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# A novel role for primary cilia in airway remodeling

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**ABSTRACT**

Primary cilia (PC) are solitary cellular organelles that play critical roles in development, homeostasis, and disease pathogenesis by modulating key signaling pathways such as sonic hedgehog and calcium flux. The antenna-like shape of PC enables them also to facilitate sensing of extracellular and mechanical stimuli into the cell, and a critical role for PC has been described for mesenchymal cells such as chondrocytes. However, nothing is known about the role of PC in airway smooth muscle cells (ASMC) in the context of airway remodeling. We hypothesized that PC on ASMCs mediate cell contraction and are thus integral in the remodeling process. We found that PC are expressed on ASMC in asthmatic lungs. Using pharmacological and genetic methods we demonstrated that PC are necessary for ASMC contraction in a collagen gel 3D model both in the absence of external stimulus and in response to the extracellular component hyaluronan. Mechanistically, we demonstrate that the effect of PC on ASMC contraction is to a small extent due to their effect on Sonic hedgehog signaling, and to a larger extent due to their effect on calcium influx and membrane depolarization. In conclusion, PC are necessary for the development of airway remodeling by mediating calcium flux and sonic hedgehog signaling.

## INTRODUCTION

Wound healing after injury is characterized by an intricately coordinated process of tissue regeneration, mesenchymal cell expansion and scar formation (29). Chronic lung injury promotes an imbalanced wound healing response leading to airway remodeling (5, 35) which is characteristic of chronic airway diseases such as asthma and COPD (2, 4). The extracellular matrix mediates the wound healing response to injury by providing signals to mesenchymal cells. For example, during tissue injury, hyaluronan (HA) is fragmented into short fragments (short HA, sHA), which promote inflammation, cell migration, contraction, and tissue remodeling (16, 27, 41). Therefore, understanding the communication between mesenchymal cells and matrix components such as sHA may provide novel treatments for airway remodeling in asthma and COPD.

Communication between mesenchymal cells and the extracellular matrix requires a means of sensing changes within the matrix that occur as result of injury or disease development. Primary cilia (PC) have emerged as major contributors to cellular sensing of the microenvironment (13). PC are structurally related to motile cilia, but unlike motile cilia, PC are solitary (one per cell), ubiquitous (almost every cell can develop a PC at some point in its lifetime) (59, 60), and play crucial roles in cell division, differentiation and migration (14, 28, 36). Mutations in PC-related genes are a common genetic cause of polycystic kidney disease and syndromes such as Bardet-Biedl, Joubert, Meckel disease and others (8, 28).

Recent literature supports a connection of PC to lung biology, since several of the genetic syndromes of PC-related mutations have associated lung phenotypes, such as bronchiectasis (23). In addition, in vertebrates PC play a crucial role in Sonic hedgehog (Shh) signaling (28, 37), which is a an important regulator of lung morphogenesis (42, 47), is activated in lung fibrosis (9), and has recently been shown to mediate cellular quiescence and repair after lung injury (53). PC also have important

roles in mechanotransduction and regulation of calcium flux (19, 45, 69). It has long been known that mechanical loading is closely linked to remodeling (56). Persistent airway smooth muscle cell (ASMC) contraction contributes to airway remodeling through mechanotransduction pathways (56), and ASMC contraction is mediated in part by changes in intracellular calcium flux (7, 34). Since PC modulate calcium influx in response to mechanical stimuli (45, 55, 69), connections between PC and ASMC contraction, calcium flux, and airway remodeling are possible.

PC have been described in many mesenchymal cells, e.g. chondrocytes, osteocytes, and fibroblasts (33, 40, 72), but only a single report has described the presence of PC in ASMCs (71). In that report, mechanotransduction sensory molecules such as α2-, α5-, β1-integrins and EGFR were localized on PC, and PC loss resulted in deficient migration and wound repair *in vitro,* suggesting an important role of PC in airway repair and remodeling. ASMCs are central players of airway remodeling (reviewed in (51)). We thus hypothesized that PC in ASMCs are necessary for cell contractility either at baseline or in response to stimuli in their microenvironment. We found that PC are expressed in asthmatic remodeled airways. Furthermore, inhibition of PC by pharmacological or genetic means significantly curtails the ability of human ASMCs to contract in a 3D gel model. PC mediation of cell contractility occurs to a lesser degree through the Shh pathway, but mainly because PC are necessary for calcium flux and membrane depolarization of ASMC. Thus, we provide for the first time evidence linking PC with ASMC function in remodeling.

## MATERIALS AND METHODS

*Detection of PC in ASMCs and asthmatic lung tissue.* Normal human ASMCs (Lonza, Walkerville, MD) were grown to confluence in smooth muscle cell growth media (Lonza; SmGM-2), then serum starved overnight (18-24 h) in basal media. Briefly, cells were

fixed with acetone:methanol (1:1 v/v), then blocked for 1 h at room temperature with 1%BSA, 1% milk, 10% normal donkey serum (Jackson ImmunoResearch, West Grove, PA) in Tris-buffered saline withTween-20 (TBST). Following incubation with primary antibodies (mouse anti-acetylated tubulin, 1:1000, rabbit anti-γ-tubulin, 1:800 (both from Sigma-Aldrich, St. Louis, MO); rabbit anti-Arl13b, 1:500 (Proteintech; Chicago, IL), mouse anti-rootletin, 1:500 (Santa Cruz Biotechnology; Santa Cruz, CA), or rabbit anti-IFT88, 1:250 (Proteintech), or with matching host isotype control 1-2 h at RT, primary cilia were detected with secondary antibodies, including AlexaFluor 594 donkey anti-rabbit and donkey anti-mouse, AlexaFluor 488 donkey anti-rabbit and donkey anti-mouse (Molecular Probes, ThermoFisher Scientific, Hudson, NH). Four subjects with severe asthma were recruited from the Durham, NC community. These patients fulfilled criteria for asthma (50) exhibiting a provocative concentration of methacholine resulting in a 20% fall in the forced expiratory volume in one second ($PC_{20}$) of < 8 mg/ml and reversibility, as demonstrated by at least a 12% and 200 ml increase in the $FEV_1$ or the forced vital capacity (FVC) with inhaled albuterol. All subjects provided consent in this Duke University Institutional Review Board-approved protocol (Pro00010753). Subjects underwent bronchoscopy with endobronchial biopsy. Formalin-fixed sections of normal human lung were procured from Abcam (Cambridge, MA; n=1), OriGene (Rockville, MD; n=3), and Amsbio (Cambridge, MA; n=1). Sections of endobronchial biopsies from asthmatic patients and normal lung were deparaffinized in xylenes and rehydrated to TBST. Following antigen retrieval in a pressure cooker (Biocare Medical, Concord, CA) in 1XEDTA, tissues were blocked as described above and stained with rabbit anti-α-Smooth Muscle Actin, 1:800 (Abcam; secondary AlexaFluor 594 donkey anti-rabbit) or rabbit anti-Arl13b (1:500, secondary AlexaFluor 488 donkey anti-rabbit) combined with anti-Rootletin (1:500, secondary AlexaFluor 594 donkey anti-mouse) in adjacent

sections to identify primary cilia localization in airway smooth muscle. Cover slips were affixed with Prolong Gold plus DAPI (Life Technologies, ThermoFisher Scientific, Hudson, NH) and PC expression was imaged using an Olympus BX51 microscope (Olympus Scientific Solutions Americas, Inc, Waltham, MA) fitted with an Olympus DP10 digital camera, or captured on a Zeiss LSM780 (Carl Zeiss Inc, Oberkochen, Germany) using a Plan-Apochromat 40X/1.4 Oil DIC objective. To determine the percentage of cells expressing primary cilia in culture, nuclei (stained with DAPI) and primary cilia (stained with Arl13b and rootletin) were counted from each of four 400X images of stained cells.

*Mechanical stretch of cultured ASMC.* Six-well amino treated BioFlex culture plates (Flexcell International Corp., Hillsborough, NC) were coated with Type I Collagen solution (Sigma-Aldrich, St. Louis, MO) (250 µg/mL) 2 hours prior to plating cells. Human ASMCs (Lonza) were plated in SmGM-2 media (Lonza) and incubated overnight, then switched to basal media prior to cell stretching. Wells were cyclically stretched at 5% or 15% elongation at 0.86 Hz for 24 hours using a Flexcell Tension Plus System (Flexcell International Corp, Hillsborough, NC), with statically cultured wells as controls. To visualize PC, samples were fixed in 4% PFA then incubated with rabbit anti-Arl13b. AlexaFluor 488 goat anti-rabbit IgG (Proteintech) was used as secondary antibody. Samples were mounted in Prolong Gold with DAPI, and imaged on a Zeiss LSM 700 Confocal Microscope.

*Lentiviral knockdown of IFT88 expression in ASMC.* All lentivirus were packaged in HEK293T/17 cells (ATCC # CRL-11268) according to Barde et. al. (2010) (3). Briefly, 293T cells were transiently transfected with pMD2G, psPAX2 and transfer vector containing the shRNA-hIFT88 clone (MISSION shRNA, pLKO.1 plasmid backbone;

Sigma-Aldrich) or scrambled control using Lipofectamine 2000. ASMC cells were infected at 5 MOI for experimental assays. IFT88 expression knockdown was assessed by quantitative Real Time-PCR using human IFT88-specific KiCqStart$^{R}$ SYBR Green pre-designed primer pairs (Sigma-Aldrich, St. Louis, MO) in combination with Power Sybr Green PCR Master Mix (ThermoFisher) as well as staining for IFT88 localization in the ciliary axoneme as described above.

*Collagen gel contraction assay.* Type 1 collagen solution from bovine skin (~3 mg/ml stock, Sigma-Aldrich; St Louis, MO) was neutralized with 10X PBS and 1 N NaOH to a pH of about 6.5, then mixed 1:1 with ASMC in media supplemented with 1 M HEPES buffer to achieve a cell:collagen mixture of 300,000 cells/ml and 1.2 mg collagen/ml. The collagen/cell mixtures were polymerized 2 h in a 37$^{o}$C incubator, 300 µl media was added to the gels and images were collected using an Epson Perfection V30 scanner and Image Capture software (Epson America, Long Beach, CA). Images were collected at intervals of 0 to 40 hours, and the extent of gel contraction was determined with NIH ImageJ software. In some experiments, the gels were supplemented with high molecular weight hyaluronan (HMW HA; Healon$^{R}$ OVD; Abbott Laboratories, North Chicago, IL) or Healon sonicated to form short fragment hyaluronan (sHA; 250-500 kDA), at 0.5 mg/ml, as well as Sonic Hedgehog pathway inhibitors HPI-4 (Ciliobrevin A, R&D Systems, Minneapolis, MN) at 5 µM, 10 µM and 15 µM, or Vismodegib (LC Laboratories, Woburn, MO) at 15 µM , 30 µM , or 45 µM. Contracted gels were fixed in 4% paraformaldehyde, and paraffin sections prepared for immunofluorescent detection of PC as described above.

*Measurement of intracellular calcium levels.* Studies were performed as previously described (44). Briefly, ASMC cells, either uninfected or infected with lentiviral IFT88

shRNA or scrambled controls were seeded onto 25 mm glass coverslips in 6–well cell culture plates with normal medium and used at confluency 3-4 days post-seeding. To measure intracellular calcium the cells underwent serum starvation for 24 hours, and then were incubated for 20 minutes with Fura-2AM (3μg/mL) in HBSS containing 25 mM MOPS at pH 7.4. Cells were rinsed for an additional 20 minutes using HBSS + MOPS buffer to remove excess Fura-2AM. Fresh HBSS + MOPS was added to the cells upon transfer of the coverslip to the attofluor chamber. Data was acquired using a Nikon Eclipse Ti inverted microscope fitted with a 40x oil immersion objective and Nikon Elements software. Changes in intracellular calcium are reported as the change in the emission ratio of calcium bound Fura (340nm) to calcium unbound Fura (380 nm). The presence of primary cilia was confirmed under these growth conditions by immunofluorescence detection as described above.

*Measurement of ASMC membrane potential ($V_m$).* Serum-starved ASMC were seeded on glass coverslips and transferred to a recording chamber on the stage of an inverted microscope (Olympus IMT2) and perfused at a rate of 1 ml/min with a solution containing (in mM) 135 NaCl, 5.4 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 5.5 glucose, 10 HEPES, pH 7.4 (following addition of 1 N NaOH) at room temperature. $V_m$ was measured with a Molecular Devices amplifier (Sunnyvale, CA; Axopatch 200B) in fast current-clamp conditions. The procedure consists of establishing a stable GiGa-seal (10–20 GΩ) between a glass pipette and the cell membrane, followed by rupture of the membrane patch under the pipette. $V_m$ values were read on the digital display of the patch amplifier and simultaneously recorded and stored on a hard drive of a computer equipped with pClamp software (Molecular Devices). The pipette resistance varied from 2 to 3 MΩ when filled with a solution containing (in mM) 135 KCl, 10 NaCl, 2 $MgCl_2$, 10 glucose, 0.1

EGTA, 0.2 $Na_2ATP$, 10 HEPES, pH 7.2 (1 N KOH). The data was digitized with a Digidata 1440A low-noise digitizer (Molecular Devices) connected to a computer equipped with pClamp software (Molecular Devices).

*Statistics.* Data was analyzed with Student's t-test or one-way ANOVA with Tukey's post-hoc testing using GraphPad Prism software, version 7b.

**RESULTS**

*Human ASMCs express PC.* In order to develop experimental protocols to assess the functional role of PC in airway remodeling, we first determined whether PC were expressed in primary human ASMC. When stained for proteins expressed in either the axoneme (acetylated tubulin and Arl13b) or basal body (γ-tubulin and rootletin) of the PC, most primary human ASMC were found to express PC in culture (Figure 1A,B). The percentage of ciliated nuclei was determined from four 400X images of Arl13b plus rootletin-stained cells. About 80% of ASMC nuclei are ciliated when grown under these conditions.

Primary cilia, as marked by Arl13b and rootletin, are present in smooth muscle cells in airways from severe asthmatics (Figure 1C-F), and, as was previously published (71), in normal human lung (Figure 1G-F). PC were found in smooth muscle cells in the airways in 4 out of 4 asthmatic lung sections, and in 3 out of 5 normal lung sections, although PC were comparatively less abundant in normal lung. This provides the first evidence of PC expression in human pulmonary disease, localizing them to a region characterized by extensive smooth muscle expansion typical of airway remodeling.

*PC elongate in response to cyclic tensile stress.* PC are mechanosensory organelles (11, 67, 69). Because mechanical strain, and smooth muscle shear stress are central hallmarks of airway remodeling in pulmonary disease, the effect of mechanical stretch

on PC was tested in cultured ASMC. Cells seeded onto collagen-coated BioFlex plates were serum starved overnight, and then subjected to cyclical stretch at a 5% and 15% elongation over a 24-hour period. PC were identified in fixed ASMC based on Arl13b expression (Figure 2A). Compared to static controls, PC length increased significantly at both 5% ($p<0.01$) and 15% ($p<0.05$) stretch (Figure 2B). The percentage of ciliated nuclei significantly increased at 5% stretch ($p<0.05$) but decreased at 15% ($p<0.05$) as compared to static controls (Figure 2C), suggesting that the increasing cyclic tension may result in a trend toward cilia disassembly. These data show that PC respond in a dynamic fashion to mechanical strain exerted upon ASMC, with possible consequences on cellular responses to airway remodeling.

*Pharmacological inhibition of PC reduces contractile activity in collagen.* To test the role of PC in airway remodeling, the collagen gel contraction assay was used as an *in vitro* method of determining how contractile cells such as SMC functionally interact with the extracellular matrix. Because PC are critical for the activation of the Shh pathway, which is associated with lung injury response, the effect of inhibition of Shh on ASMC function and PC expression was tested. ASMC were prepared with media containing the Shh pathway inhibitors, Vismodegib (which affects localization of Smoothened (Smo) to the ciliary membrane (1, 62) or HPI-4 (which blocks Shh signaling downstream of Smo, but also directly impacts ciliogenesis (24, 38)). Based on results of a dose response test of both inhibitors for ASMC contractility in collagen gels (Table 1), we used a dose of 15 μM for both HPI-4 and Vismodegib for experimental assays. Both inhibitors significantly reduced the contraction of collagen gels ($p<0.01$ and 0.05, HPI-4 and Vismodegib, respectively), but gels prepared with HPI-4 exhibited significantly decreased contraction compared to those prepared with Vismodegib (Figure 3A and B, and Table 1). PC were found in ASMC cells in gels prepared with DMSO vehicle and Vismodegib, but were not

detectable in the presence of HPI-4 (Figure 3C). These data suggest that while Shh signaling may mediate SMC contractility to some extent, total PC ablation has more profound impact on ASMC-mediated collagen gel contraction. This data supports potential PC effects on airway remodeling which are independent of Shh signaling.

*shRNA-mediated knockdown of IFT88 in ASMC results in reduced collagen gel contractility.* Because pharmacological inhibitors of cilia formation may have off-target effects, we confirmed our observations by genetically disrupting ciliogenesis. This was performed by shRNA-mediated knockdown of the essential ciliary protein, IFT88 (46, 52). IFT88 was expressed in ASMC (Figure 4A) and the level of IFT88 mRNA expression was reduced by approximately 50% in shRNA-transfected cells compared to controls (data not shown). However, staining for IFT88 showed a marked decrease in the IFT88 knockdown (KD) population compared to Scrambled control cells (Figure 4B,C), demonstrating that IFT88 expression knockdown was affected at the ciliary axoneme level. In addition, expression of the gene Gli1, which is downstream of PC (Shh) signaling was also decreased by 50%, demonstrating that the IFT88 KD had led to a decrease in PC function. Interestingly, the percentage of ciliated nuclei was similar between scrambled and IFT88 KD cells (Figure 4D), and PC in the KD cells were slightly longer ($p$<0.0001) compared to controls (Figure 4E-G). Elongated PC in response to genetic perturbations of ciliary genes have been described before (66). There was also a significant effect of IFT88 knockdown on ASMC morphology (Figure 4H,I), and a lower cell yield from flasks containing IFT88 KD cells compared to similarly seeded and cultured control cells (data not shown).

Next, the effect of IFT88 KD on ASMC contractility in collagen gels was determined (Figure 4J). Over a 40-hour period, gels containing IFT88 KD cells were significantly less contracted than those with scrambled control cells ($p$<0.0002),

indicating that the presence of PC is necessary for constrictive remodeling within the collagen gel matrix. Taken together, both pharmacological and genetic disruption of PC function results in decreased contractility in collagen gels, demonstrating that PC are an important functional component in this process.

*PC mediate hyaluronan-induced gel contraction.* In asthma, airway smooth muscle layer expansion is accompanied by deposition of extracellular matrix (ECM) components in the subepithelial space, including the glycosaminoglycan hyaluronan (HA) (25). HA is fragmented into smaller molecular weight species and can be detected in the bronchial alveolar lavage fluid of patients with virtually every chronic lung disease (10, 43, 58). HA in remodeled lung tissue is associated with airway inflammation and hyperresponsiveness (26, 43). While PC can interact directly with the ECM through integrins (48, 49, 71), no association has been made between PC and HA. The hypothesis was tested that HA effects on ASMC contractility is mediated by PC, using both high molecular weight HA (HMWHA; >5000 kDa) and short fragment HA (sHA; <500 kDa) to determine if differences exist between normal and pathological HA.

Both pharmacological inhibition, with HPI-4, and genetic knockdown of IFT88 was used to test the effect of disrupted PC on HA mediated ASMC contractility in collagen matrices (Figure 5). sHA consistently increased contractility in ASMC (Figure 5A). The effect of HMW HA was more variable, but overall, there was decreased contraction over time in gels supplemented with HMWHA (Figure 5A), confirming prior observations that sHA acts as an ASMC constrictor, while HMWHA inhibits ASMC contractility (25, 44). PC formation was not affected by HMWHA or sHA (Figure 5B). When PC were disrupted with either HPI-4 or IFT88 expression knockdown, gel contraction was reduced, but the addition of either HMWHA or sHA in the presence of

disrupted PC failed to affect contraction (Figure 5C, D). These data indicate that PC are at least partly necessary for the ASMC contractility response to HA.

*PC mediate HA effects on calcium flux.* Recent work has demonstrated that sHA induces membrane depolarization and increases intracellular calcium concentration in human ASMC (44), which is associated with increased ASMC contractility (44). Therefore, the effect of disrupted PC on sHA-induced membrane depolarization and calcium flux was investigated to determine if this pathway provides a potential mechanism through which PC mediate sHA effects on ASMC contractility. PC were detected in ASMC grown under conditions necessary for depolarization and calcium flux experiments (Figure 6A). ASMC in basal media have a resting membrane potential of -62.64 mV, which, upon exposure to sHA, was depolarized to -41.26 mV (Figure 6B). The same effect was observed in ASMC grown in basal media supplemented with DMSO as the vehicle control for HPI-4 (-58.79 mV resting to -41.42 mV upon sHA exposure; $p< 0.0001$). HPI-4 significantly attenuated the effect of sHA exposure on cell membrane depolarization, indicating that functional PC are necessary for sHA-mediated membrane depolarization (Figure 6B). IFT88 KD cells also had a significantly attenuated depolarization response to sHA compared to scrambled shRNA-treated cells, (Figure 6C), strengthening the conclusion that functional PC facilitate sHA-induced membrane depolarization.

To investigate further the role of PC in HA-mediated effects on ASMC contractility, the effect of disrupted PC function on HA-mediated influx of calcium ($Ca^{2+}$) into the intracellular space was assessed. Previous work demonstrated that sHA, but not HMWHA, induced an increase in cytosolic $Ca^{2+}$ concentration (44). In our hands sHA also induced a significant increase in cytosolic $Ca^{2+}$ compared to control (Figure 6D,E). The sHA effect on $Ca^{2+}$ flux was attenuated both in HPI-4 treated ASMC and in IFT88 KD cells (Figure 6D,E), indicating that, as with sHA-increased ASMC contraction in

collagen gels and sHA-mediated membrane depolarization, functional PC are necessary for sHA-induced $Ca^{2+}$ current flux in ASMC. Taken together, these data support the hypothesis that PC play an important role in airway SMC remodeling: PC show a dynamic response to mechanical strain, are necessary for cell contraction in unstimulated cells and in response to the pathological ECM component sHA, and are necessary for sHA-induced $Ca^{2+}$ flux and membrane depolarization, which are the cellular events leading up to ASMC contraction.

## DISCUSSION

In aggregate, these data suggest a mechanistic connection between PC, cell-matrix interactions, and ASMC contraction, which provides important insights into the pathogenesis of airway remodeling. Our results provide evidence suggesting that PC mediate the response of ASMCs to its microenvironment.

Even though PC are in principle expressed in every cell type, there is remarkably little knowledge about their expression in the lung. Jain et al. (39) showed that PC are expressed in airway epithelia after cell injury, and indicated that PC expression was a marker of undifferentiated, proliferating epithelial cells (39). Wu, et. al. (2009) however demonstrated PC on ASMC *in situ* in human airways, and showed that PC on ASMC mediate cell migration. Our findings expand on these results and suggest further mechanisms for PC involvement in airway remodeling.

A primary function of PC is to sense extracellular strain or shear stress, and this function has been described in diverse cells such as renal tubular epithelia (61, 63), chondrocytes (32, 67), and osteoblasts (18). Increased mechanical strain is a central component of airway remodeling (20, 21). Our results suggest that mechanical strain may activate ASMC PC assembly, because PC on ASMC subjected to cyclic tensile

strain responded with increased PC length compared to static cultured cells. In addition, the number of ciliated nuclei increased at low-grade stretch, again supporting a dynamic effect on cilia assembly. IFT88 mRNA expression was not changed with cyclic stretch, (data not shown), indicating that stress effects on cilia length are post-translational in nature. This finding supports the notion that PC become activated in response to strain, which is common in airway constriction or remodeling, and are thus primed to mediate the cellular response to their environment. Our results suggest a positive feedback mechanism between airway remodeling and PC. Remodeling activates PC through mechanical strain, and activated PC are necessary for remodeling. Thus, inhibiting PC activity in ASMC may be a way to disrupt the vicious cycle of remodeling and airway strain.

PC mediate cell responses to both chemical and mechanical triggers (6). HA is an abundant component of the cellular microenvironment and HA metabolism is altered in airway remodeling. Significantly, sHA expression is upregulated in many diseases in which airway remodeling is a central feature, such as asthma, COPD, idiopathic pulmonary fibrosis, and lung transplant rejection (25, 43). Because sHA in the pathological matrix promotes cell proliferation and contraction, we studied whether PC mediate sHA effects. Both pharmacological and genetic inhibition of PC function significantly abrogated the effect of HA on ASMC contraction. Importantly, PC seem to mediate both the contractile response to sHA, as well as the relaxation response to HMWHA.

Interestingly, in our hands IFT88 KD cells did not have a decrease in PC abundance, and had a slight increase in PC size. Although perhaps counterintuitive, this result is not without precedent. There are several examples of mutations in primary cilia genes that lead to increased primary cilia length, e.g. mutations or deficiency in Kif7 (31), Mks1 and Mks3 (65), Bbs1 (17), and DYNC2LI1 (66). There also may be a cell

specific response to the effect of a genetic perturbation of primary cilia genes on cilia formation. For example, *Mks1*$^{krc}$, *Mks1*$^{del64\text{-}323}$ and *B9d1*-null mutant mice have disrupted cilia on the embryonic node and limb bud mesenchyme, while tracheal and bile duct cilia numbers are not affected (15, 22, 70). Also, cilia size and function may not always be correlated. For example, embryonic fibroblasts isolated from *B9d1*-null mice possess structurally normal cilia but still demonstrate reduced Shh signalling capacity (22). Our results suggest that IFT88 knockdown can lead to an inhibition of PC function, even though size is not reduced.

Since PC mediate the ASMC response to its pericellular HA matix, the possible mechanism was investigated. PC are essential hubs for Sonic hedgehog signaling (28, 37). Shh genes (HHIP, PTCH, CROCC) have recently been implicated in large gene-wide association studies as determinants of decreased lung function (30, 47, 57) and susceptibility to obstructive airway disease (12, 68, 74), and Shh genes have also been implicated in lung fibrosis (73). Recent evidence provides a direct role for Shh in injury, repair, and cellular quiescence in the adult mouse lung (53). Taken together, these studies support a role for PC in lung function and response to injury, since PC are integral part of Shh signaling. Our results, however, suggest that the effect of PC in ASMC contraction is only partly mediated through Shh signaling. Inhibition of Shh signaling through Vismodegib had a modest effect on collagen gel contraction, while pharmacological (through HPI-4) or genetic (through IFT88 shRNA) disruption of PC caused a strong inhibition of contraction. Thus, PC may mediate other signaling pathways that are important for smooth muscle contractility, such as calcium flux.

Calcium flux is important for many mesenchymal cell functions such as cell contractility and migration, and is necessary for the development of airway remodeling and hyperresponsiveness. For example, we recently showed that oxidative lung injury

through inhalation of chlorine gas leads to the release of sHA, which promotes ASMC contraction through the induction of calcium influx into the cell and subsequent membrane depolarization (44). Recent research has suggested that PC are necessary for orderly calcium signaling (19, 54, 64), which prompted investigation into how PC modulate calcium flux into the smooth muscle cell as induced by sHA. Our data suggest that PC are necessary for sHA-mediated membrane depolarization and changes in intracellular calcium that ultimately results in SMC contraction. Both pharmacological and genetic disruption of PC function significantly decreased membrane depolarization and completely abolished sHA-induced calcium flux into ASMCs, suggesting that PC must be included in the calcium signaling pathway that mediate airway remodeling and contractility. This does not mean that sHA is directly activating PC, or that PC are directly interacting with sHA. However, our findings do suggest that PC are an integral part of the signaling pathway downstream of sHA engagement with its receptors, which leads to calcium flux, membrane depolarization and ultimately cell contraction.

In conclusion, our results show that PC are expressed by ASMC both *in vivo* and *in vitro*; are necessary for ASMC contraction in the three-dimensional matrix; mediate the contractile response of ASMC to microenvironmental stimuli such as extracellular sHA; and are necessary for calcium flux into the ASMCs in response to sHA. In aggregate, our data suggest a possible mechanistic role for PC in the cellular response to extracellular matrix perturbations (i.e. hyaluronan degradation), and implicate PC in the pathogenesis of airway disease.

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## DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

## AUTHOR CONTRIBUTIONS

C.S.T. and S.G. conception, design of research, experimentation, data analysis, and manuscript preparation; W.S., A.L. Z.Y, J.R.C, and S. M. performed calcium related experiments, analyzed data, interpreted results of experiments, and manuscript preparation; B.M.Y and R.L.H. conducted mechanical strains experiments, immunostaining, data analysis, and manuscript preparation; Y.R.Y., R.M.T., and J.L.I. contributed human lung samples, contributed to data interpretation and manuscript preparation.

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## FIGURE LEGENDS

**Figure 1. Primary cilia are expressed in human airway smooth muscle cells.** Primary cilia (PC) were assessed in ASMC in culture and in airway smooth muscle in human normal and asthmatic lung. (*A* and *B*) Normal ASMC (Lonza) were grown to confluence on 8-well glass chamber slides, then serum starved overnight. PC were detected with antibodies to acetylated tubulin (green) and γ-tubulin (red) (*A*), or with antibodies to Arl13b (green) and rootletin (red). Arrows point to PC (*B*). Scale bars = 20 μm and 5 μm for insets. (*C-J*) Adjacent sections of formalin-fixed human asthmatic (C-F) or healthy (G-J) lung biopsies were stained with antibodies to either α-Smooth Muscle Actin (red, C, E, G, I), or Arl13b and rootletin (green and red respectively; D, F, H, J). Arrows point to PC, to show localization of PC to regions positive for α-Smooth Muscle Actin staining. Nuclei for all images is marked by DAPI (blue).

**Figure 2. Primary cilia length increases in response to mechanical stretch of ASMC.** Cells were cultured on collagen-coated Bioflex culture plates, serum starved overnight, then subjected to cyclic tensile stretch at a 5% or 15% change in surface area. (*A*) PC were visualized with an antibody to Arl13b (green); nuclei stained with DAPI (blue). (*B*) Percent change in cilia length was determined using ImageJ software to measure individual cilia from confocal images, to compare 5% stretch (*n*=41 cilia) and 15% stretch (*n*=47 cilia) to static controls (*n*=82 cilia). (*C*) Percentage of ciliated nuclei were determined, counting the number of nuclei that expressed primary cilia per confocal image (51.6%, 69.1%, and 41.7% for static, 5%, and 15%, respectively). Data are presented as mean +/- SEM. Scale bar = 20 μm.

**Figure 3. Pharmacological inhibition of PC reduces ASMC contractility.** (*A*) ASMC were prepared in basal media supplemented with 15 μM Sonic Hedgehog pathway inhibitors Vismodegib (Smoothened inhibitor) or HPI-4 (inhibits downstream of Smoothened), then combined with neutralized collagen and allowed to contract for up to 20 hours. (*B*) Gel areas were measured with ImageJ software (*n*=3 gels per group), data presented as mean +/- SEM. (*C*) Contracted collagen gels were fixed in 4% PFA and paraffin sections stained with acetylated tubulin (red; nuclei stained with DAPI (blue)). Images are 1000X magnification.

**Figure 4. Knockdown of IFT88 expression in ASMC reduces contractile activity in collagen gels.** (*A*) Confluent and serum starved ASMC were stained with antibodies to acetylated tubulin (green) and IFT88 (red) to assess IFT88 expression and localization in PC. IFT88 co-localizes in the ciliary axoneme with acetylated tubulin (arrows). Image is at 400X magnification. (*B* and *C*) ASMC were infected with 5 MOI of lentiviral vector containing shRNA to IFT88 or with Scrambled control shRNA. Cells were stained with an antibody to IFT88 (red) to determine levels of expression and localization to PC. Nuclei are shown in blue (DAPI) and images are 400X magnification. (*D*) Percentage of ciliated nuclei was determined using four 400X images of Arl13b and rootletin staining to indicate cilia, and using DAPI to localize nuclei, comparing Scrambled control (82.4%) to IFT88 KD (87.6%). (*E* and *F*) To assess PC morphology in ASMC following knockdown of IFT88 expression, cells were stained with antibodies to Arl13b (green) and rootletin (red). Arrows indicate cilia, images are 400X magnification. (*G*) Cilia length was measured with ImageJ software, using four 400X images of Arl13b and rootletin staining to determine relative cilia length comparing Scrambled control (*n*=139 cilia measured) to IFT88 KD (*n*=103 cilia measured). Data is presented as mean +/- SEM. (*H* and *I*) Scrambled control and IFT88 KD cells were grown for 3 weeks in culture and light

783 microscope images taken (100X magnification) to assess cellular morphology. Inset
784 shows non-infected wild type (WT) cells, at 100X magnification. (*J*) IFT88 KD and
785 Scrambled control ASMC were combined with neutralized collagen and allowed to
786 contract for 40 hours. Gel areas were measured (*n*=6 IFT88 KD and *n*=6 Scrambled
787 control) and percent contraction from time zero was determined. Data presented as
788 mean +/- SEM.
789
790 **Figure 5. Hyaluronan-induced ASMC contraction is mediated by PC.** (*A*) To
791 determine the effect of high molecular weight hyaluronan (HMW HA) and short fragment
792 HA (sHA) on ASMC contraction in collagen gels, cells were prepared in basal media
793 alone or supplemented with 0.5 mg/ml HMW HA (*n*=3) or sHA (*n*=3) and combined with
794 neutralized collagen. Gels contracted over 40 hours, and gel areas were measured at
795 time zero and at 40 hours to calculate percent contraction over time. Data is presented
796 as mean +/- SEM. (*B*) Contracted gels were fixed in 4% PFA, paraffin embedded, and
797 sections stained with acetylated tubulin (red; nuclei stained with DAPI in blue) to
798 visualize PC (arrows); images at 400X magnification. (*C*) ASMC were prepared in basal
799 media supplemented with DMSO vehicle control (*n*=3), or supplemented with either 0.5
800 mg/ml sHA plus DMSO (*n*=3), or 0.5 mg/ml HMW HA plus DMSO (*n*=3; left side of
801 graph). Another set of gels was prepared with ASMC in basal media plus DMSO as
802 vehicle control (*n*=9), with 15 μM HPI-4 plus 0.5 mg/ml sHA (*n*=6), or with 15 μM HPI-4
803 plus 0.5 mg/ml HMW HA (*n* = 6; right side of graph). Percent contraction over 40 hours
804 was determined, data presented as mean +/- SEM. (*D*) Collagen gels were prepared
805 with either Scrambled control of IFT88 KD ASMC in basal media alone (*n*=12), or
806 supplemented with either sHA (*n*=6), or HMW HA (*n*=9; left side of graph) and allowed to
807 contract for 40 hours. Percent contraction was determined at 40 hours, data presented
808 as mean +/- SEM.

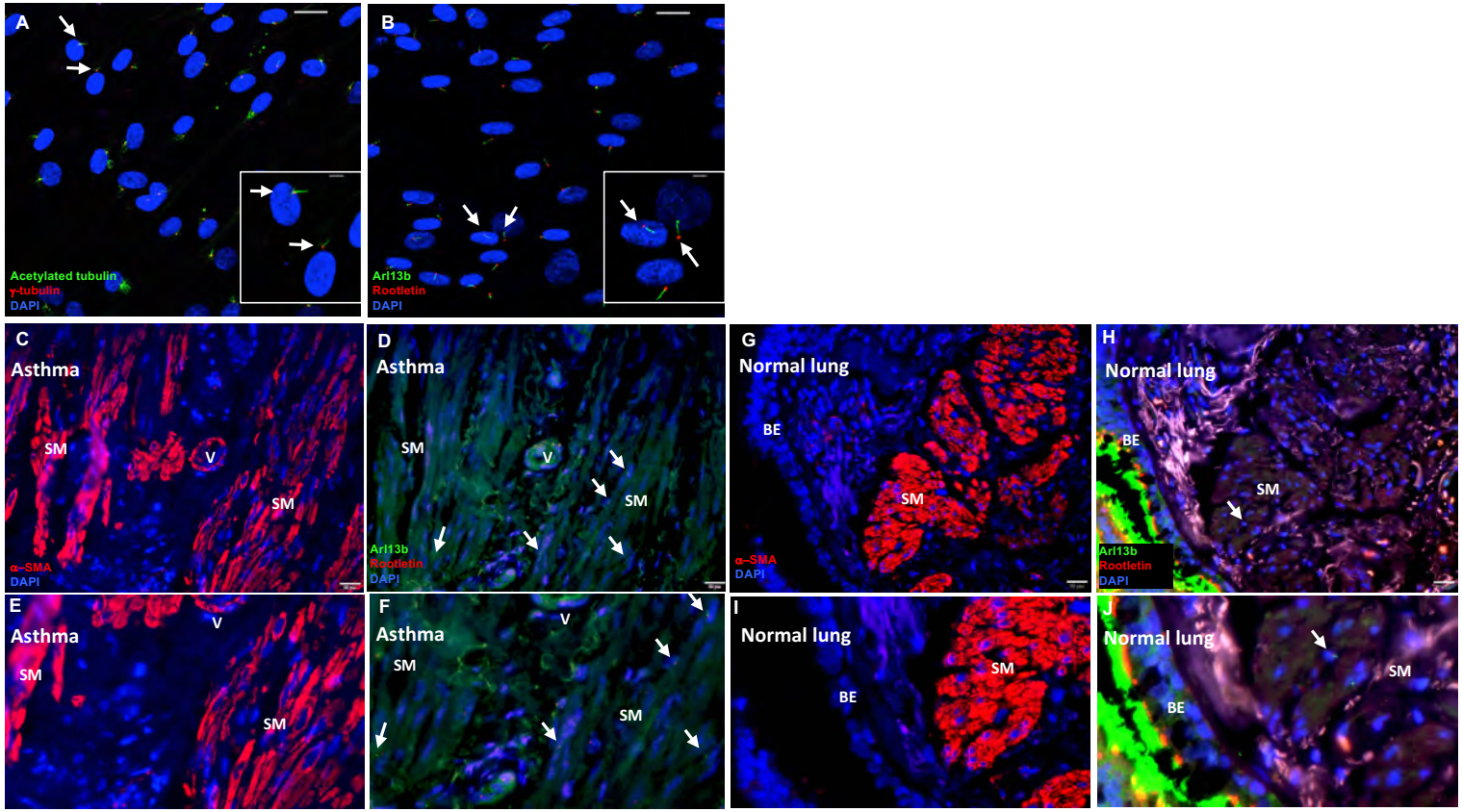
**Figure 6. Hyaluronan effects on calcium flux are mediated by PC.** (*A*) ASMC were grown on 25 mm cover slips in 6-well dishes to confluence, serum starved, then fixed cells stained with antibodies to Arl13b (green) and rootletin (red). Scale bar = 20 μm. (*B* and *C*) Membrane depolarization was measured in: (*B*) ASMC in basal media or basal media plus sHA, basal media plus DMSO vehicle control or DMSO + sHA, or basal media plus HPI-4 or HPI-4 plus sHA; (*C*) Scrambled control or IFT88 KD ASMC with and without sHA. (*D* and *E*) Changes in the ratio of calcium-bound to calcium-unbound Fura-2AM were used to determine intracellular calcium flux in: (*D*) ASMC prepared in basal media plus DMSO or DMSO + sHA, compared to HPI-4 or HPI-4 plus sHA; (*E*) Scrambled control or IFT88 ASMC with and without sHA. Data presented as mean +/- SEM.

**Table 1. Dose response of HPI-4 and Vismodegib on ASMC in the collagen gel contraction assay**

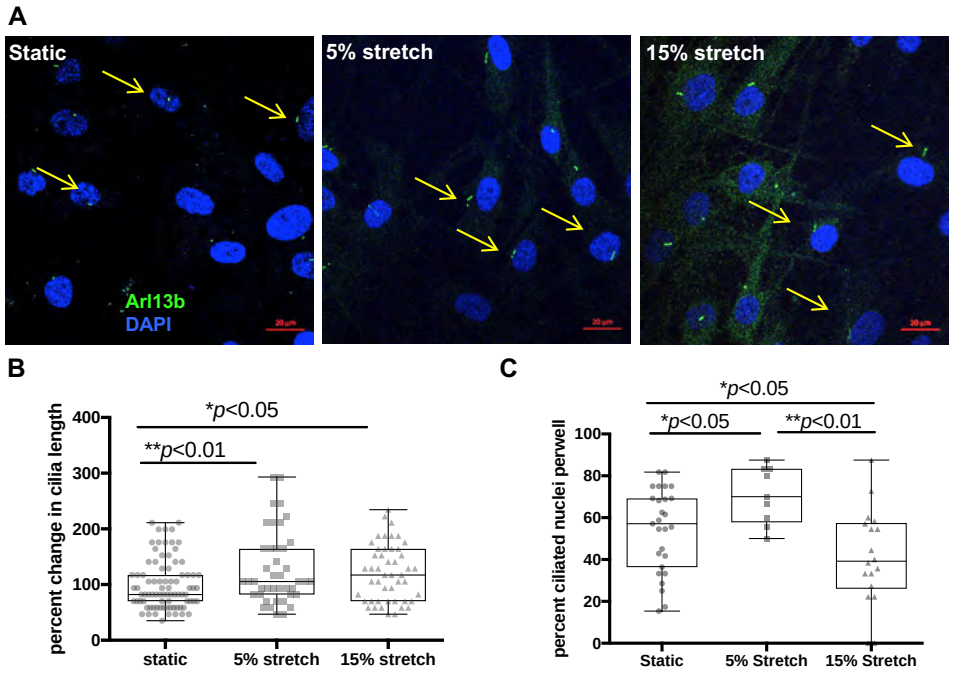
| **Drug** | **% contraction at 24 hr (+/- SEM)** | **% contraction at 40 hr (+/- SEM)** | ****percent contraction 0-24 hours** | ****percent contraction 0-40 hours** |
|---|---|---|---|---|
| DMSO control | 60.896 +/- 2.322 | 65.595 +/- 2.464 | | |
| HPI-4 5 μM | 58.033 +/- 2.550 | 56.229 +/- 2.971 | ns | *p<0.05* |
| HPI-4 10 μM | 56.501 +/- 1.521 | 61.011 +/- 1.825 | ns | ns |
| HPI4 15 μM | 41.134 +/- 3.115 | 48.655 +/- 1.769 | *p<0.001* | *p<0.001* |
| | | | | |
| DMSO control | 60.832 +/- 1.525 | 73.952 +/- 1.620 | | |
| Vismodegib 15 μM | 55.219 +/- 0.4298 | 66.982 +/- 1.323 | *p<0.05* | *p<0.01* |
| Vismodegib 30 μM | 49.281 +/- 3.609 | 62.673 +/- 4.078 | *p<0.05* | *p<0.05* |
| Vismodegib 45 μM | 60.465 +/- 2.295 | 68.383 +/- 3.207 | ns | ns |

**Statistical comparisons made using one-way ANOVA, Holm-Sidak post-hoc testing, using Prism GraphPad Prism software, version 7.0b. Comparisons were made between DMSO control and dose groups. Number of gels analyzed per group = 3

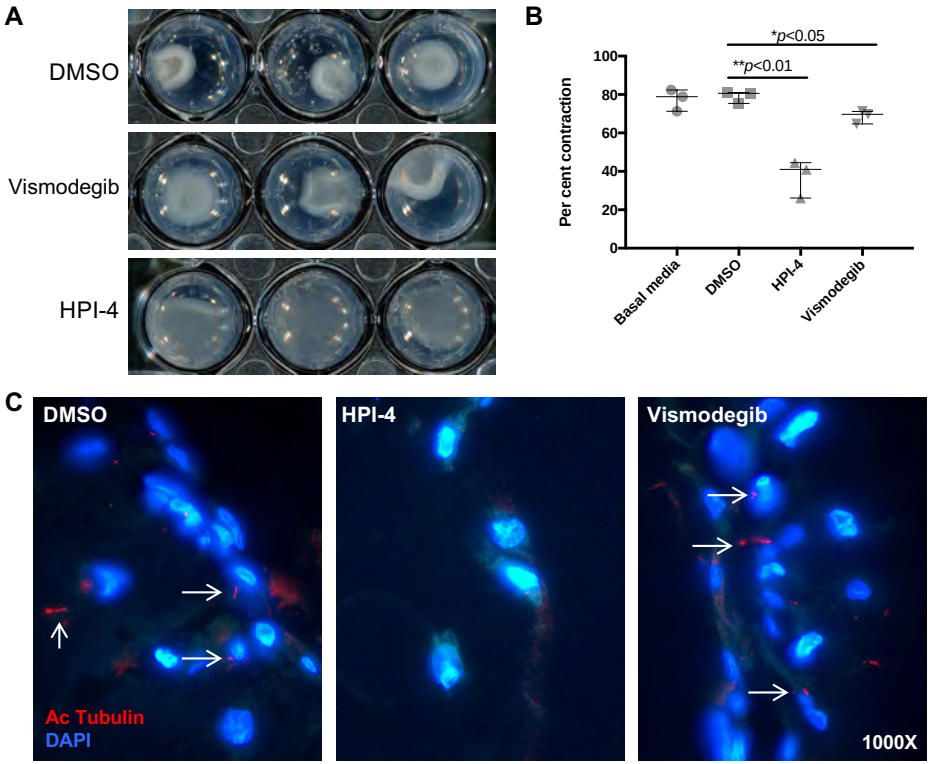
Trempus et al Figure 1



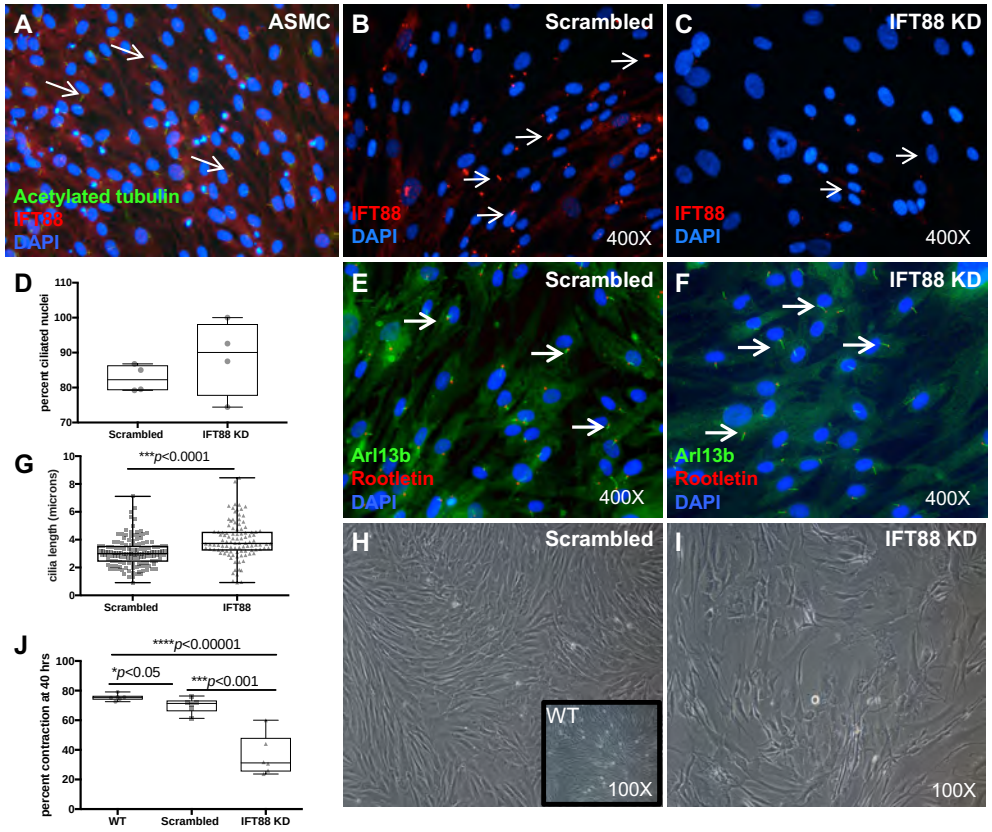
Trempus et al Figure 2



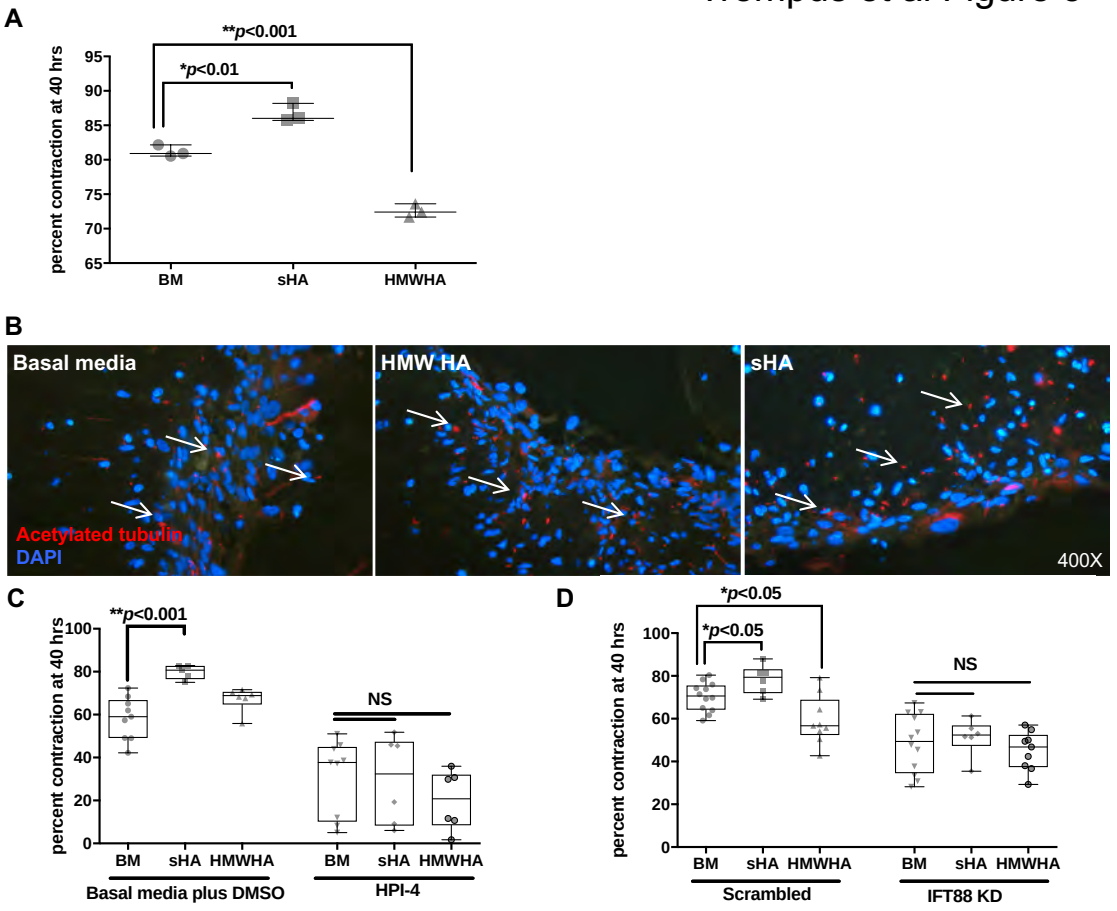
Trempus et al Figure 3



Trempus et al Figure 4



Trempus et al Figure 5



Trempus et al Figure 6