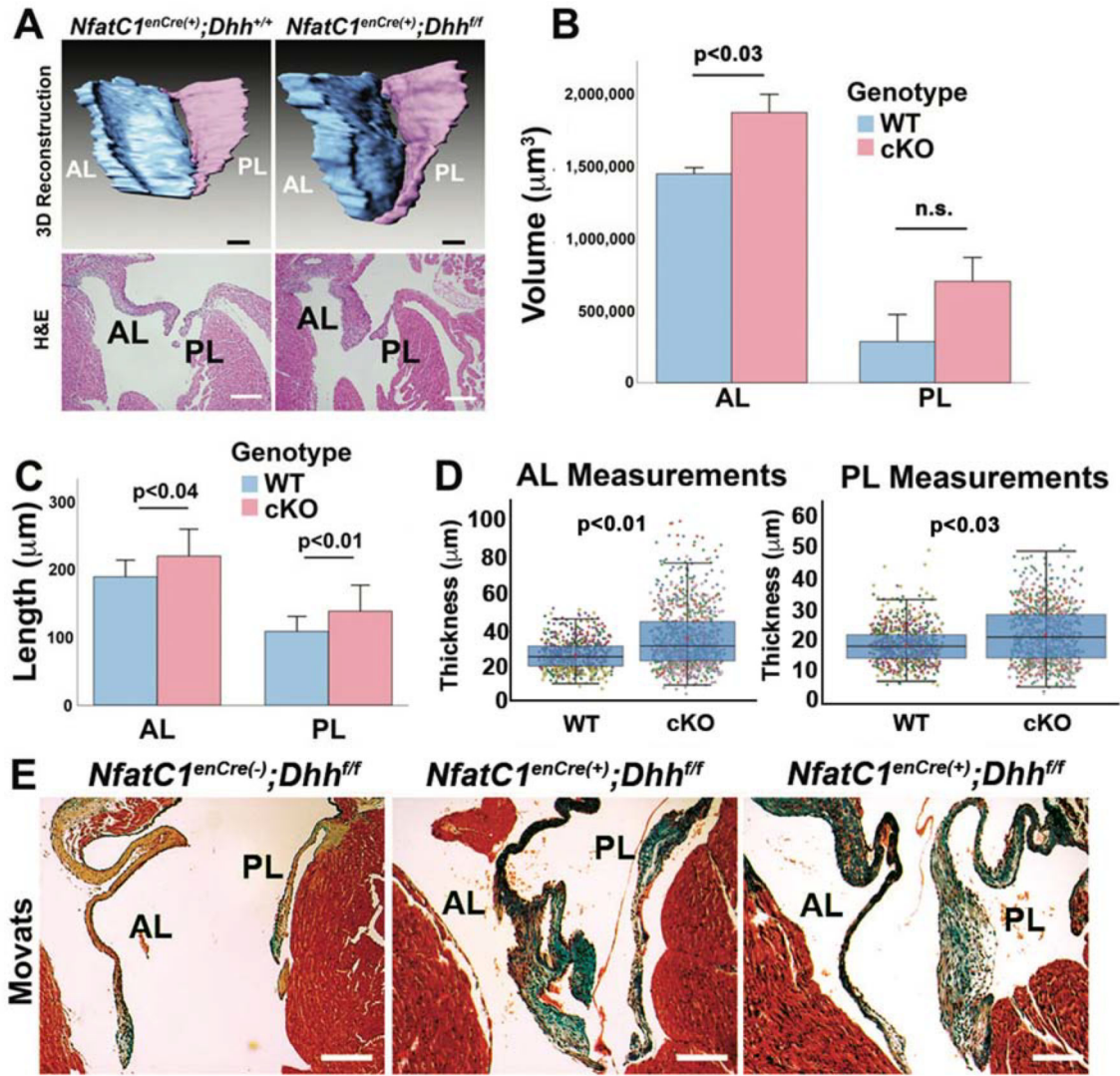
**Figure 1. DHH ligand is expressed by the endocardium and activates Smoothened ciliary translocation in interstitial cells**

(A) RT-PCR analyses of cultured murine VICs showing expression of *Smo*, *Gli1*, *Gli2*, *Gli3* and *Patched 1* (P1) but not *Shh*, *Ihh*, or *Dhh*. *Hprt* was used as a control for the RT reaction.

(B) mRNA reads from RNAseq datasets of isolated anterior mitral valve leaflets at P0 showing *Dhh* is the only hedgehog ligand present within the mitral valves.

(C) Section in situ for *Dhh* mRNA at E15.5 and P0 showing expression in valve endocardium (arrows), primary atrial septum (PAS), epicardium (epi), ventricular endothelium (blue arrowheads) and endothelium of forming chordae tendineae (black arrowheads).

(D) IHC shows the expression of DHH protein by endocardial cells of the developing mitral valve (white arrows), epicardium (epi) and ventricular endothelium (white arrows). Expression within the valve is more concentrated along the atrialis. No discernible expression is observed at adult timepoint (\*\*). (E) High resolution confocal microscopy and 3D reconstruction of IHC of the anterior leaflet of P0 mitral valves show Smoothened (red) translocation onto ciliary axoneme (green-acetylated tubulin) indicating active HH signaling (arrowheads). AVC = atrioventricular cushion, AL = anterior leaflet, PL = posterior leaflet.



**Figure 2. DHH signals through a paracrine mechanism and its loss results in myxomatous degeneration.**

(A) 3D reconstructions of P0 mitral valves (top row) created from H&E images (bottom row) showed that conditional knockout of *Dhh* from endocardial cells (*NfatC1<sup>enCre(+)</sup>;Dhh<sup>ff</sup>*) resulted in enlarged valves compared to controls. AL, PL = anterior and posterior leaflets, respectively. (B) Quantification of mitral valve volumes from panel A revealed a statistically significant increase in conditional knockout (cKO) volume compared to controls (WT). (C) The length of both the anterior and posterior leaflets were significantly increased in cKOs compared to WT. (D) Box plots of mitral valve thickness values between wild type and cKO. The blue boxes show the main distribution of the thickness values. The bottom of the box is the 25<sup>th</sup> percentile, the middle line is the median value, and the top of the box is the 75<sup>th</sup> percentile. Red dot denotes the mean thickness measurement. Conditional knockouts (cKO) showed an increased range of thicknesses in both anterior and posterior leaflets, and the average thickness was statistically increased in both leaflets compared to controls. Each dot is an individual thickness measurement and the different colors



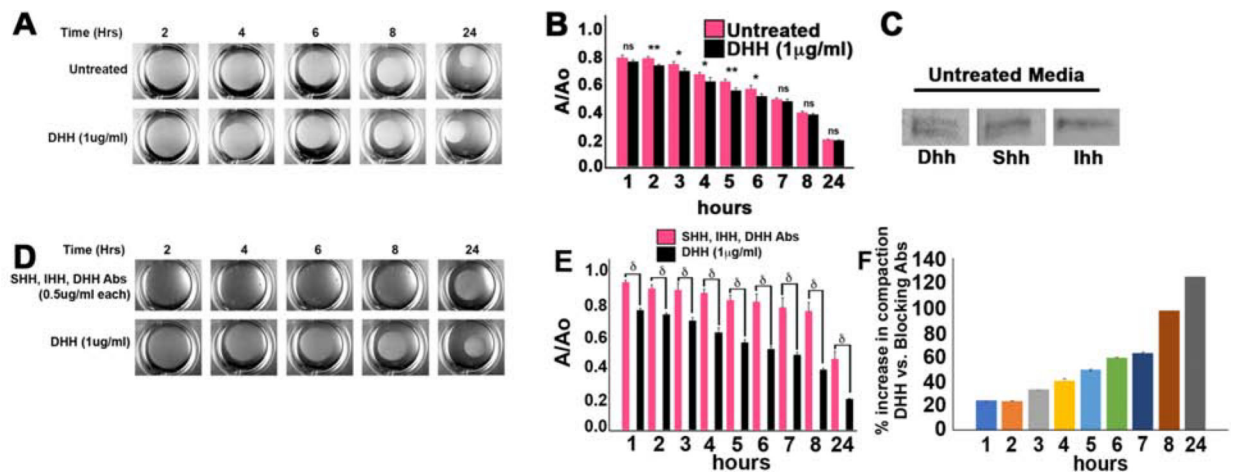
correspond to measurements taken from individual mice. n=5 animals/genotype. **(E)** Adult *Dhh* conditional knockout mice (*NfatC1<sup>enCre(+)</sup>;Dhh<sup>f/f</sup>*) and control littermates were stained for Movats Pentachrome (proteoglycans (blue), collagen (yellow), nuclei & elastin (black), muscle (red), and fibrin (bright red)). *NfatC1<sup>enCre(+)</sup>;Dhh<sup>f/f</sup>* mice showed enlarged anterior (AL) and posterior (PL) leaflets with blurring of molecular boundaries, increased proteoglycans and enlarged/redundant valve tissue compared to controls, indicating a myxomatous phenotype.

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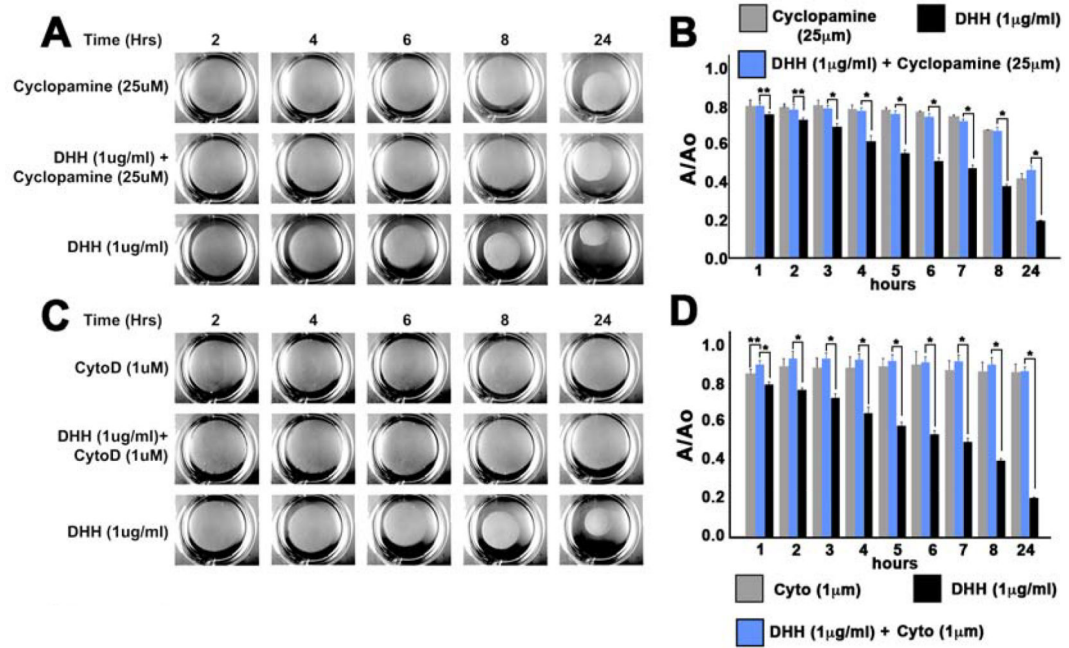
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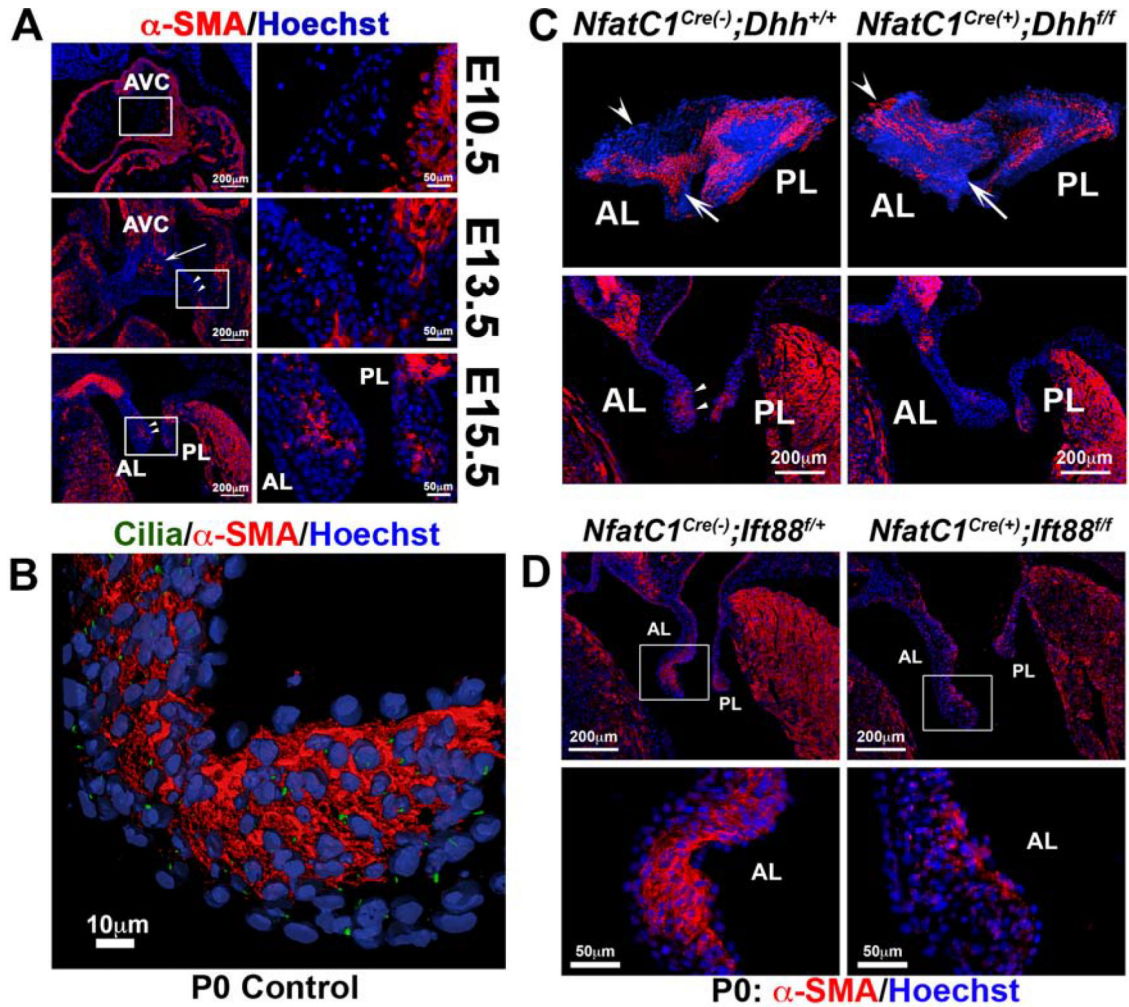
**Figure 3. DHH treatment promotes VIC contraction in 3D collagen hydrogel *in vitro* assays.**

(A) Representative images of Untreated and DHH treated hydrogels seeded with embryonic chicken VICs (cVICs, HH30–31) over the course of 24 hours. (B) Graph of hydrogel diameter change from (panel A) over time shows DHH treatment increased hydrogel contraction from hours 2–6 compared to control, but there was no statistical (n.s.) difference by 24 hours. ns=not significant,  $*=p<0.05$ ,  $**=p<0.005$  (C) Westerns revealed that HH ligands were present in serum samples from untreated media. (D) Antibodies against HH ligands blunted compaction of collagen gels compared to the DHH treated group. (E) Quantification of hydrogel diameters from panel D showed antibodies against HH ligands significantly reduced contraction rate compared DHH treatment.  $\delta=p<0.0005$  (F) Quantification of percent increase in compaction compared between DHH stimulated and antibody treated showing that over time, DHH treated gels compact significantly more at every timepoint evaluated.



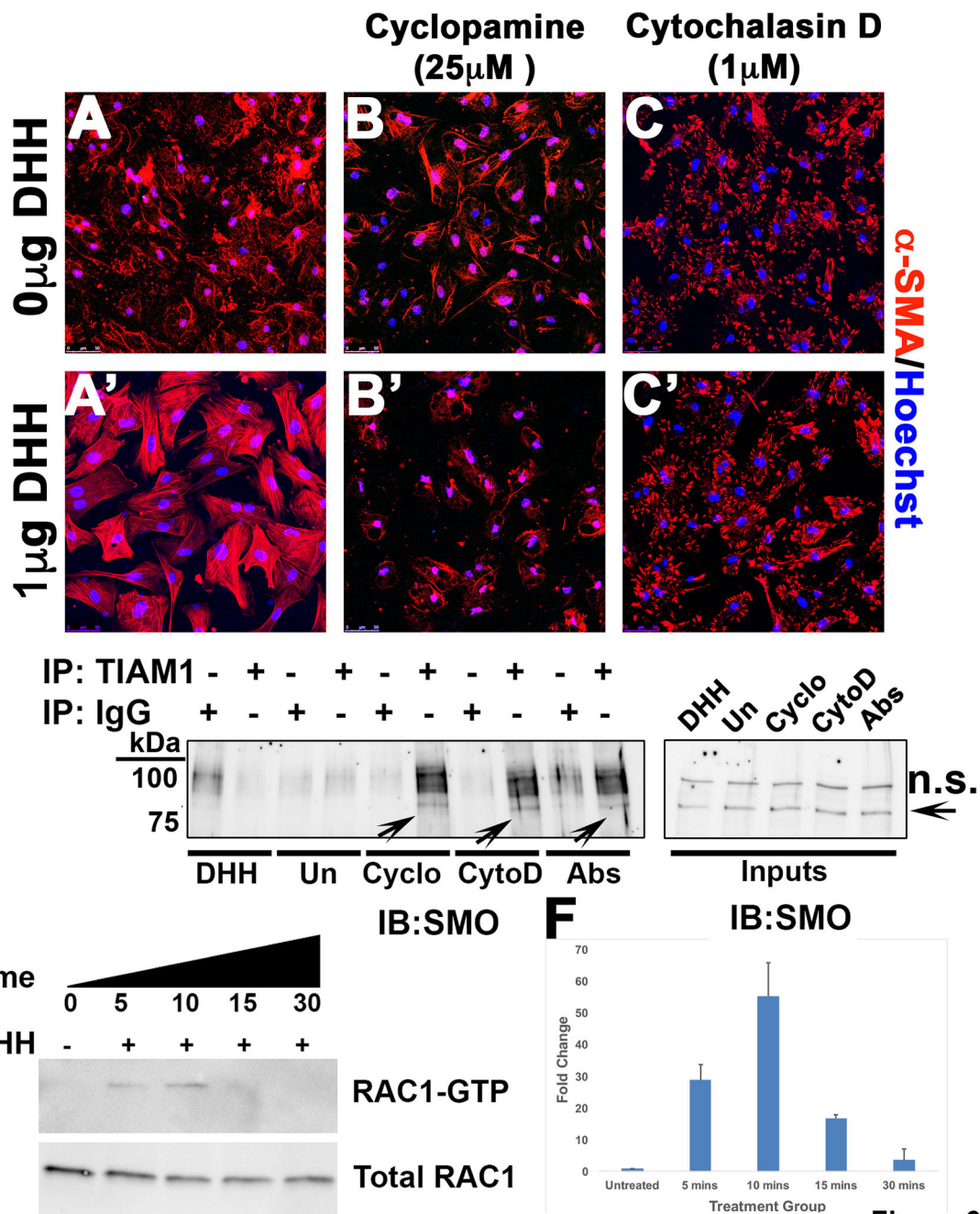
**Figure 4: ECM remodeling is dependent on cilia/smoothened signaling.**

(A) Cyclopamine, a Smoothened-specific HH inhibitor, reduced hydrogel contraction compared to the DHH treated group. (B) Graph depicting the quantification of hydrogels from the groups in panel A. There was no significant difference between cyclopamine alone versus cyclopamine plus DHH ligand.  $*=p<0.05$ ,  $**=p<0.005$  (C) Treatment with the actin polymerization inhibitor, cytochalasin D (CytoD), prevented hydrogel contraction. (D) Quantification of CytoD vs DHH treatment from gels in panel C shows CytoD significantly reduced contraction of hydrogels, demonstrating that collagen remodeling is dependent on an intact and active actin cytoskeleton.  $*=p<0.05$ ,  $**=p<0.005$



**Figure 5. Temporal-spatial  $\alpha$ -SMA is regulated by *Dhh* and primary cilia *in vivo*.**

(A) IHC showed that  $\alpha$ -smooth muscle actin begins to be expressed in the atrioventricular cushion (AVC) by E13.5 and continues to increase in later stages of valvulogenesis.  $\alpha$ -SMA is robustly expressed within the myocardium at all timepoints analyzed. The expression within the valves is restricted to a core of cells localized to the fusion region of the superior and inferior cushions (arrow) as well as within the tip of the anterior and posterior leaflets (arrowheads). (B) 3D reconstruction created from z-stack of high-resolution confocal image of wildtype anterior leaflet shows primary cilia (green) are present throughout  $\alpha$ -SMA rich regions (red) within the valve tip. (C) 3D reconstruction (top row) of control (*NfatC1<sup>Cre(-)</sup>;Dhh<sup>+/+</sup>*) and *Dhh* conditional knockout (*NfatC1<sup>Cre(+)</sup>;Dhh<sup>ff</sup>*) P0 mitral valves show reduced  $\alpha$ -SMA (red) expression compared to wildtype controls. White arrow heads indicate areas of high  $\alpha$ -SMA expression on the wildtype anterior leaflet on IHC stains from which the 3D reconstructions were created (bottom row). (D) Mitral valves of cilia conditional knock outs (*NfatC1<sup>Cre(+)</sup>;Ift88<sup>ff</sup>*) also show an apparent reduction in  $\alpha$ -SMA expression (red) compared to controls. AVC = atrioventricular cushion, AL = anterior leaflet, PL = posterior leaflet

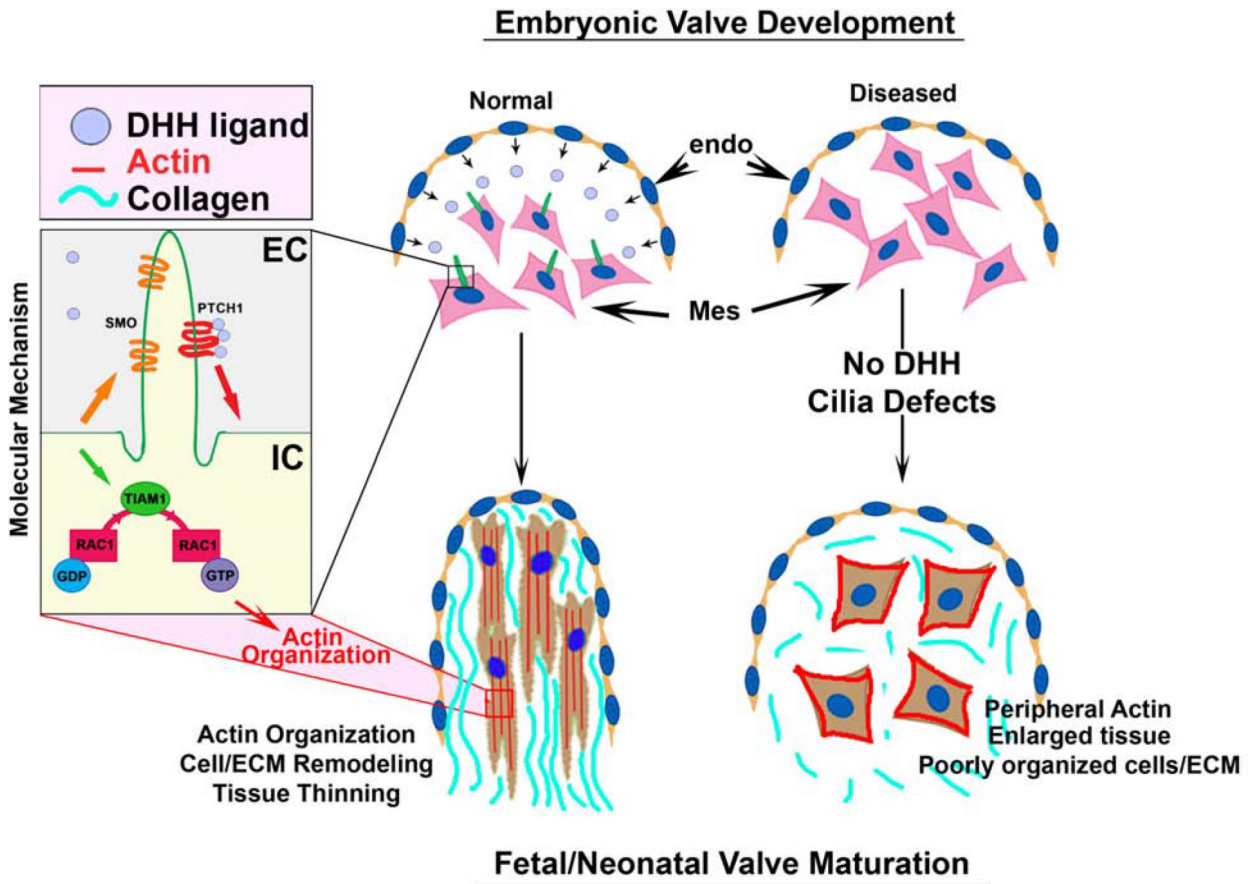


**Figure 6**

**Figure 6. DHH stimulates  $\alpha$ -SMA organization through a Smoothed-dependent TIAM1-RAC1 activation.**

(A, A') Serum starved cVICs (HH30–31) display  $\alpha$ -SMA expression along the cell periphery. Addition of DHH (1  $\mu$ g/ml) to cVICs induces a rapid reorganization of  $\alpha$ -SMA stress fibers. (B, B') Addition of the smoothed inhibitor completely blocks this DHH-mediated actin effect as does depolymerization of the smooth muscle actin cytoskeleton with cytochalasin D (C, C'). (D) Co-immunoprecipitation showed that HH treatment from DHH ligand or HH-containing untreated media results in the loss of Smoothed (SMO) and

TIAM1 binding. This effect is inhibited by cyclopamine (Cyclo), cytochalasin D (CytoD), or HH antibodies (Abs). Inputs for each of the co-IP experiments are shown to the right and display relatively equal presence of smoothened protein in the lysate (**E**) DHH treatment activates the TIAM1 target kinase RAC1 (RAC1-GTP) in cVICs, inducing a greater than 50-fold increase within 10 minutes.



**Figure 7: Model of DHH-cilia signaling in valve development.**

In normal embryonic valves, the endocardium (endo) releases DHH ligand, which acts on ciliated mesenchyme (mes). Upon binding to patched (PTCH1), smoothed is de-repressed, which leads to TIAM1 activation of RAC1 (RAC1-GTP). RAC1-GTP consequentially activates actin, which promotes its organization, collagen remodeling and tissue thinning and elongation during fetal gestation. In the absence of DHH or in the context of cilia gene variants as seen in patients with MVP, the hedgehog signal is not received, and  $\alpha$ -SMA organization fails to occur resulting in a defective valve remodeling and poor cellular and ECM organization.