PNAS
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Supplementary Information for

Primary cilia in satellite cells are the mechanical sensors for muscle hypertrophy

Weijun Li[1,4], Zhenhong Zhu[1,5], Kai He[3], Xiaoyu Ma[3], Robert Pignolo[1], Gary C. Sieck[2], Jinghua Hu[3]*, Haitao Wang[1]*

1. Division of Geriatric Medicine & Gerontology, Departments of Internal Medicine, Department of Physiology and Biomedical Engineering, Mayo Clinic, Rochester, Minnesota, USA

2. Department of Physiology and Biomedical Engineering, Mayo Clinic, Rochester, Minnesota, USA

3. Department of Biochemistry and Molecular Biology, Division of Nephrology and Hypertension, Mayo Clinic, Rochester, MN, USA

4. Department of Orthopedic Surgery, the Second Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, Zhejiang Province, China

5. Orthopaedics department, Shanghai Sixth People's Hospital, Shanghai Jiaotong University, Shanghai, China

* Correspondence to:

Haitao Wang, Ph. D

Email: Wang.Haitao@mayo.edu

Jinghua Hu, Ph.D

Email:Hu.jinghua@mayo.edu

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Supplementary Materials and Methods

Mechanical Stimulation System

The Juvent 1000 Dynamic Motion Platform was used for mechanical stimulation. Amplitude was delivered at a sinusoidal frequency of 32–37 Hz (displacement frequency), acceleration “g” force of 0.3 g (peak to peak) (+/− 20%), and vertical displacement of ~ 85 μm with a continuous duty cycle. Myoblasts were stimulated by mechanical stimulation for 10 min at room temperature and then incubated for 24 hrs at 37°C prior to collection of RNA.

Cyclic Mechanical Stretch Platform

Cells were stretched with 10% magnitude at 0.25 Hz frequency, one h/day. The system was described and validated in detecting the mechanical stretching of cells (1, 2). Myoblasts were seeded on collagen type Iα-coated elastic silicon membrane (Flexcell). A stretch of 10% magnitude was used to closely mimic the mechanical strain induced by moderate exercise without provoking cell injury (3).

Immunofluorescence Staining.

Myotubes and frozen muscle sections were fixed with 4% PFA for 15 min at room temperature and then permeabilized with 0.2% Triton X-100 in PBS for 10 min. Cells were blocked with 4% BSA in PBS for one h and incubated with mouse anti-MyHC (ABCAM, Cambridge, MA), rabbit anti-laminin (Sigma, St. Louis, MO), ARL13B (17711-1-AP, Proteintech, Rosemont), Glutamylated tubulin GT335 (AG-20B-0020-C100, Adipogen, San Diego), IFT88 (13967-1-AP, Proteintech, Rosemont, IL), or GLI-3 (AF3690, R&D Systems, Minneapolis), overnight at 4°C

and goat anti-mouse Alexa Fluor 488 and Goat-anti-rabbit Alex Fluor 555 at room temperature for one h. Cells were counterstained with DAPI. Stained cells were photographed and analyzed using an inverted fluorescent microscope (Nikon Eclipse TE 2000-U), a digital camera (Digital Sight DS-Fi1), and Elements BR 3.00 software (Nikon, Melville, NY). An average of 300 cilia was measured in each group (5 cell lines for each genotype). The percentage of $GLI3^+$ cells was calculated from the ratio of $GLI3^+$ cells to total ciliated cells observed in each sample. The length is measured within the ciliated cells (magnification 200X).

Western Blotting

Western blotting was performed by standard procedures as previously described (4). Cultured myoblasts or myotubes were washed with PBS and lysed in RIPA buffer (Sigma, St Louis, MO, USA) with 1x proteinase inhibitor cocktail (Sigma, St Louis, MO, USA). Antibody against GAPDH (Santa Cruz Biotech, Dallas, TX), ARL3 (ABCAM, Cambridge, MA), and IFT88 (13967-1-AP, Proteintech, Rosemont, IL) were all used at dilutions of 1:1000. Appropriate anti-mouse or anti-rabbit HRP-conjugated secondary antibodies (Santa Cruz Biotech, Dallas, TX) were used at a dilution of 1:5000.

RNA Isolation And QRT–PCR.

Total RNA was isolated from cultured myoblast or skeletal muscle tissues of mice using the RNeasy Mini Kit (Qiagen, Germantown, MD). The quantity of RNA was analyzed using NanoDrop instrumentation (NanoDrop Technologies, Wilmington, DE). Purified RNA (1–4 µg) was used to synthesize the first strand of cDNA by reverse transcription system (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The sequence of the primers is presented in Table S1. Quantification of mRNA was done with the SYBR Green method using ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA). All reactions were carried out in duplicate to reduce variation. The data were analyzed using SDS software version 2.0, and the results were exported to Microsoft Excel for further analysis. Data normalization was accomplished using

the endogenous control GAPDH, and the normalized values were subjected to a $2^{-\Delta\Delta Ct}$ formula.

Secreted protein analysis with mass-spectrometry

A portion of the supernatant was collected for Mass-spectrometry of protein and peptides analysis (Dataset S1). Gel fragments subjected to mass-spectrometry were analyzed using standard in-house proteomics software (Mayo Clinic Proteomics Core). The proteins where the total spectrum count (TSC) ratios are four or higher, with at least four peptides, were sorted for analyses, with the exclusive unique peptide counts (EUPC) to human entries.

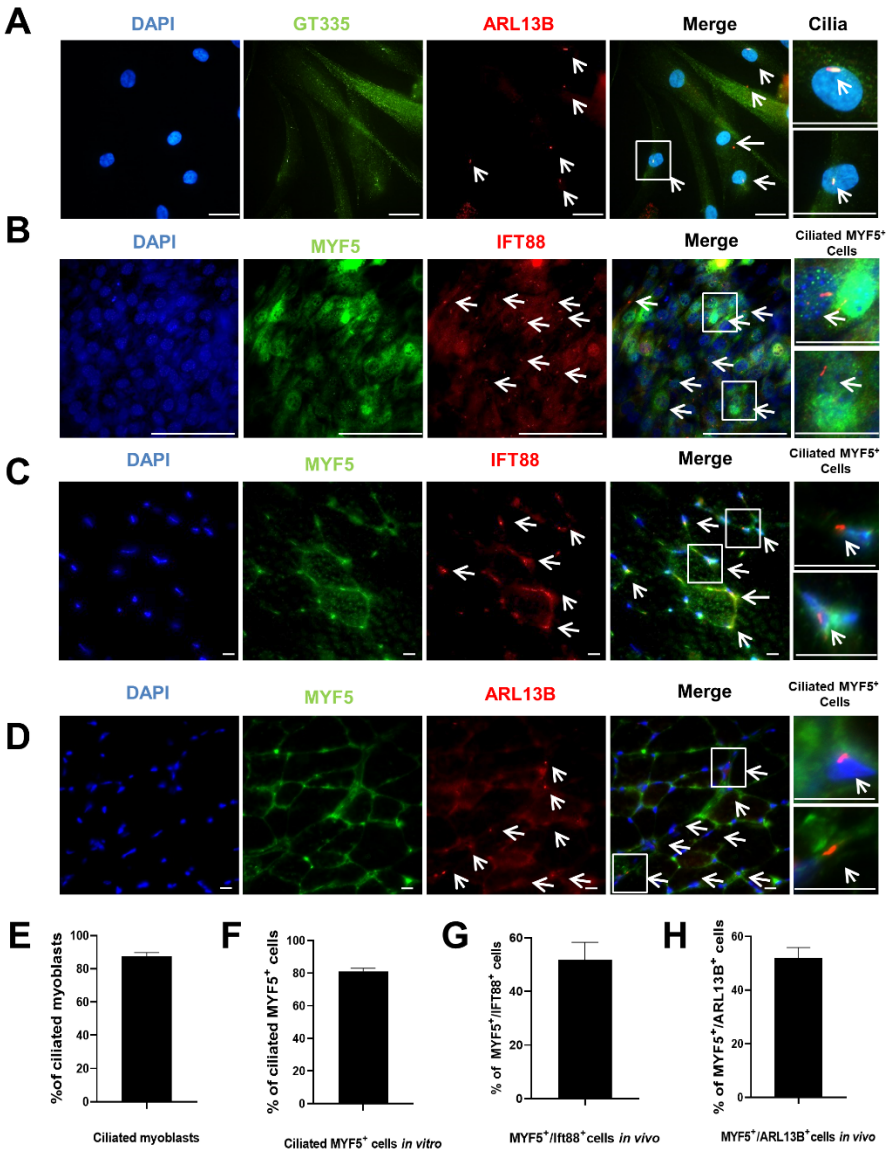

Fig. S1. Ciliated MYF5 positive cells *in vitro* and *in vivo*.

A. Primary cilia were identified by staining for glutamylated tubulin (GT335) (green), and the ciliary membrane marker ARL13B (red) and counterstained with DAPI (blue). Merged GT335, ARL13B, and DAPI indicate the ciliated cells (white arrows) B. MYF5 (green) and IFT88 (red) staining with DAPI counterstain (blue) in myoblasts. Merged MYF5, IFT88, and DAPI indicate the ciliated MYF5-positive cells (white arrows). C. MYF5 (green) and IFT88 (red) staining with DAPI counterstain (blue) in tissue slides from WT mouse tibialis anterior. Merged MYF5, IFT88, and DAPI indicate the ciliated MYF5- positive cells (white arrows). D. MYF5 (green) and ARL13B (red) staining with DAPI counterstain (blue) in slide from WT mouse tibialis anterior. Merged MYF5, ARL13B, and DAPI indicate the ciliated MYF5- positive cells (white arrows). Percentage of ciliated MYF5 positive cells in WT mouse tibialis anterior. E, F, G, and H. Percentage of A, B, C, and D, respectively. Scale bars indicate 10μm. An average of 300 cilia was measured in each group of E, F, G, and H.

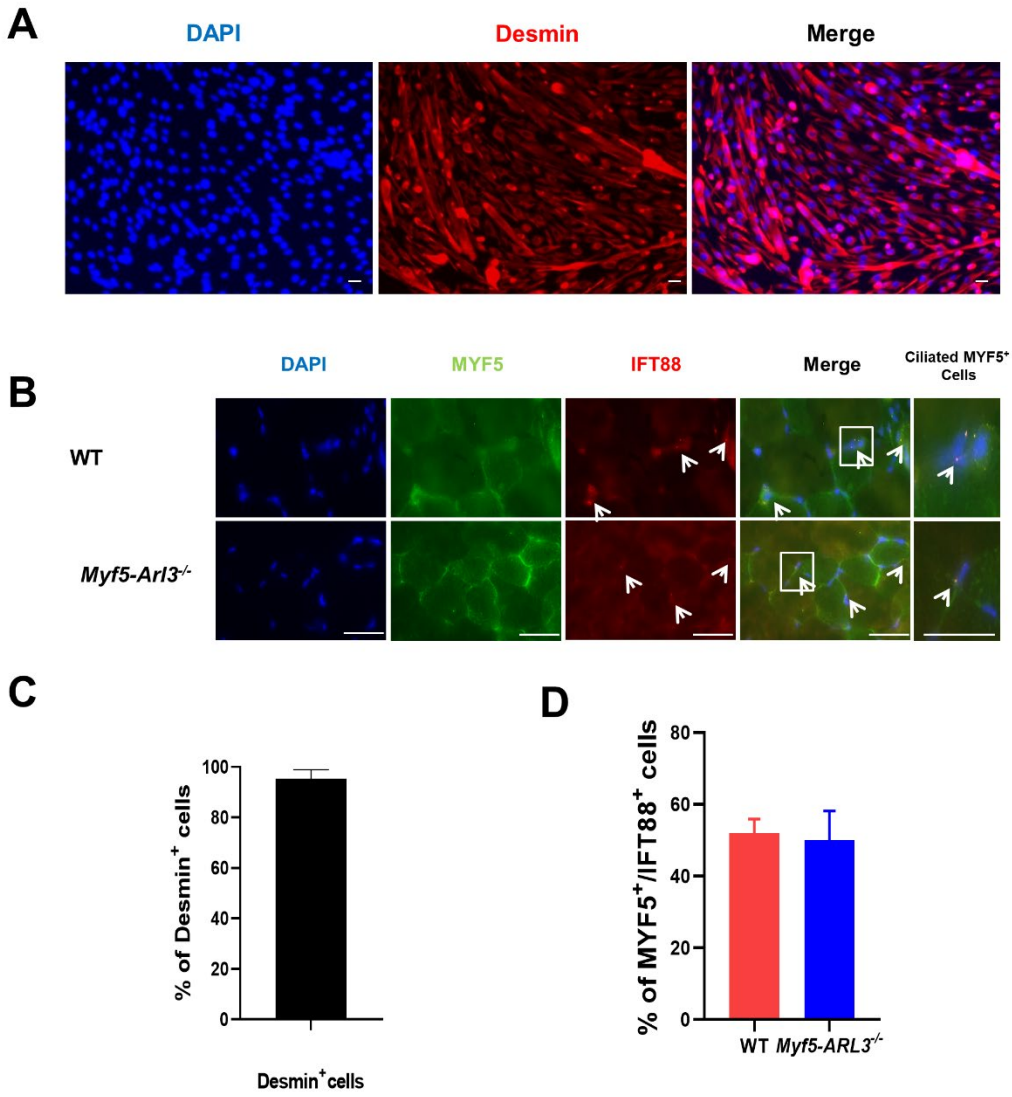

Fig. S2. The isolated myoblasts are dominantly desmin-positive cells, and the percentage of ciliated myoblasts was not changed between WT and *Myf5-Arl3*$^{-/-}$.

A.The isolated cells were identified by staining for a myoblast marker, desmin (red), and counterstained with DAPI (blue). B. MYF5 (green) and IFT88 (red) staining with DAPI counterstain (blue) in tissue slides from WT or *Myf5-Arl3*$^{-/-}$ mouse tibialis anterior. Merged MYF5, IFT88, and DAPI indicate the ciliated MYF5- positive cells (white arrows). C. The percentage of desmin-positive cells in isolated cells. D. The Percentage of ciliated MYF5 positive cells in WT and *Myf5-Arl3*$^{-/-}$ mouse tibialis anterior. Scale bars indicate 10μm. An average of 300 cilia was measured in each group of D.

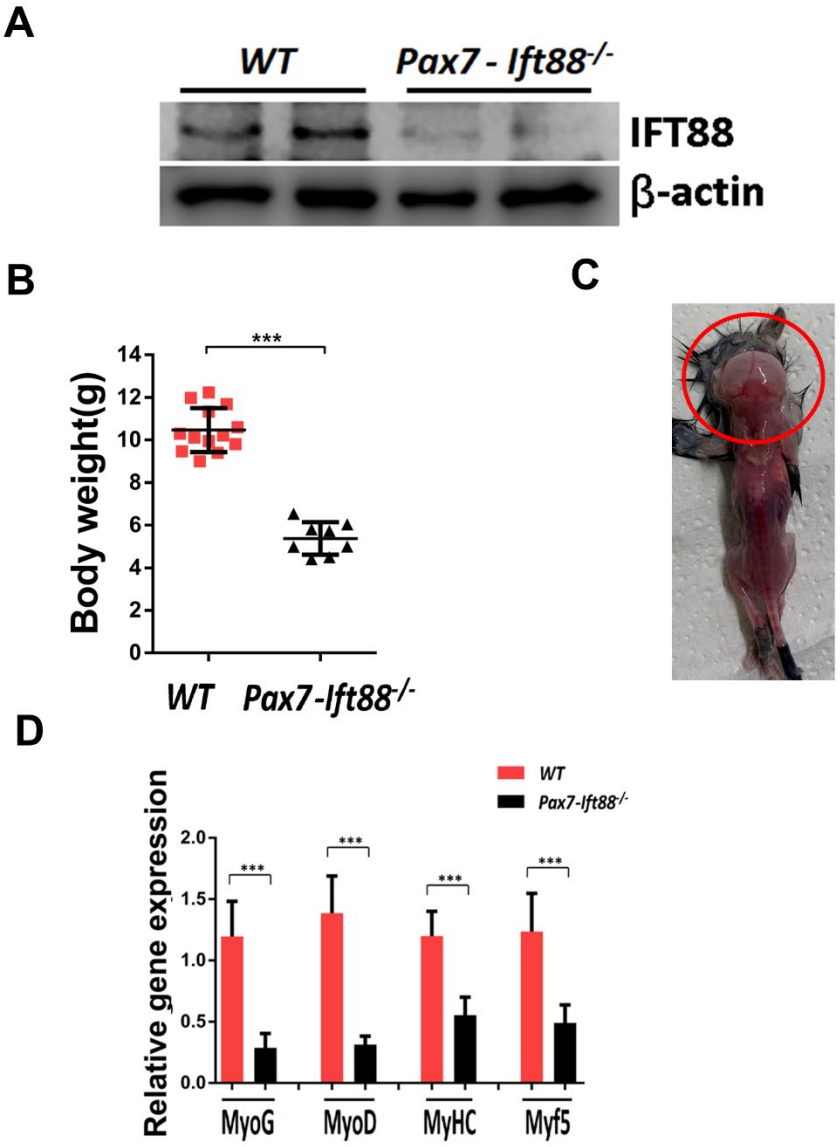

Fig. S3. The characterization of the *Pax7-Ift88*$^{-/-}$ mouse model.

A. The IFT88 protein was successfully abolished in *Pax7-Ift88*$^{-/-}$ myoblasts. B. The bodyweight of *Pax7-Ift88*$^{-/-}$ mice is decreased compared with WT mice at the age of 2 weeks. C. Hydrocephalus was developed around the age of 2-3 weeks in *Pax7-Ift88*$^{-/-}$ mice (red circle). D. Relative expression of MyoG, MyoD, and MyHC expression decreased in TA from *Pax7-Ift88*$^{-/-}$ mice compared with WT mice at the age of 2 weeks using Real-Time PCR test. Each dot represents the data from one mouse. (One-way ANOVA with Tukey's multiple comparisons tests, ***, $p < 0.001$).

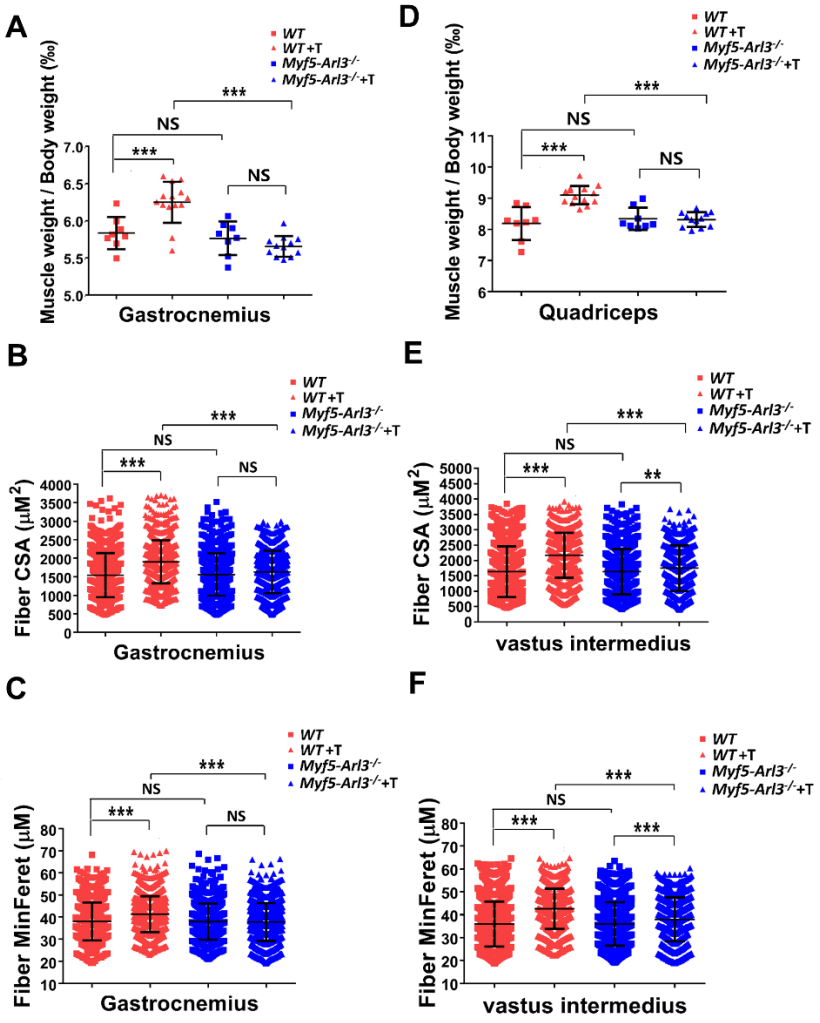

Fig. S4. Dysfunctional primary cilia impair exercise-induced muscle hypertrophy.

A. The weights of gastrocnemius were compared between WT and *Myf5-Arl3*$^{-/-}$ mice before and after treadmill training (T). B. Gastrocnemius muscle fiber cross-sectional area was quantified using Image J and MyoVision software. C. Fiber MinFere was quantified for muscle fibers from the gastrocnemius using Image J and MyoVision software. D. The weights of quadriceps were compared between WT and *Myf5-Arl3*$^{-/-}$ mice before and after treadmill training (T). E. Vastus intermedius muscle fiber cross-sectional area was quantified using Image J and MyoVision software. F. Fiber MinFere was quantified for muscle fibers from the vastus intermedius using Image J and MyoVision software. Each dot represents the data from one mouse. NS, not significant. Scale bars indicate 50 μm. At least 3000 fibers were measured for each sample. (One-way ANOVA with Tukey's multiple comparisons tests, **, $p<0.01$, ***, $p<0.001$, NS; not significant).

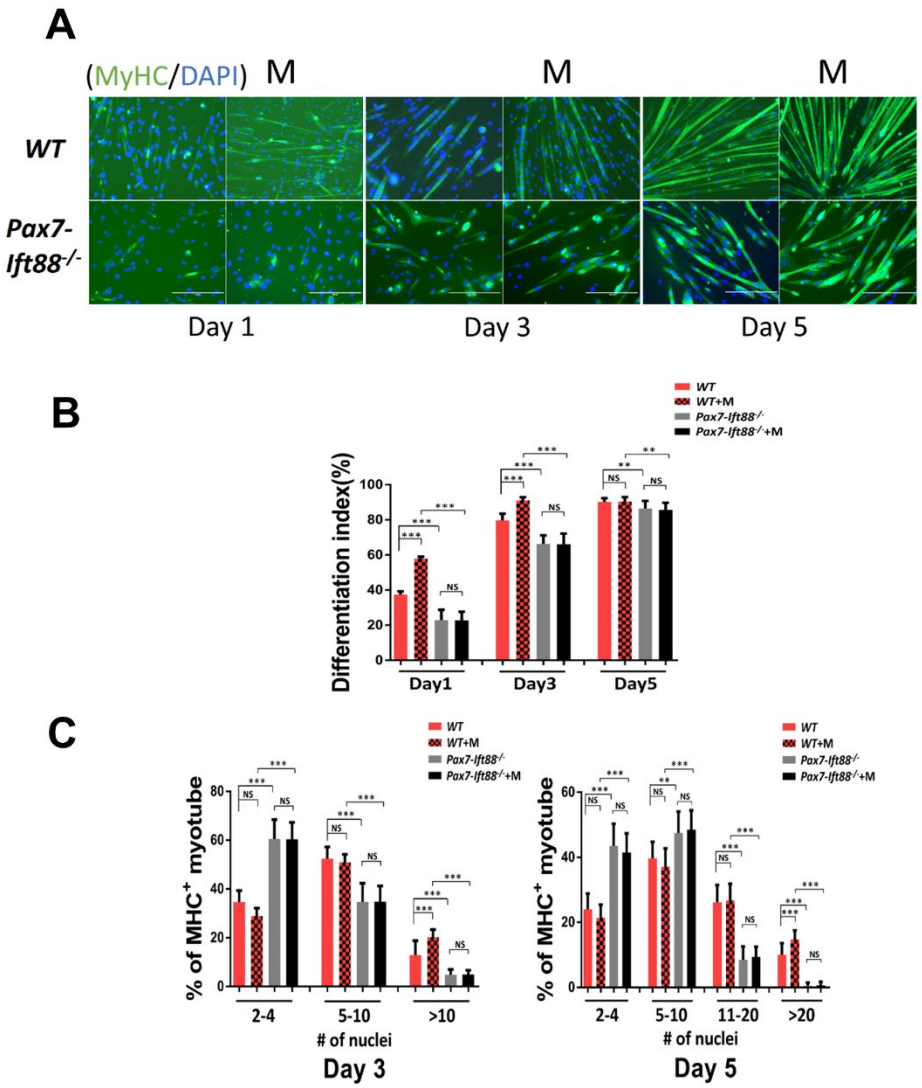

Fig. S5. Primary cilia are the mechanical sensors for myoblast differentiation *in vitro*.

A. MyHC (green) was stained at different days after mechanical stimulation (M) in myoblasts isolated from either WT or *Pax7-Ift88*$^{-/-}$ mice. B. Differentiation index of myotubes (day 1, 3, and 5) were measured after mechanical stimulation in myoblasts. C. Fusion index of myotubes (day 3 and 5) was measured after mechanical stimulation in myoblasts. Each sample represents the data from 1000 myoblasts. Scale bars: 100 μm. (One-way ANOVA with Tukey's multiple comparisons tests, **, $p<0.01$, ***, $p<0.001$, NS; not significant).

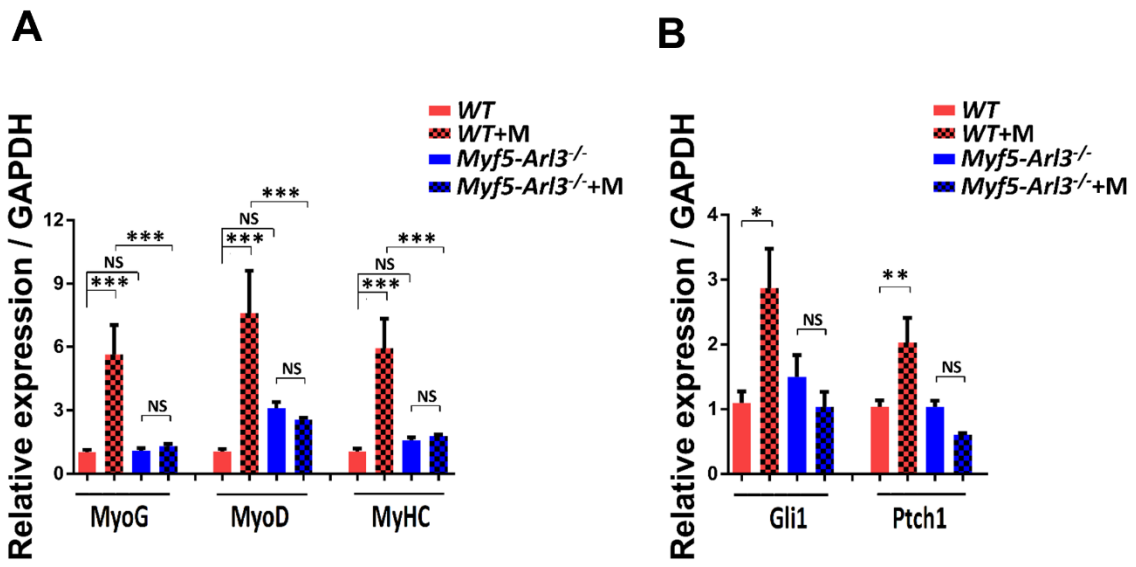

Fig. S6. The induced expression of myogenic gene markers by mechanical stimulation in serum-free medium.

A. Relative expression of *MyoG*, *MyoD*, and *MyHC* in *Myf5-Arl3*$^{-/-}$ myoblasts after mechanical stimulation (M) *in vitro* using Real-Time PCR. B. Relative expression of *Gli1* and *Ptch1* in myoblasts derived from WT and *Myf5-Arl3*$^{-/-}$ mice after mechanical stimulation (M) in vitro using Real-Time PCR. (One-way ANOVA with Tukey's multiple comparisons tests, *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$, NS; not significant).

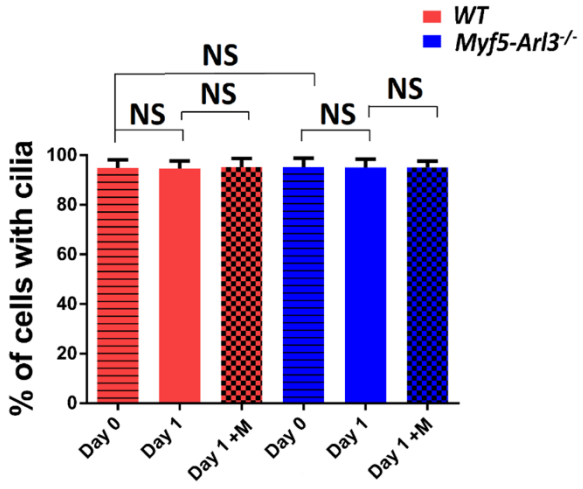

Fig.S7. The percentage of ciliated myoblasts was not changed with mechanical stimulation.

The percentage of ciliated myoblasts derived from WT and Myf5-Arl3$^{-/-}$ mice were counted after mechanical stimulation. (One-way ANOVA test, NS; not significant). An average of 300 cilia was measured in each group (5 cell lines for each genotype).

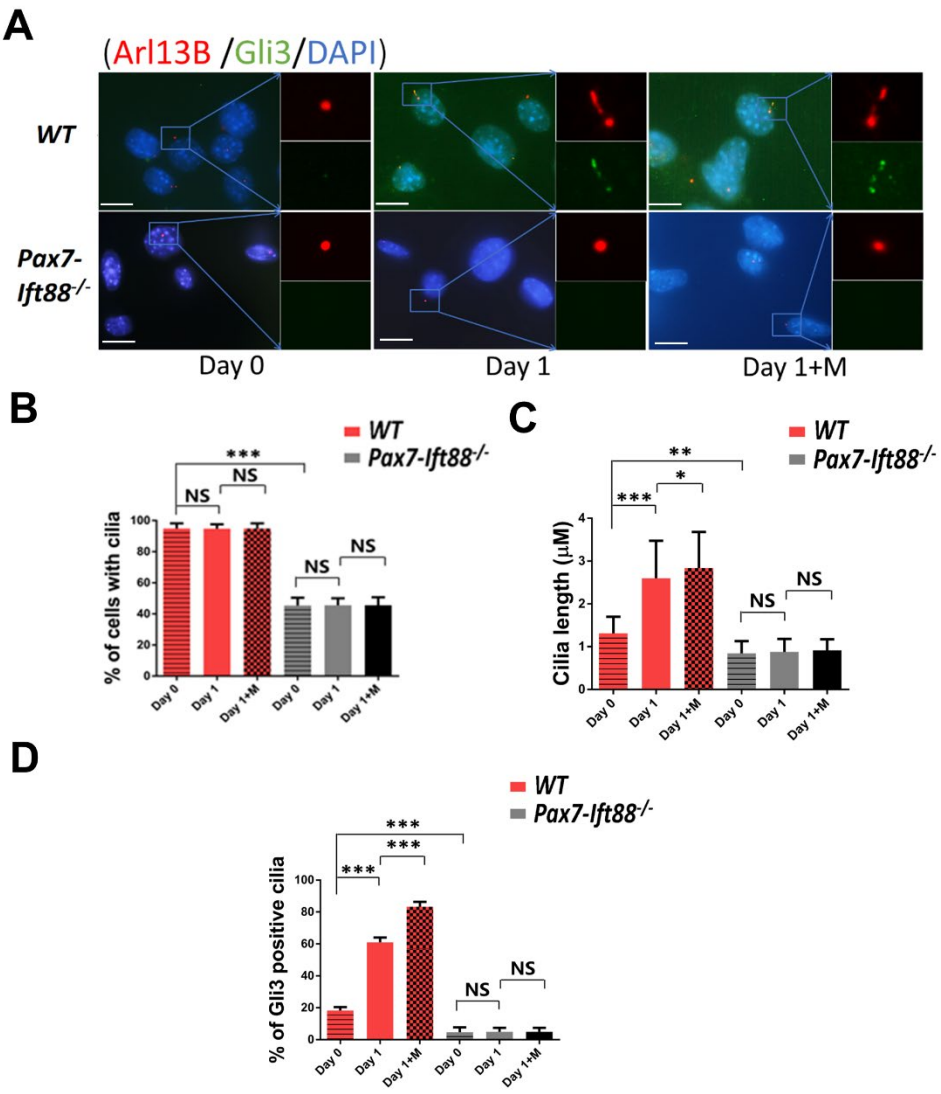

Fig. S8. The activation of the Hh signaling pathway by mechanical stimulation depends on functional primary cilia.

A. GLI3 translocates to cilia after mechanical stimulation in WT but not *Pax7-Ift88*$^{-/-}$ myoblasts. Immunofluorescence staining of cilia with ARL13B (red) and GLI3 (green) and counterstained with DAPI (blue). Scale bars: 5μm. B. The percentage of ciliated myoblasts decreased in *Pax7-Ift88*$^{-/-}$ myoblasts. C. The length of cilia was measured using a Nikon ECLIPSE Ti with MetaMorph software. D. Quantification of A. The numerator is GLI3 positive cilia, and the denominator is total ciliated cells per low power field (magnification 200X). Scale bars: 5μm. An average of 300 cilia was measured in each group of B, C, and D (5 cell lines for each genotype). (One-way ANOVA with Tukey's multiple comparisons tests, *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$, NS; not significant).

Table S1

The following forward (F) and reverse (R) primers were used:

Gene Name (F/R)	Sequences (5'-3')
GAPDH F	TGAAGGTCGGTGTGAACGGATTTGGC
GAPDH R	CATGTAGGCCATGAGGTCCACCAC
MyoD F	CTGCTCTGATGGCATGATGGAT
MyoD R	ACTGTAGTAGGCGGTGTCGT
MyoG F	CAACCAGGAGGAGCGCGATCTCCG
MyoG R	GCTGGGTGTTAGCCTTATGTGAATGG
MyHC F	AAGTTGCATCCCTAAAGGCAG
MyHC R	TCCACCACAAACACCGATGA
Gli1 F	GGTGCTGCCTATAGCCAGTGTCCTC
Gli1 R	GTGCCAATCCGGTGGAGTCAGACCC
Ptch1 F	CTCTGGAGCAGATTTCCAAGG
Ptch1 R	TGCCGCAGTTCTTTTGAATG

Dataset S1

Secreted protein analysis with mass-spectrometry from a portion of supernatant under mechanical stimulation (M) and non-stimulation (CON) cells.

SI References

1. R. W. Stroetz, N. E. Vlahakis, B. J. Walters, M. A. Schroeder, R. D. Hubmayr, Validation of a new live cell strain system: characterization of plasma membrane stress failure. *J Appl Physiol (1985)* **90**, 2361-2370 (2001).
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4. H. Wang *et al.*, R-Spondin 1 promotes vibration-induced bone formation in mouse models of osteoporosis. *J Mol Med (Berl)* **91**, 1421-1429 (2013).