**SUPPORTING INFORMATION APPENDIX**

# Arhgap36-dependent activation of Gli transcription factors

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## MATERIALS AND METHODS

### Statistics

All experimental samples represents biological replicates, and all statistical analyses utilized standard two-sided *t*-tests to determine the indicated *p* values.

### Reagents

Shh-EGFP (1, 2) and Shh-LIGHT2 cells (3) have been previously described. *Smo*$^{-/-}$ MEFs (4) were provided by P. Beachy, and *Sufu*$^{-/-}$ MEFs (5) were provided by R. Toftgård. *Gli2*$^{-/-}$ and *Gli3*$^{-/-}$ MEFs (6) were provided by W. Bushman. *Kif3a*$^{-/-}$ and *Ift88*$^{-/-}$ MEFs were provided by J. Reiter (7, 8). NIH-3T3, C3H10T1/2, and HEK-293T cells were purchased from the American Type Culture Collection.

The human ORFeome collection (v5.1) was purchased from Open Biosystems; pcDNA3.1/nV5-DEST, pcDNA3.2/V5-DEST, pUC19, and pDONR207 from Invitrogen; pCL-ECO from Imgenex; phRL-SV40 from Promega; p3xFLAG-CMV-14 and p3xFLAG-CMV-26 from Sigma; pSP64Ts from Addgene; and pEGFP-C1 from Clontech. Murine *Arhgap6* cDNA and *ARHGAP36* (isoform 2) were purchased from OpenBiosystems (pENTR223.1 vector; Clone ID: 100015404 and MGC Clone: BC063790.1). Human *ARHGAP36* (isoform 1) was purchased from Origene (RC211571) and used as the template to generate constructs of isoforms 4 and 5. pBMN-I-GFP was provided by G. Nolan; pRSET-mCherry was provided by R. Tsien; pDONR223 was provided by J. Hartley and D. Esposito; pCS2+-EGFP-DEST (9) was provided by V. Pasque; pMT-Rev$^{AB}$neo (10) was provided by M. Scott; and the EGFP-Sufu plasmid (11) was provided by A. Liu; The Gli-dependent firefly luciferase reporter (p8xGliBS-FL) and

pEGFP-C1-derived *Smo-Myc₃* and *SmoM2-Myc₃* expression vectors have been previously described (3); an analogous *Sufu-HA* expression construct was generated by amplifying *Sufu* cDNA using the primers listed in Table S2, digesting the PCR product with NheI/HindIII, and ligating it into the pEGFP-C1-derived vector. pSP64Ts-dnPKA was generated by amplifying cDNA encoding the *PKAR1a* AB mutant subunit (12) from the pMT-Rev$^{AB}$neo plasmid with the primer listed on Table S2, digesting with BamH1/Xba1 ligating into pSP64Ts (Addgene) (13) digested with BglII/SpeI. The 3xFLAG-Gli2 expression plasmid was generated as previously described by cloning murine *Gli2* into the p3xFLAG-Myc-CMV-26 vector (Sigma) (2) The 3xFLAG-Gli3 and 3xFLAG-Gli3R expression plasmid were generated by amplifying full length murine *Gli3* and a construct harboring amino acids 1-699 of murine *Gli3* respectively, using the primers and templates on Table S2. These products were digested with Not1/Kpn1 and ligated into p3xFLAG-Myc-CMV-26. Wildtype and mutant murine *Arhgap36* and murine *Kif3a* were obtained by PCR using the primers and templates listed in Table S2. For Gateway recombination-mediated cloning, the PCR products were amplified further with the primers 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTCA -3' and 5'-GGGGACCACTTT GTACAAGAAAGCTGGGTC-3' to add attB adapter sequences. The clones were then transferred into pDONR223 or pDONR207 in a BP recombination reaction using BP Clonase II (Invitrogen). pDONR223 or pDONR207 entry constructs were then transferred to either pcDNA3.1/nV5-DEST, pcDNA3.2/V5-DEST, pBMN-3xFLAG-IRES-mCherry-DEST, or pBMN-mCherry-DEST or pCS2+-EGFP-DEST vectors using LR Clonase II (Invitrogen) according to the manufacturer's protocols. All PCR products were generated with Phusion polymerase (New England Biolabs) and all plasmids were sequence-verified. siRNA reagent

sources are listed in Table S3. Antibody sources and working dilutions are listed in Table S4. Cyclopamine was provided by Infinity Pharmaceuticals.

**Generation of retroviral sub-libraries of the human ORFeome collection**

The pBMN-3xFLAG-IRES-mCherry-DEST vector was generated through the following steps. *mCherry* cDNA was amplified from pRSET-mCherry with the primers listed in Table S2 and the PCR product was cut with PciI/XhoI and ligated into pBMN-I-GFP previously digested with NcoI/SalI to remove the *EGFP* sequence. 5’-Phosphorylated oligonucleotides encoding a 3xFLAG sequence (5’-TCGAGGGGTTCAGGAGACTACAAAGA CCATGACGGTGATTAT AAAGATCATGACATCGACTACAAGGATGACGATGACAAGTAGTGAGCGGCC-3’ and 5’-GGCCGCTCACTACTTGTCATCGTCATCCTTGTAGTCGATGTCATGATCTTTATAAT CACCGTCATGGTCTTTGTAGTCTCCTGAACCCC-3’) were then annealed and cloned into the XhoI/NotI sites of plasmid, which was then cut with XhoI, blunted with Klenow, and ligated with a Gateway RfB recombination cassette (Invitrogen). pBMN-mCherry was generated by excising the *IRES-EGFP* sequence from pBMN-I-GFP via a XhoI/Sal restriction digest and ligating together the vector. This vector was cut with BamHI/EcoRI and a BamHI/EcoRI mCherry fragment excised from pRSET-mCherry was ligated in, resulting in pBMN-mCherry. This vector was cut with BamHI, the ends were digested with Mung Bean Nuclease, and a Gateway RfA recombination cassette was inserted, generating pBMN-mCherry-DEST.

96-well plates containing sub-libraries of the human ORFeome collection (v5.1) were thawed, and 5 µL of the bacterial glycerol stock in each well was transferred into 1 mL of Superior Broth (SB, Athena) with spectinomycin (25 µg/mL) in a 96-well block. The bacteria were cultured for 24 hours at 37 °C with 100-rpm shaking, and the contents of each block were

then combined. DNA was isolated from 10 mL of each mixed bacterial culture using Qiagen miniprep kits according to the manufacturer's protocol, except the DNA was eluted in a 0.5X EB buffer. 50 ng of each DNA preparation was combined with 100 ng of pBMN-3xFLAG-IRES-mCherry-DEST retroviral vector and brought up to 4 μL with Tris-EDTA, pH 8.0 buffer. 1 μL of LR Clonase II (Invitrogen) was added to the reaction, which was incubated for 24 hours at room temperature. 0.5 μL of proteinase K (2 μg/mL; Invitrogen) was then added, and the reaction was incubated at 37 °C for 10 minutes. Each LR Clonase reaction was purified over a Qiagen miniprep column and eluted in 30 μL of 0.5X EB buffer. 5 μL of each purified construct was used to transform 25 μL of MegaX DH10B™ T1R Electrocomp™ cells (Invitrogen), with the addition of 75 μL of 10% glycerol. Each reaction was immediately transferred to 20 mL of SB containing ampicillin (75 μg/mL), and 100 μL of each culture was plated on Luria Broth-ampicillin plates to confirm the presence of at least 2000 colony-forming units per reaction, which corresponds to greater than 20X gene coverage for each pool. The SB-ampicillin cultures were grown overnight at 30 °C, and DNA was isolated from 10 mL of each culture. cDNA pools for hit deconvolution were made in the same manner, using the appropriate subset of wells.

Retroviral medium was generated in the following manner: 12.2 μL of 2 M $CaCl_2$, 1.33 μg of the pBMN-3xFLAG-IRES-mCherry-DEST vector containing a human ORFeome cDNA sub-library, and 0.67 μg of pCL-ECO retrovirus packing vector were diluted into 100 μL of nuclease-free water and added to 100 μL of 2X HBS buffer (280 mM NaCl, 1.5 mM $Na_2HPO_4$, 50 mM HEPES, pH 7.1). After a 1-minute incubation at room temperature, the mixture was added dropwise to one well of a 6-well plate with HEK-293T cells at 30% confluency, cultured in DMEM containing 10% fetal bovine serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. After 12 hours, the medium was replaced with DMEM containing 2%

fetal bovine serum, 8% calf serum, and the antibiotics. Retrovirus-containing supernatant was then collected 48 hours later, passed through a 0.45-µm filter, and either used immediately or stored at -80 °C.

**Statistical validation of the FACS-based Shh-EGFP cell assay**

Shh-EGFP cells were seeded in four 24-well plates at a density of 40,000 cells/well and cultured in DMEM containing 10% calf serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin and cultured to confluency. The Shh-EGFP cells were then treated for 30 hours with DMEM containing 0.5% calf serum and antibiotics (n = 48) or 0.5% calf serum with the addition of ShhN (1:20 dilution) conditioned medium (n = 48) for 30 hours. Shh-EGFP cells were washed with room-temperature PBS, dissociated with 125 µL TrypLE (Invitrogen) for 3-5 minutes at 37 °C, suspended in an additional 125 µL of 2X FACS buffer (PBS containing 2% calf serum), and filtered into round-bottom FACS tubes through a cell strainer (BD Biosciences). Cytometry was performed on a BD LSRII (configured with a 488-nm laser, a 505-nm long pass filter, and a 525/50-nm band pass filter for EGFP detection) and data was collected with FACSDiva software (BD Biosciences) and analyzed using FlowJo (TreeStar). The Z' factor for the FACS-based EGFP cell assay was then calculated as previously described (14).

**FACS-based screen of retroviral cDNA pools**

Shh-EGFP cells were seeded into a 24-well plate at a density of 40,000 cells/well and cultured in DMEM containing 10% calf serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. After 24 hours, the cells were infected with retroviral supernatants containing 4 µg/mL polybrene for 8 hours, the medium was replaced, and the cells were cultured for an

additional 2 days. The Shh-EGFP cells were then treated for 30 hours with DMEM containing 0.5% calf serum and antibiotics.

To ascertain Hh pathway activity and infection rates with single-cell resolution, each well of Shh-EGFP cells were washed with room-temperature PBS, dissociated with 125 μL TrypLE (Invitrogen) for 3-5 minutes at 37° C, suspended in an additional 125 μL of 2X FACS buffer (PBS containing 2% calf serum), and filtered into round-bottom FACS tubes through a cell strainer (BD Biosciences). Fluorescence cytometry was performed on either a BD Digital Vantage (configured with a 488-nm laser, a 505-nm long pass filter, and a 530/30-nm band pass filter for EGFP detection and a 570-595 tunable dye laser, a 600-nm long pass filter, and a 610/20-nm band pass filter for mCherry detection) or a BD LSRII (configured with a 488-nm laser, a 505-nm long pass filter, and a 525/50-nm band pass filter for EGFP detection and a 532-nm laser, a 600-nm long pass filter, and a 610/20-nm band pass filter for mCherry detection). Data was collected with FACSDiva software (BD Biosciences) and analyzed using FlowJo (TreeStar). For screening samples, at least 20,000 mCherry-positive cells were analyzed, and at least 5,000 mCherry-positive cells were analyzed for experiments involving the retroviral transduction of single genes.

In addition to the *ARHGAP36*-containing retroviral pool, a sub-library with *GLI1* increased reporter expression in Shh-EGFP cells. We note that the Hh pathway activators *SHH*, *DHH*, *SMO*, *GRK2*, and *GLI2* are not included in the human ORFeome collection that we screened (v5.1), whereas *IHH* is a library member. We speculate that the non-cell autonomous nature of IHH function could explain why this Hh pathway activator did not score as a hit in our study.

**Cell-based assays of Hh pathway activity**

All *Arhgap36* and *ARHGAP36* constructs used in Hh pathway assays encoded C-terminal V5 tags, except for the *Arhgap36-C* vector and its N-terminal V5 sequence. To evaluate overexpression phenotypes in NIH-3T3 cells, the fibroblasts were seeded into 24-well plates at a density of 35,000 cells/well and cultured in DMEM containing 10% calf serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin for 1 day. Each well was then transfected with 50 µL of OptiMEM containing 95 ng of p8XGliBS-FL, 5 ng of phRL-SV40, 100 ng of pUC19, 200 ng of the designated gene expression construct, unless otherwise indicated, and 1.2 µL of Transit LT-1 reagent (Mirus) according to the manufacturer's protocols. After 1 day, the cells were cultured in 750 µL of fresh medium until they became confluent (2 days) and then treated with DMEM containing 0.5% calf serum, antibiotics, and either various doses of ShhN-conditioned medium, 200 nM SAG, or 5 µM cyclopamine for 30 hours. The cells were then lysed and luciferase activities were measured using a Dual-Luciferase Reporter system (Promega) on a Veritas luminometer.

Overexpression phenotypes in *Smo*$^{-/-}$, *Sufu*$^{-/-}$, *Gli2*$^{-/-}$, *Gli3*$^{-/-}$, and *Kif3a*$^{-/-}$, *Ift88*$^{-/-}$, and *Ift88* wildtype littermate control MEFs were conducted as described for NIH-3T3 cells, except for the following modifications. *Smo*$^{-/-}$ MEFs were cultured in DMEM containing 10% fetal bovine serum, antibiotics, and 1 mM sodium pyruvate. *Sufu*$^{-/-}$ and *Kif3a*$^{-/-}$ MEFs were cultured in the same manner with the addition 10 µg/mL gentamicin or non-essential amino acids (NEAA) supplement (Invitrogen), respectively. *Gli2*$^{-/-}$ and *Gli3*$^{-/-}$ MEFs were cultured in DMEM containing 10% calf serum, antibiotics, and 1 mM sodium pyruvate and NEAA. *Ift88*$^{-/-}$ MEFs were cultured in DMEM containing 15% fetal bovine serum, antibiotics, 1 mM sodium pyruvate and NEAA. For the *Smo* add-back control, each well was transfected with 5 ng of *Smo*-

*Myc*$_3$ cDNA supplemented with 195 ng of *EGFP* cDNA; Hh ligand-independent rescue was achieved with 200 ng/well of *SmoM2-Myc*$_3$ cDNA. For the *Kif3a* add-back control, *Kif3a* cDNA was transfected at the designated amounts with or without 150 ng of *Arhgap36* cDNA as indicated and supplemented with *EGFP* cDNA for a total of 200 ng in all conditions. For the *Sufu* add-back control, 5 ng *Sufu-HA* cDNA was co-transfected with either 195 ng of *EGFP* cDNA, 45 ng of *EGFP* cDNA and 150 ng of *Arhgap36* (WT) cDNA, or 45 ng of *EGFP* cDNA and 150 ng of *Arhgap36 (1-214)* (N) cDNA. For all MEFs other than the *Ift88*$^{-/-}$ line and its wildtype littermate control, 50,000 cells/well were reverse-transfected with the indicated amounts of cDNA constructs with FugeneHD, plated in 24-well plates, grown to confluency in full growth medium, treated with Hh pathway agonists or antagonists as necessary, and assayed for luciferase reporter activity. To evaluate phenotypes in *Ift88*$^{-/-}$ and wildtype control MEFs, cells were seeded in 24-well plates at a density of 50,000 cells/well. Cells were transfected the following day with 200 ng of each of the indicated constructs, using Lipofectamine LTX with Plus Reagent (Invitrogen) as per the manufacturer's instructions. The cells were then grown and assayed for luciferase activity as above.

To evaluate overexpression phenotypes in C3H10T1/2 cells, the pluripotent mesenchymal line was seeded into 96-well plates at a density of 2,500 cells/well and cultured for 24 hours in DMEM containing 10% fetal bovine serum, antibiotics, and 1 mM sodium pyruvate. The cells were then transduced with the designated retroviruses as described above for Shh-EGFP cells. Two days after infection, the cells were treated with DMEM containing 0.5% fetal bovine serum, antibiotics, and 1 mM sodium pyruvate, with or without 5% ShhN-conditioned medium (1:20 dilution) for 48 hours. Each well of cells was treated with 50 μL of lysis buffer (250 mM NaCl, 25 mM $MgCl_2$, 1% Triton X-100, 100 mM Tris-HCl, pH 9.5) for 45 minutes,

and alkaline phosphatase activities were measured using CDP-Star reagent (PerkinElmer) on a Veritas luminometer.

To evaluate knockdown phenotypes C3H10T1/2 cells were reverse-transfected with the designated siRNAs in 96-well plates as follows. For each well, 1.2 μL of INTERFERin (PolyPlus) was diluted in 25 μL of OptiMEM (Invitrogen) and mixed with the siRNA reagent diluted in another 25-μL aliquot of OptiMEM. After 15-20 min 10,000 cells were added to each well for a total volume of 150 μL and a final siRNA concentration of 50 nM. The cells were transfected 4 hours, after which they were cultured in fresh medium for 48 hours. Cells were treated with DMEM containing 0.5% fetal bovine serum and antibiotics, with or without 5% ShhN conditioned medium (1:20 dilution) for 24 hours. After treatment, cells were treated with lysis buffer, and cDNA was prepared using the two-step Cells-to-CT kit (Ambion). qRT-PCR was performed on a Roche Lightcycler 480 II using the following TaqMan probes: *Arhgap36*-Mm01311376_m1, *Gli1*-Mm00494645_m1, *Beta-2-Microglobulin*-Mm00437762_m1 (Applied Biosystems). Gene expression levels were normalized to *beta-2-microglobulin.*

**Zebrafish experiments**

All zebrafish (*Danio rerio*) procedures were performed on embryos obtained from wildtype AB fish, in compliance with protocol 10511 approved by the Institutional Animal Care and Use Committee of the Stanford University School of Medicine. Embryos were randomly selected from each clutch for individual experimental conditions, and the resulting phenotypes were assessed in a non-blinded manner.

To determine the *Arhgap36* overexpression phenotype in zebrafish embryos, pCS2+-Arhgap36-EGFP (derived from Gateway recombination-mediated cloning with pCS2+-EGFP-

DEST) and pSP64Ts-dnPKA vectors were linearized with NotI and XbaI, respectively, and their corresponding *Arhgap36-EGFP* and *dnPKA* transcripts were generated using mMESSAGE mMACHINE SP6 kit (Ambion) according to the manufacturer's protocols. 150 pg of each capped mRNA was microinjected into zebrafish zygotes, and the embryos were fixed at 24 hpf. Expression of the Hh target gene *ptch2* (previously named *ptc1*) was detected by whole-mount *in situ* hybridization with a digoxigenin riboprobe (15) according to standard protocols (15, 16).

TBLASTN searches of the zebrafish genome (Zv9, gene build 75) revealed two genes that encode proteins homologous to Arhgap36, annotated as *zgc:136763* (25% protein sequence identity and 17% similarity) and *arhgap6* (24% identity and 16% similarity). To determine the expression patterns of these genes, total RNA was isolated from a mixture of 10 hpf, 13 hpf, and 22 hpf embryos (approximately 25 embryos of each stage) using the RNAqueous Micro Kit (Ambion) according to the manufacturer's instructions. cDNA was generated using the SuperScript III First-Strand System (Invitrogen), using approximately 400 ng of total RNA. PCR was performed using the Phusion High-Fidelity PCR Master Mix (New England Biolabs), using the following conditions: 98 °C for 30 s; 98 °C for 10 s, 63 °C for 30 s, 72 °C for 35 s; 72 °C for 10 min (35 cycles). A *zgc:136763* fragment was amplified from the cDNA using the following primers: 5'-TAGCCAAACACGCGGAGAAT-3' and 5'-CGtaatacgactcactatagggTGCTGATTG GCTGACAGGAG-3'. An *arhgap6* fragment was similarly amplified using the following primers: 5'-ACAGTGTTCATGACGTGGCT-3' and 5'-CGtaatacgactcactatagggGGACCTCGG AGGATCGTTTC-3'. Using the T7 promoter appended to the reverse primer (lowercase letters), digoxigenin-labeled antisense RNA probes were generated using the MEGAscript T7 Transcription Kit (Invitrogen), substituting the kit-provided nucleotides with DIG-RNA Labeling Mix (Roche). The riboprobes were then used to detect *zgc:136753* and *arhgap6* transcripts by

whole-mount *in situ* hybridization according to standard protocols (15, 16). Expression of *ptch2* at the same developmental stages was also assessed to identify Hh pathway-responsive tissues.

To determine loss-of-function phenotypes for *zgc:136753* and *arhgap6*, splice junction- and translational start site-targeting antisense MOs (Gene Tools) (Table S5) were microinjected into one- to four-cell stage embryos according to standard protocols. The resulting *ptch2* expression levels at 8 and 24 hpf were then assessed by whole-mount *in situ* hybridization as described above. The efficacy of splice-blocking MOs *zgc:136763* MO1 and *arhgap6* MO1 were validated by performing dose titrations and generating cDNA for each condition (20-25 embryos; 500 ng total RNA). PCR amplification of the misspliced products was subsequently conducted using the following primers: *zgc:136763*, 5'-CAGGAAGTGGCCCTTTACCA-3 and 5'-TCTCCAGCTCATTGGGGTGA-3'; *arhgap6*, 5'-TCTCCTGATGCAGTCGGTGT-3' and 5'-CATGAACACTGTGACGGTCG-3'. Misspliced products were also band-purified using the QIAquick Gel Extraction Kit (Qiagen), sequenced, and aligned with reference sequences for *zgc:136763* (NM_001044906.1) and *arhgap6* (XM_005174683.1) using ClustalW2.

**Tissue distribution of *Arhgap36* transcript levels**

TaqMan primers/probes for *Arhgap36* were used to probe the Origene TissueScan Mouse Normal cDNA Array according to the manufacturer's protocols. qRT-PCR was performed on a Roche Lightcycler 480 II using the following TaqMan probes: *Arhgap36*-Mm01311381_g1 and *GAPDH*-Mm99999915_g1 (Applied Biosystems). Gene expression levels were normalized to *GAPDH*. To quantify *Arhgap36* levels in Shh-LIGHT2 and C3H10T1/2 cells, total RNA was isolated using Trizol (Invitrogen), and the corresponding cDNA was prepared using

SuperscriptIII (Invitrogen) according to the manufacturer's protocols. qRT-PCR was then performed as described above.

**Immunofluorescence microscopy**

To quantify Gli2 levels by immunofluorescence, Shh-EGFP cells were seeded into 6-well plates at a density of 100,000 cells/well and infected as above. One day after infection, the cells were trypsinized and seeded into 24-well plates containing poly-D-lysine-coated 12-mm glass coverslips and cultured for 24 hours in DMEM containing 10% calf serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. The cells were then treated with DMEM containing 0.5% calf serum and the antibiotics overnight. Following the overnight treatment, media on the cells was replaced with this low-serum medium in the absence or presence of 5% ShhN-conditioned medium for 1 hour. Cells were then fixed in PBS containing 4% paraformaldehyde for 10 minutes at room temperature, washed 3 x 5 minutes with PBS, fixed in ice-cold methanol for 2 minutes, and washed 2 x 5 minutes with PBS. The cells were permeabilized with PBS containing 0.3% Triton X-100 for 5 minutes and blocked overnight at 4 °C in PBS containing 1% bovine serum albumin, 0.1% Triton X-100, and 0.05% sodium azide. Gli2 and the primary cilia were detected with the antibodies listed in Table S4. Nuclei were stained with DAPI, and the coverslips were rinsed briefly in water and mounted onto slides using Prolong Gold Antifade reagent (Invitrogen). Imaging was performed using a Leica DM4500B microscope equipped with a HCX Plan-Apo 63x/1.4 NA oil-immersion objective, a Retiga-SRV CCD camera (QImaging), and Metamorph software (Molecular Devices). Ciliary Gli2 levels were quantified using ImageJ software (NIH) by determining total pixel intensities within a 4-pixel radius of each ciliary tip

and subtracting background fluorescence in an adjacent region of equivalent size. At least 50 cilia were analyzed for each experimental condition.

To determine the subcellular localization of Arhgap36, Arhgap36-N (1-214), and ARHGAP36 isoforms, NIH-3T3 fibroblasts were seeded into 6-well plates as above and transduced with the appropriate FLAG-tagged retrovirus as described above for Shh-EGFP cells. One day after infection, the cells were trypsinized, and seeded into 24-well plates containing poly-D-lysine-coated 12-mm glass coverslips and cultured for 24 hours in DMEM containing 10% calf serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. The cells were then treated with DMEM containing 0.5% calf serum and the antibiotics for 24 hours, fixed, and blocked in as described for the Gli2 localization experiments. The FLAG-tagged proteins were detected with the primary and secondary antibodies listed in Table S4. Nuclei were stained with DAPI, the coverslips were rinsed briefly in water and mounted onto slides using Prolong Gold Antifade reagent (Invitrogen), and imaging was performed as described above. Image brightness levels were adjusted using Adobe Photoshop in the same manner, except for the micrographs of ARHGAP36 isoform 1. Since the anti-immunofluorescence intensities were uniformly low for this isoform, we increased the brightness levels to accurately depict its subcellular localization.

To determine the effect of the addition of exogenous *Kif3a* on *Kif3a*$^{-/-}$ MEFs, the cells were plated onto poly-D-lysine-coated coverslips as described for the Gli2 and Arhgap36 immunofluorescence experiments and then reverse-transfected with the indicated constructs as described for cell–based assays for Hh activity with the exception that the 50 ng *V5-Kif3a* condition was supplemented with 150 ng of pUC19 instead of *V5-EGFP*. 48 hours post-transfection, the cell media was switched to DMEM containing 0.5% FBS supplemented with 100 U/mL penicillin, and 0.1 mg/mL streptomycin overnight. The cells were fixed and treated as

above, and V5-EGFP, V5-Kif3a and Arl13b were detected using the antibodies and concentrations listed in Table S4. As the immunofluorescence intensity for *V5-Kif3a* transfected cells was lower than that the *V5-EGFP* overexpression control, brightness levels were increased to determine V5-Kif3a subcellular localization.

**Western blot analyses**

To quantify Gli3 processing and Gli1 and Gli2 protein levels, either Shh-EGFP cells or NIH-3T3 cells were seeded into a 24-well plate at a density of 40,000 cells/well and cultured in DMEM containing 10% calf serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. After 24 hours, the cells were infected with the designated retroviruses as described above and cultured for an additional 2 days. The Shh-EGFP or NIH-3T3 cells were then cultured in DMEM containing 0.5% calf serum and antibiotics, with or without 5% ShhN-conditioned medium for 24 hours. ShhN-stimulated cells were also treated with 5 μM cyclopamine or an equivalent amount of DMSO vehicle. The cells were subsequently washed in ice-cold PBS and lysed for 30 minutes at 4 °C in buffer containing 300 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.2 mM PMSF, 10 mM Tris-HCl, pH 7.5, Complete Mini EDTA-free protease inhibitors (Roche), and PhoSTOP phosphatase inhibitors (Roche). The lysates were centrifuged for 10 min at 10,000 *g*, and SDS-PAGE loading buffer was added to a final concentration of 8% glycerol, 2% SDS, 100 mM DTT, 2% β-mercaptoethanol 0.0025% bromophenol blue, and 20 mM Tris-HCl, pH 6.8. The samples were heated to 100 °C for 5 minutes, resolved on 3-8% Criterion XT Tris-acetate polyacrylamide gels (Bio-Rad), and transferred onto PVDF membranes. Gli1, Gli2, Gli3, Sufu and importin β proteins were detected using the antibodies listed in Table S4, SuperSignal West Dura Extended Duration (Gli2, Gli3, importin β, and Sufu) or SuperSignal West Femto

substrates (Gli1) (Pierce), and a ChemiDoc XRS imaging system (Bio-Rad). Band intensities were quantified using Quantity One software (Bio-Rad).

To detect V5-tagged proteins expressed in NIH-3T3 cells, the fibroblasts were seeded into 24-well plates and cultured as described for the Hh signaling assays. Each well was transfected with 400 ng of the appropriate pcDNA3.1/nV5-DEST- or pcDNA3.2/V5-DEST-derived construct, which were generated by Gateway recombination cloning of the corresponding PCR product (see Table S2 and reagents described above). After 2 days the resulting cell lysates were resolved on 10% Criterion XT Bis-Tris polyacrylamide gels and transferred to PDVF membranes. The V5 tag was then detected using the antibodies listed in Table S4, SuperSignal West Dura or Femto reagents, and the ChemiDoc XRS imaging system.

**Co-immunoprecipitation experiments**

For each sample, two wells of a 6-well plate were transfected with 1.3 µg EGFP-Sufu plasmid, 1.3 µg p3xFLAG-CMV-14, and either 4 µg pBMN-Arhgap36-3xFLAG or 4 µg pBMN-ARHGAP26-3xFLAG using calcium phosphate as previously described. The culture medium was replaced the next day and 24 hours later, protein lysates were collected by treating cells with NP-40 Buffer for 30 minutes at 4˚C followed by sonication using a Branson Sonifier. Samples were then clarified by centrifugation at 15,000 *g* for 10 minutes. Protein assays were performed using the Pierce BCA Protein Assay and snap frozen in liquid nitrogen and stored at -80 °C.

20 µL of Protein G Dynabeads (Invitrogen) were washed with three times with NP-40 Buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.3% NP-40) and then incubated with 3 µg of anti-GFP monoclonal antibody (3E6, Invitrogen) for 2 hours at 4˚C. The bead-antibody complexes were washed three times with NP-40 Buffer and then incubated overnight with

400 µg of protein lysates at 4˚C. The beads were again washed three times with NP-40 Buffer, resuspended in 60 µL of SDS-PAGE loading buffer and heated to 100 °C for 10 minutes. Samples were loaded to a 3-8% Tris-Acetate Gel (BioRad) and resolved by electrophoresis. The gels were blotted to PVDF membranes, and FLAG and GFP epitopes were detected using antibodies listed on Table S4. Chemiluminescent detection was performed with SuperSignal West Dura reagent (anti-GFP) or SuperSignal West Femto (anti- DYKDDDDK) and a Chemidoc XRS imaging system.

**Human medulloblastoma microarray and RNA-seq analyses**

Transcriptional profiling data was compiled from previously published series (17-20) and a new series generated at the German Cancer Research Center (DKFZ) (Gene Expression Omibus accession numbers GSE37418, GSE49243, and GSE10327), for a total of 436 medulloblastomas and 18 normal cerebellum samples. This merged dataset was restricted to gene expression data generated on Affymetrix U133 Plus 2.0 arrays. Data was MAS5.0-normalized and each sample assigned to a molecular subtype as previously described (21, 22). *ARHGAP36* (probe ID 238047_at), *GLI1* (probe ID 206646_at), and *PTCH1* (probe ID 209815_at) transcripts were visualized using the GenePattern software suite (http://www.genepattern.org) and the R2 visualization platform (http://r2.amc.nl).

Early access to RNA sequencing data for *ARHGAP36* was provided by the PedBrain Tumor Project/International Cancer Genome Consortium (P. Lichter and S. Pfister; European Genome-Phenome Archive accession number EGAS00001000771). Paired-end RNA-seq libraries were prepared with a ribosomal RNA-depleted RNA fraction, preserving the strand specificity. In brief, 0.2 µg of total RNA was prepared using the RiboZero™ Gold kit

(Epicentre) for removing ribosomal RNA and following the manufacturer's instructions. The resulting RNA was further processed following a previously reported library preparation protocol (23), but by starting at the fragmentation stage of the procedure (second step). Sequencing was carried out on an Illumina HiSeq 2000 system by running 2 x 51 cycles according to the manufacturer's instructions. RNA-seq data was aligned to the RefSeq database using STAR (24) and visualized using IGV (25).

Fresh frozen medulloblastoma and normal cerebellum samples used in these transcriptional profiling and RNA-seq studies were obtained after institutional review board approval and appropriate patient/family consent through the PedBrain Tumor Project/International Cancer Genome Consortium (http://www.pedbraintumor.org).

**Murine medulloblastoma microarray analysis**

Transcriptional profiling data for *Arhgap36* (probe ID 1454660_at) was obtained from a previously published series (26), using the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/geo2r/) interface (GEO accession number: GSE19657; Platform GPL1261).

**Cerebellar granule precursor cells experiments**

To transduce cerebellar granule precursor cells (GNPs) with *Arhgap36* or *ARHGAP36* (*isoform 2*), retroviral vectors were generated in the following manner: 10 µg of the pBMN-3xFLAG-IRES-mCherry-DEST harboring *Arhgap36*, *ARHGAP36*, or a pBMN-mCherry control vector combined with 2.6 µg pLP/VSVG (Invitrogen), 4 µg pCMV-Gag-Pol, 1.7 µg pAdvantage (Clontech) in 500 µL of OptiMEM-I + GlutaMAX-I (Gibco). 30 µL of Lipofectamine 2000

(Invitrogen) was added to 500 µL of OptiMEM-I + GlutaMAX-I and incubated for 5 minutes at room temperature. The DNA/OptiMEM mix was added to the Lipofectamine 2000/OptiMEM mix and incubated for 30 minutes at room temperature to form complexes.

The complexes were then added dropwise to a 10-cm dish of HEK-293T cells seeded the previous day (2 x $10^6$cells) and switched to 5 mL of OptiMEM-I + GlutaMAX-I media. The cells were incubated with the complexes for 4 hours at 37 °C, and the media was then changed to 10 mL of DMEM containing 10% FBS, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 1 mM sodium pyruvate and 1 mM NEAA. Retrovirus-containing supernatant was collected 48 hours post-transfection, followed by a full media replacement, and again at 72 hours post-transfection. The supernatants were centrifuged at 1500 *g* for 10 minutes to remove cell debris, pooled, concentrated using Retro-X (Clontech) as per the manufacturer's directions, and stored at 4°C.

Primary cultures of postnatal day P4 *CD1* mouse cerebellar GNPs were established as described previously(27). Briefly, cerebella were dissected into calcium-free Hank's buffered saline solution (HBSS, Life Technologies), and meninges were removed. Pooled cerebella were treated with trypsin-EDTA and dissociated in HBSS by trituration. Cells were pelleted and resuspended in DMEM-F12 containing 15 mM HEPES, L-glutamine, N2 supplement, 10% fetal bovine serum, 25 mM KCl, and penicillin/streptomycin. The cell suspensions were passed through a 40-µm cell strainer (Falcon) and then plated at a density of $10^6$ cells/well in 24-well plates coated with poly-DL-ornithine. Following 6 hours of incubation at 37 °C in 5.0% $CO_2$, serum-containing medium was replaced with serum-free medium containing 3.0 mg/mL ShhN and allowed to incubate overnight. Cells were then transduced with retrovirus for 48 hours in serum-free medium containing 3 µM cyclopamine.

Total RNA was isolated from GNPs in 750 mL TRIzol (Life Technologies) and purified with the RNeasy Mini Kit (Qiagen). Reverse transcription was performed on isolated RNAs with the High Capacity cDNA Reverse Transcription Kit (Life Technologies). Quantitative reverse transcription-PCR (qRT-PCR) was performed with SYBR Green master mix (Roche) in a LightCycler 480 (Roche). β-Actin was used as a reference gene to calculate $2^{-\Delta\Delta Ct}$. The following primers were used: *Gli1*, 5'-ACAGCGGGGGCAGAAGTCG-3' and 5'-CCTCAGCCCCAGTATCCCCAGTCG-3'; β-Actin, 5'-ATATCGCTGCGCTGGTCGTC-3' and 5'-AGGATGGCGTGAGGGAGAGC-3'.

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**Table S1. Rho GAP family members in the hORFeome collection (v5.1)**

| Gene Symbol | Aliases |
|---|---|
| *ARHGAP1* | *CDC42GAP, RHOGAP, RHOGAP1, p50rhoGAP* |
| *ARHGAP5* | *GFI2, RhoGAP5, p190-B, p190BRhoGAP* |
| *ARHGAP9* | *10C, RGL1* |
| *ARHGAP10* | *GRAF2, PS-GAP, PSGAP* |
| *ARHGAP11A* | |
| *ARHGAP12* | |
| *ARHGAP15* | *BM-024, BM046* |
| *ARHGAP17* | *hCG_1984576, MST066, MST110, MSTP038, MSTP066, MSTP110, NADRIN, PP367, PP4534, RICH1, RICH1B, WBP15* |
| *ARHGAP19* | |
| *ARHGAP20* | *RARHOGAP* |
| *ARHGAP21* | *RP11-324E23.2, ARHGAP10* |
| *ARHGAP22* | *RhoGAP2, RhoGap22* |
| *ARHGAP24* | *FILGAP, RC-GAP72, RCGAP72, p73, p73RhoGAP* |
| *ARHGAP25* | *KAIA0053* |
| *ARHGAP26* | *GRAF, GRAF1, OPHN1L, OPHN1L1* |
| *ARHGAP27* | *hCG_1992862, CAMGAP1, PP905, SH3D20, SH3P20* |
| *ARHGAP28* | |
| *ARHGAP29* | *PARG1, RP11-255E17.1* |
| *ARHGAP31* | *AOS1, CDGAP* |
| *ARHGAP32* | *GC-GAP, GRIT, PX-RICS, RICS, p200RhoGAP, p250GAP* |
| *ARHGAP33* | *NOMA-GAP, SNX26, TCGAP* |
| *ARHGAP36* | *RP13-102H20.1* |
| *ARHGAP44* | *NPC-A-10, RICH2* |
| *CHN1* | *ARHGAP2, CHN, DURS2, NC, RHOGAP2* |
| *FAM13A* | *ARHGAP48, FAM13A1* |
| *GMIP* | *ARHGAP46* |
| *PIK3R2* | *MPPH, P85B, p85, p85-BETA* |
| *PRR5-ARHGAP8* | |
| *RACGAP1* | *CYK4, HsCYK-4, ID-GAP, MgcRacGAP* |
| *RALBP1* | *RIP1, RLIP1, RLIP76* |
| *SH3BP1* | *ARHGAP43* |
| *STARD8* | *ARHGAP38, DLC3, STARTGAP3* |
| *SYDE1* | *7h3* |
| *TAGAP* | *FKSG15, ARHGAP47, IDDM21, TAGAP1* |

**Table S2. PCR primers for cDNA cloning and mutagenesis**

| Gene | Template | Forward | Reverse |
|---|---|---|---|
| *Arhgap36* | Mouse E10-12 cDNA* | CAAAAAAGCAGGCTCAGCCACCATGG CGTGGATGCTGGACTGCCTATTC | CAAGAAAGCTGGGTCAGGAAAGAAGC GGGTGGCAAATTTG |
| *Arhgap36* K283A | pDONR207-Arhgap36 | TTTTCGTGAGATGAAGGACCCTCTGCT GCC** | AACTCCGCGAGGAGCTCAGCCACATC ATG** |
| *Arhgap36* R287E | pDONR207-Arhgap36 | TTTTGAAGAGATGAAGGACCCTCTGCT GCC** | AACTCCTTGAGGAGCTCAGCCACATCA TG** |
| *Arhgap36* K283A R287E | pDONR207-Arhgap36 | TTTTGAAGAGATGAAGGACCCTCTGCT GCC** | AACTCCGCGAGGAGCTCAGCCACATC ATG** |
| *Arhgap36-N* (1-214) | pDONR207-Arhgap36 | CAAAAAAGCAGGCTCAGCCACCATGG CGTGGATGCTGGACTGCCTATTC | GTACAAGAAAGCTGGGTCCTTCCTCTT GGATCCCAGAAGCC |
| *Arhgap36-GAP* (215-414) | pDONR207-Arhgap36 | GTACAAAAAAGCAGGCTCAGCCACCA TGAGCCTCAACCCAATTGCTCAACAAA TC | GTACAAGAAAGCTGGGTCCACCTGGA AGAGGATGTCCCAGTTATC |
| *Arhgap36-C* (415-590) | pDONR207-Arhgap36 | GTACAAAAAAGCAGGCTCACAGGTGC CCCCCCATATTCAGAAG | CAAGAAAGCTGGGTCAGGAAAGAAGC GGGTGGCAAATTTG |
| *ARHGAP36 (isoform 1)* | human cDNA ORF clone (Origene, RC211571) | GGGGACAAGTTTGTACAAAAAAGCAG GCTCAATGGGTGGCTGCATTCCTTTTC TGAAGG | GGGGACCACTTTGTACAAGAAAGCTG GGTCAGGAAAGAAGTAGCTGACGCCA GTTTTGGC |
| *ARHGAP36 (isoform 2)* | MGC Clone: BC063790.1 | GTACAAAAAAGCAGGCTCAGCCACCA TGAGTGTCTTGGGAGGAGCCCCAG | GTACAAGAAAGCTGGGTCAGGAAAGA AGTAGCTGACGCCAG |
| *ARHGAP36 (isoform 4)* | human cDNA ORF clone (Origene, RC211571) | GGGGACCACTTTGTACAAGAAAGCTG GGTCATGGCGTGGATACTGGACTGCCT TTTCGCCTCGGCCTTTGAGCCCCGCCC CCGCCGTGTGAGTGTCTTGGGAGGAGC C | GGGGACCACTTTGTACAAGAAAGCTG GGTCAGGAAAGAAGTAGCTGACGCCA GTTTTGGC |
| *ARHGAP36 (isoform 5)* | human cDNA ORF clone (Origene, RC211571) | GGGGACAAGTTTGTACAAAAAAGCAG GCTCAATGGTATCGATACACAGCCTCT CTGAGC | GGGGACCACTTTGTACAAGAAAGCTG GGTCAGGAAAGAAGTAGCTGACGCCA GTTTTGGC |

| | | | |
|---|---|---|---|
| *mCherry* | pRSET-mCherry | AGTCACATGTTGAGCAAGGGCGAGGA GGATAAC | TCAGCTCGAGTCGACTTACTTGTACAG CTCGTCCATGC |
| *Kif3a* | Mouse E10-12 cDNA* | GTACAAAAAGCAGGCTCAGCCACCCC GATCAATAAGTCGGAGAAGCCGG | GTACAAGAAAGCTGGGTCCTTACTGAA GTAAAGAATCAATTACGGTC |
| *dnPKA* | pMT-Rev$^{AB}$neo | TTATTAGGATCCACCACCATGGCGTCT GGCAGTATGGC | TAATTATCTAGATTATCAGACGGACAG GGACACGAA |
| *Gli3* | Mouse E10-12 cDNA* | GAATGCGGCCGCGGAGGCCCAGGCCC ACAGC | GGGGTACCTCATTTCAGTCTTTGTGTTT G |
| *Gli3R* | Mouse E10-12 cDNA* | GAATGCGGCCGCGGAGGCCCAGGCCC ACAGC | GGGGTACCTTATGGCTTCTCAGCCTTG ACAG |
| *Sufu* | Mouse E10-12 cDNA* | GCCTAGCCACCATGGCGGAAC | CGCAAGCTTAGGCGTAATCTGGCACGT CGTATGGCTACTGCAGTGGACTGTCGA AC |

*Reverse-transcribed from mouse E10-12 polyA+ RNA (Ambion)
**5’ phosphorylated

**Table S3. siRNA reagents**

| Gene | Vendor | Catalog Number | Sequence |
|---|---|---|---|
| *Arhgap36* (#1) | Dharmacon | D-058136-04 | CCACAGUGAUACUCUCGAA |
| *Arhgap36* (#2) | Qiagen | Mm_1100001E04Rik_4 | CTGACAGATGACAACAATTAA |
| *Arhgap36* (#3) | Sigma | SASI_Mm01_00242115 | CUAUCUUGAUGUAAUUCCA[dT][dT] |
| *Arhgap36* (#4) | Sigma | SASI_Mm01_00242116 | GUGUGAAUGUCCAUGAUGU[dT][dT] |
| Non-targeting control (NTC) | Dharmacon | D-001210-03 | Negative control siRNA with at least 4 mismatches to any human, mouse or rat gene. Microarray tested. |
| *Smo* | Dharmacon | M-041026-01 | siGENOME pool |

**Table S4. Antibody reagents**

**Experiment: Arhgap36-3xFLAG and ARHGAP36-3xFLAG localization**

| Reagent | Source | Catalog number | Application | Dilution |
|---|---|---|---|---|
| Mouse monoclonal anti-FLAG | Sigma | F1804 | IF | 1:1000 |
| Goat anti-mouse IgG (H+L)-Alexa Fluor 488 conjugate | Invitrogen | A-11001 | IF | 1:300 |
| Rabbit polyclonal anti-Arl13b | Proteintech | 17711-1-AP | IF | 1:500 |
| Rabbit polyclonal anti-Arl13b | Caspary Lab | | IF | 1:3000 |
| Goat anti-rabbit IgG (H+L)-Alexa Fluor 594 conjugate | Invitrogen | A-11037 | IF | 1:300 |

**Experiment: ARHGAP36-V5 expression levels**

| Reagent | Source | Catalog number | Application | Dilution |
|---|---|---|---|---|
| Mouse monoclonal anti-V5 | Invitrogen | R960-25 | WB | 1:2500 |
| Goat anti-mouse IgG (H+L)-HRP conjugate | Jackson ImmunoResearch | 115-035-174 | WB | 1:5000 |

**Experiment: *V5-Kif3* add-back to *Kif3a* null MEFs**

| Reagent | Source | Catalog number | Application | Dilution |
|---|---|---|---|---|
| Mouse monoclonal anti-V5 | Invitrogen | R960-25 | IF | *** |
| Goat anti-mouse IgG (H+L)-Alexa Fluor 488 conjugate | Invitrogen | A-11001 | IF | 1:300 |
| Rabbit polyclonal anti-Arl13b | Proteintech | 17711-1-AP | IF | 1:500 |
| Goat anti-rabbit IgG (H+L)-Alexa Fluor 594 conjugate | Invitrogen | A-11037 | IF | 1:300 |

**Experiment: Gli2 trafficking**

| Reagent | Source | Catalog number | Application | Dilution |
|---|---|---|---|---|
| Goat polyclonal anti-Gli2 | R&D Systems | AF3635 | IF | 1:50 |
| Donkey polyclonal anti-goat IgG (H+L)-DyLight 488 conjugate | Jackson ImmunoResearch | 715-515-147 | IF | 3 μg/mL |
| Mouse monoclonal anti-acetylated tubulin | Sigma | T7451 | IF | 1:1000 |
| Donkey polyclonal anti-mouse IgG (H+L)-DyLight 594 conjugate | Jackson ImmunoResearch | 715-515-151 | IF | 3 μg/mL |

**Experiments: Gli3 processing and Gli1, Gli2 and Sufu protein levels**

| Reagent | Source | Catalog number | Application | Dilution |
|---|---|---|---|---|
| Goat polyclonal anti-Gli3 | R&D Systems | AF3690 | WB | 0.4 μg/mL |
| Bovine polyclonal anti-goat IgG (H+L)-HRP conjugate | Jackson ImmunoResearch | 805-035-180 | WB | 0.08 μg/mL |
| Rabbit polyclonal anti-Gli1 | Cell Signaling | 2534S | WB | 1:500 |
| Rabbit polyclonal anti-importin β | Santa Cruz Biotechnology | SC-11367 | WB | 1:2000 |
| Donkey polyclonal anti-rabbit IgG (H+L)-HRP conjugate | GE Healthcare | NA934V | WB | 1:5000 |
| Goat polyclonal anti-Gli2 | R&D Systems | AF3635 | WB | 1:1000 |
| Rabbit polyclonal anti-Sufu | Rohatgi Lab | | WB | 1:2000 |

**Experiments: GFP-Sufu and Arhgap36-3xFLAG co-immunoprecipitation**

| Reagent | Source | Catalog number | Application | Dilution |
|---|---|---|---|---|
| Mouse monoclonal $IgG_{2a}$ anti-GFP (clone 3E6) | Invitrogen | A-11120 | IP | N/A |
| Rabbit polyclonal anti-GFP | Abcam | 6556 | WB | 1:4000 |
| Rabbit polyclonal anti-DYKDDDDK | Cell Signaling | 2368 | WB | 1:1000 |
| Donkey polyclonal anti-rabbit IgG (H+L)-HRP conjugate | GE Healthcare | NA934V | WB | 1:10,000 |

**Table S5. Morpholino oligonucleotides**

| MO | Sequence* | Targeted region ** | Dose (per embryo) |
|---|---|---|---|
| *zgc:136763* MO1 | GTGCAGACTCTGTGGA AGAATATAA | Intron 3-Exon 4 | 1 – 12 ng |
| *zgc:136763* MO2 | TCACGCTCTGACCCAA CATGGTGGC | Translational start site of ENSDART00000083189 | 4 ng alone; 2 ng + *zgc:136763* MO3 |
| *zgc:136763* MO3 | CCGATCCCATGTAGAA CAAAACCAT | Translational start site of ENSDART00000130906 | 4 ng alone; 2 ng + *zgc:136763* MO2 |
| *arhgap6* MO1 | GTATGTGTAAGTTGCT GTACCCGTC | Exon 3-Intron 3 | 1 – 12 ng |
| *arhgap6* MO2 | GACTCTGGATGGACAC CGACTGCAT | Translational start site of ENSDART00000128142 | 8 ng |

*Bases complementary to start codons are underlined.

**Intron/exon assignments are based upon the zebrafish genome assembly Zv9, gene build 75 (April 2014).

## SUPPLEMENTARY FIGURES

**Figure S1. Statistical validation of the cDNA overexpression screen.** (*A*) Representative FACS histogram plots of Shh-EGFP cells cultured in the absence or presence of ShhN. (*B*) EGFP fluorescence intensities for each cell culture condition. Data are the average ± SD, N = 48, yielding a coefficient of variation of 4.8% and Z' factor of 0.83.

**Figure S2. Deconvolution of the *ARHGAP36*-containing retroviral sub-library.** (*A*) Human ORFeome plate 31014 was deconvoluted by generating retroviral pools representing each column (1-12) and row (A-H) of the 96-well array. FACS histogram plots for Shh-EGFP cells infected with each pool and cultured in ShhN-free medium for 30 hours are shown. A subset of cells infected with retroviral pools derived from column 1 and row E exhibited increased EGFP expression, indicating that *ARHGAP36* cDNA in well E1 is the active component. (*B*) FACS histogram plots of Shh-EGFP cells retrovirally transduced with either *mCherry* (control) or *ARHGAP36-IRES-mCherry*. The histograms were gated for mCherry fluorescence to select for retrovirus-infected cells. ShhN-stimulated Shh-EGFP cells subjected to forward and side scatter gates were used as a comparison control.

**Figure S3. Evaluation of Arhgap36 homologs as Hh pathway agonists.** FACS histogram plots of Shh-EGFP cells retrovirally transduced with *mCherry*, *Arhgap36*, *ARHGAP36*, and other human Rho GAP genes in the human ORFeome collection (v5.1). The closest *Arhgap36* homolog, *Arhgap6*, was evaluated as well. Each Rho GAP family member was transduced with either an IRES:mCherry reporter (*Arhgap36*) or as a C-terminal mCherry fusion (all other

constructs), and the histograms were gated for mCherry fluorescence to select for retrovirus-infected cells.

**Figure S4. Arhgap36-dependent Hh pathway activation in NIH-3T3 cells.** Hh pathway activities in NIH-3T3 cells co-transfected with Gli-dependent firefly luciferase and SV40-driven *Renilla* luciferase reporters and either *EGFP* or *Arhgap36*. The cells were then cultured with various doses of ShhN-conditioned medium for 30 hours. Data are the average ± SEM, N = 3.

**Figure S5. Tissue distribution of *Arhgap36* expression.** *Arhgap36* transcript levels in individual murine tissues, as determined by real-time quantitative PCR analysis of the OriGene TissueScan$^{TM}$ Mouse Normal cDNA Array.

**Figure S6. Exogenous *Kif3a* rescues ciliogenesis in *Kif3a* null cells.** *Kif3a*$^{-/-}$ MEFs transfected with either *V5-EGFP* (*A*) or *V5-Kif3a* (*B*), using experimental conditions comparable to the 50 ng cDNA transfection in Fig. 2F. Representative immunofluorescence micrographs are shown as shifted overlays with staining for the V5 epitope, Arl13b (primary cilia), and DAPI (nuclei). The inset highlights an individual cilium in *V5-Kif3a*-transfected cells. Scale bar: 10 µm.

**Figure S7. Arhgap36-dependent Hh pathway activation requires Ift88.** Hh pathway activities in MEFs derived from *Ift88*$^{-/-}$ mice or wildtype littermates. The cells were co-transfected with Gli-dependent firefly luciferase and SV40-driven *Renilla* luciferase reporters and the designated cDNAs and then cultured for 30 hours. Data are the average ± SEM, N = 4. Statistical analyses:

double asterisks indicate *P* < 0.01 (*Arhgap36*, *SmoM2,* or *Gli1* versus *EGFP* for each cell culture condition).

**Figure S8. Arhgap36 GAP domain mutants can activate the Hh pathway.** Hh pathway activities in NIH-3T3 cells co-transfected with Gli-dependent firefly luciferase and SV40-driven *Renilla* luciferase reporters and either *EGFP*, wildtype *Arhgap36*, or the indicated *Arhgap36* mutants. The cells were then cultured for 30 hours. *SmoM2* was used to verify the Hh pathway-competence of the cells. Data are the average ± SEM, N = 3. Statistical analyses: double asterisks indicate *P* < 0.01 (wildtype *Arhgap36*, mutant *Arhgap36*, or SmoM2 versus *EGFP*).

**Figure S9. Regulation of Hh pathway components in *Arhgap36*- and *Arhgap36-N*-overexpressing cells.** (*A*) Gli1, Gli2, and Sufu protein levels in NIH-3T3 cells retrovirally transduced with *mCherry*, *Arhgap36*, or *Arhgap36-N* (amino acids 1-214) and then cultured in the absence or presence of ShhN for 24 hours. Representative western blots are shown, and importin β levels were used as gel-loading controls. (*B*) Quantification of Gli1, Gli2, and Sufu protein levels for each experimental condition. Data are the average ± SEM, N = 3 independent western blots. The single and double asterisks indicate *P* < 0.05 and *P* < 0.01, respectively (*Arhgap36* or *Arhgap36-N* versus *mCherry* for each cell culture condition).

**Figure S10. Full-length Arhgap36 is epistatic to its N-terminal domain.** Hh pathway activities in NIH-3T3 cells co-transfected with Gli-dependent firefly luciferase and SV40-driven *Renilla* luciferase reporters and the designated amounts (ng) of *EGFP*, full-length *Arhgap36*, or

*Arhgap36-N* expression vectors. The cells were then cultured in the absence or presence of 200 nM SAG for 30 hours. Data are the average ± SEM, N = 3.

**Figure S11. Subcellular distributions of mouse Arhgap36 and human ARHGAP36 proteins.** (*A*) Localization of FLAG-tagged wildtype Arhgap36 and its N-terminal domain in NIH-3T3 cells. Ciliary localization for the N-terminal domain was observed in 48% of the cells (N = 50). (*B*) Localization of FLAG-tagged ARHGAP36 isoforms. Ciliary localization for isoform 3 was observed in 68% of the cells (N = 50). Representative immunofluorescence micrographs are shown as shifted overlays with staining for the FLAG epitope, Arl13b (primary cilia), and DAPI (nuclei). Insets highlight individual cilia. Scale bars: 20 µm.

**Figure S12. Endogenous *Arhgap36* function in cell-based models of Hh signaling.** (*A*) Relative *Arhgap36* transcript abundance in Shh-LIGHT2 cells (NIH-3T3 cells stably transfected with Gli-dependent firefly luciferase and SV40-driven *Renilla* luciferase reporters), C3H10T1/2 cells, and murine embryos (E10-12) as determined by qRT-PCR. Data are the average ± SEM, N = 3. (*B*) *Arhgap36* transcript levels in C3H10T1/2 cells transfected with siRNAs targeting *Smo* or *Arhgap36* for 48 hours. A non-targeting siRNA was used as a negative control (*NTC*). The cells were then cultured in the absence or presence of ShhN for 24 hours. (*C*) *Gli1* transcript levels in the siRNA-transfected cells. Data are the average ± SEM, N = 3. Statistical analyses: Single and double asterisks indicate $P < 0.05$ and $P < 0.01$, respectively (*A*: C3H10T1/2 cells or murine embryos versus Shh-LIGHT2 cells; *B*: designated siRNA versus *NTC* for each cell culture condition). The triple asterisk indicates $P < 0.01$ and decreased *Gli1* expression (designated siRNA versus *NTC* for each cell culture condition).

**Figure S13. Expression patterns of zebrafish homologs of *Arhgap36*.** TBLASTN searches of the zebrafish genome (Zv9, gene build 75) revealed two genes that encode proteins homologous to Arhgap36, annotated as *zgc:136763* (25% protein sequence identity and 17% similarity) and *arhgap6* (24% identity and 16% similarity). Wildtype expression patterns of *ptch2* (*A*), *zgc:136763* (*B*), and *arhgap6* (*C*) are shown at the 1000-cell (3 hpf), bud (10 hpf), 8-somite (13 hpf), and Prim-5 (24 hpf) stages. Bud and 8-somite stages are shown in dorsal, posterior, anterior, and lateral views; the Prim-5 stage is shown in lateral and dorsal views. Embryo orientations depicted in (*A*) are maintained in (*B*) and (*C*). Scale bars: 200 µm.

**Figure S14. Validation of MOs targeting splice junctions within zebrafish homologs of *Arhgap36*.** (*A*) Dose titration of *zgc:136763* MO1 and its effect on transcript splicing. The wildtype PCR product is observed at 508 bp, and misspliced transcripts yield PCR products A and B. (*B*) Sequence alignments of products A and B against the *zgc:136763* reference sequence reveal 38-bp and 281-bp deletions of exon 4, respectively. (*C*) Sequence chromatogram of Product A comparing the effects of 4-ng and 8-ng doses of *zgc:136763* MO1. The grey line indicates the sequence position shown in (*B*). (*D*) Dose titration of *arhgap6* MO1 and its effect on transcript splicing. The wildtype PCR product is observed at 767 bp. and misspliced transcripts yield PCR products A and B. (*E*) Sequence alignments of products A and B against the *arhgap6* reference sequence reveal retention of intron 3 (producing an early stop codon) and a 210-bp deletion of exon 3, respectively.

**Figure S15. MOs targeting zebrafish homologs of Arhgap36 do not disrupt embryonic Hh signaling.** (*A*) Expression patterns of *ptch2* in *zgc:136763* and *arhgap6* single morphants. 8-

somite (13 hpf) and Prim-5 (24 hpf) stages are shown. (*B*) Expression patterns of *ptch2* in *zgc:136763* and *arhgap6* double morphants at the Prim-5 stage. Splice-blocking *zgc:136763* MO1 and *arhgap6* MO1 were used at doses confirmed to induce transcript missplicing (see Fig. S14), and translation-blocking *zgc:136763* MO2, *zgc:136763* MO3, and *arhgap6* MO2 were used at maximum tolerated doses. Batch (scale bars: 1 mm) and single-embryo (scale bars: 200 μm) views are shown for each experimental condition.

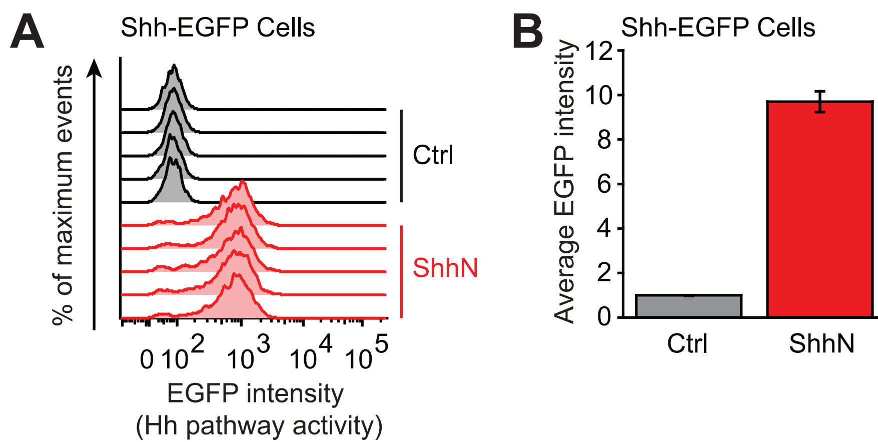
**Figure S16. ARHGAP36 and SHH target gene expression are not correlated in human medulloblastomas at the time of tumor resection.** (A) Comparison of *ARHGAP36* and *PTCH1* transcript levels across Group 3 and 4 medulloblastomas. (B) Comparison of *ARHGAP36* and *GLI1* transcript levels in the same tumors.

**Figure S17. Sequence alignment of full-length murine Arhgap36 and human ARHGAP36 isoforms.** N-terminal, GAP, and C-terminal domains are depicted in blue, green, and orange, respectively. The conserved threonine that replaces the “arginine finger” associated with catalytic GAP activity is boxed.

**Figure S18. RNA-seq analysis of *ARHGAP36* transcripts in medulloblastomas.** Alignments of the paired-end read coverage for three medulloblastoma samples, the *ARHGAP36* genomic locus (Genome Reference Consortium GRCh37/hg19), and exon-intron structures for the predicted *ARHGAP36* isoforms. Sequence junctions are indicated for pair-end read.

**Figure S19. Expression levels of ARHGAP36 isoforms.** (A) Representative western blot showing the relative levels of ARHGAP36 isoforms in the NIH-3T3-based Gli reporter assay shown in Fig. 4E. The ARHGAP36 isoforms were detected by immunoblotting for their C-terminal V5 tags, and importin β levels were used as gel-loading controls. (B) Quantification of ARHGAP36 isoform levels. Data are the average of two independent western blots ± SEM.

# Figure S1



# Figure S2

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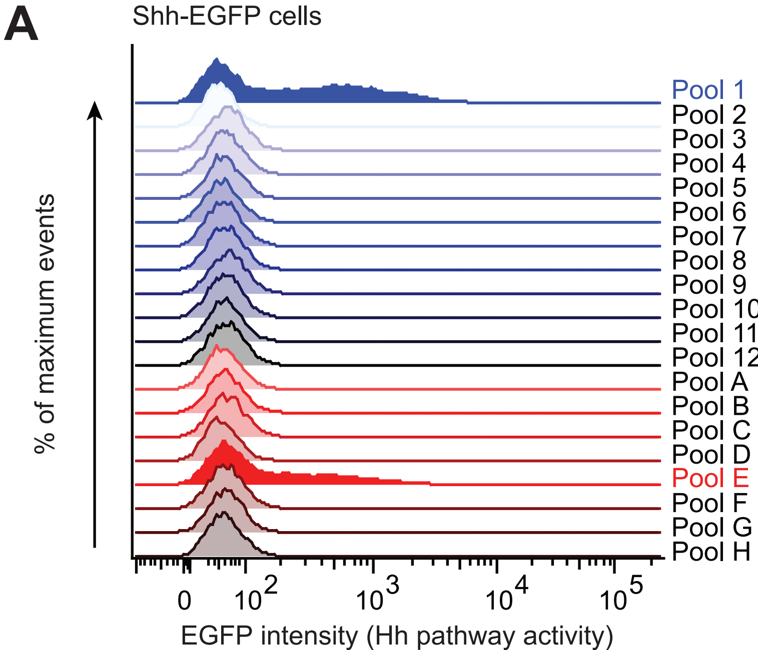


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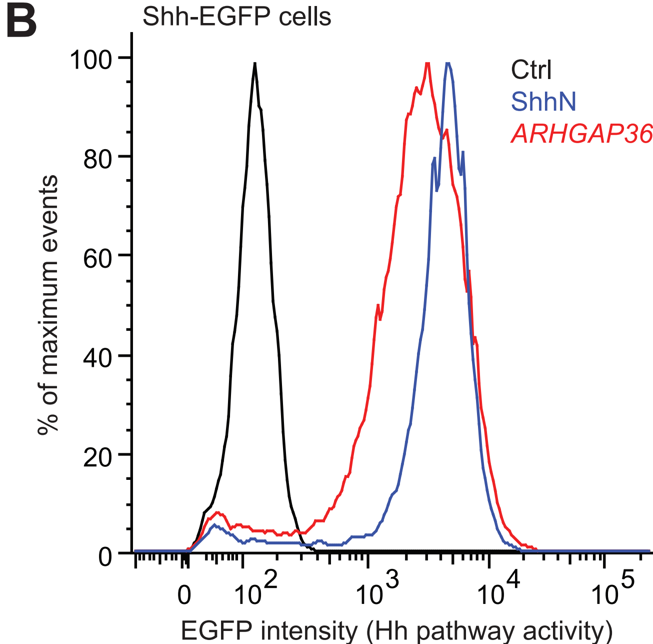

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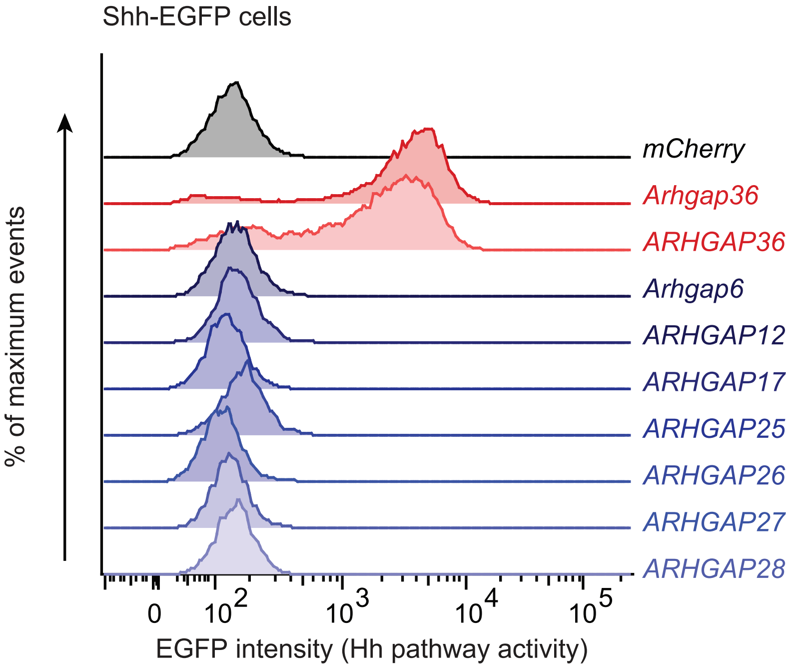

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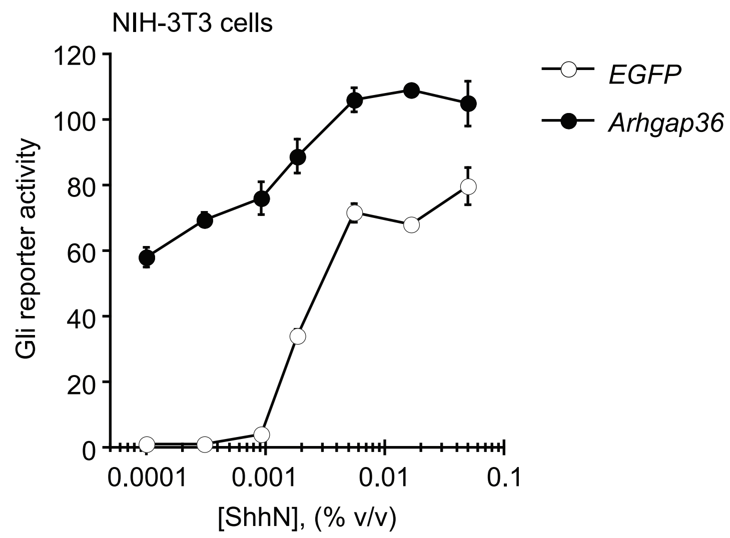

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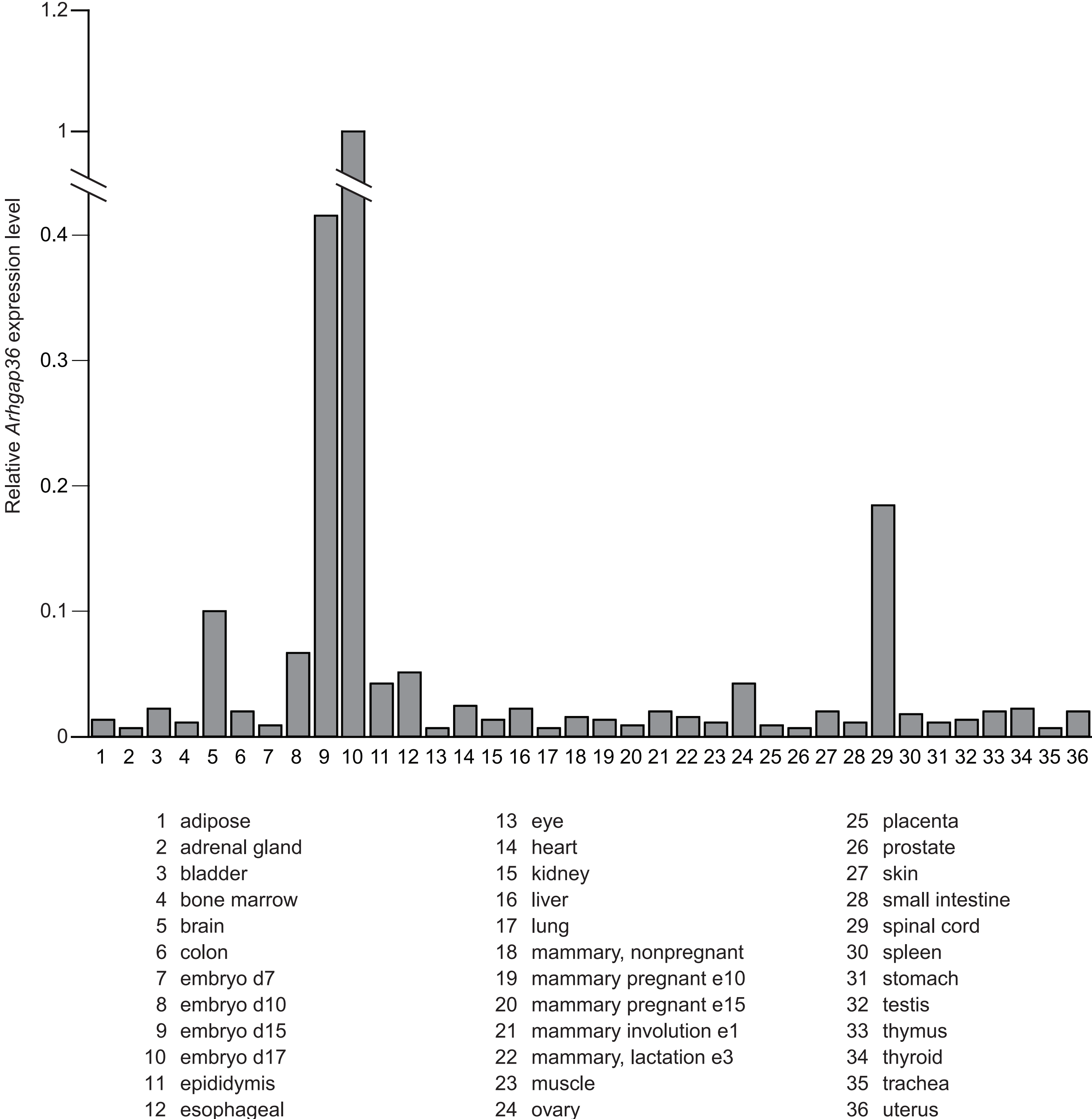

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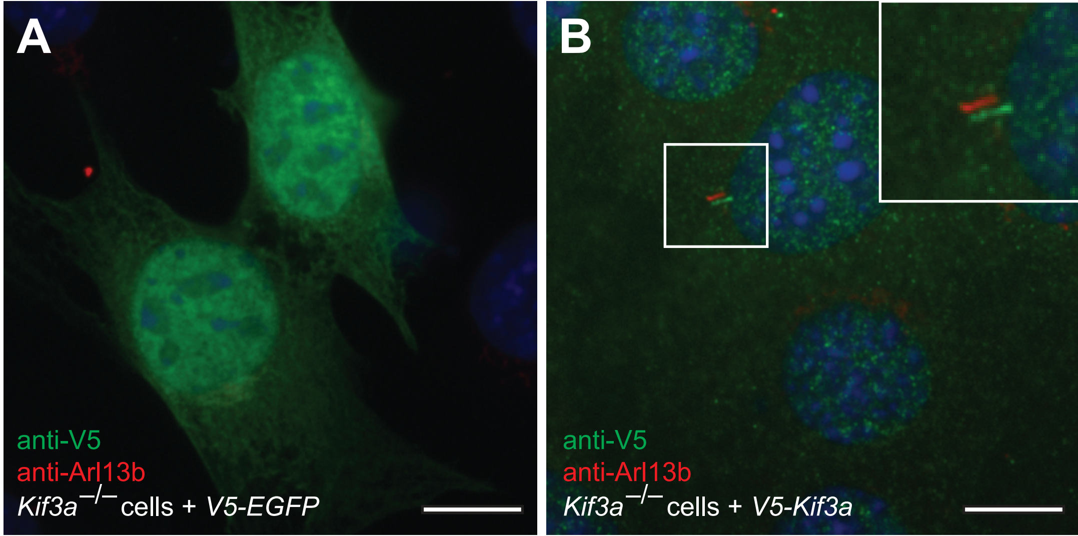

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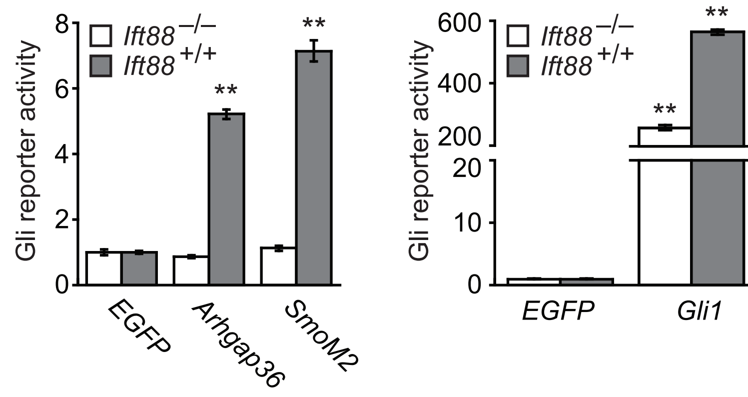

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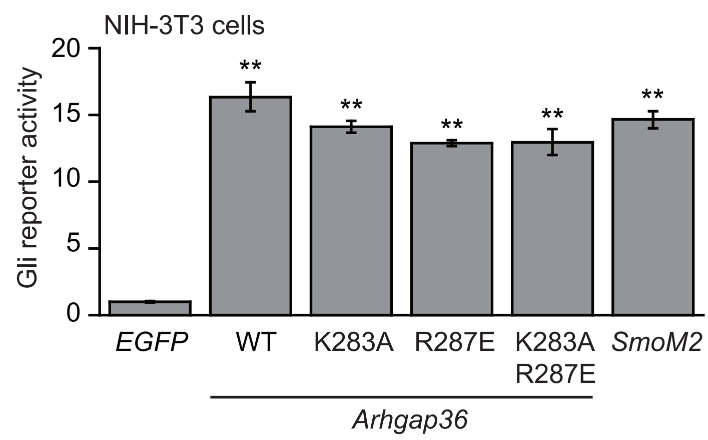

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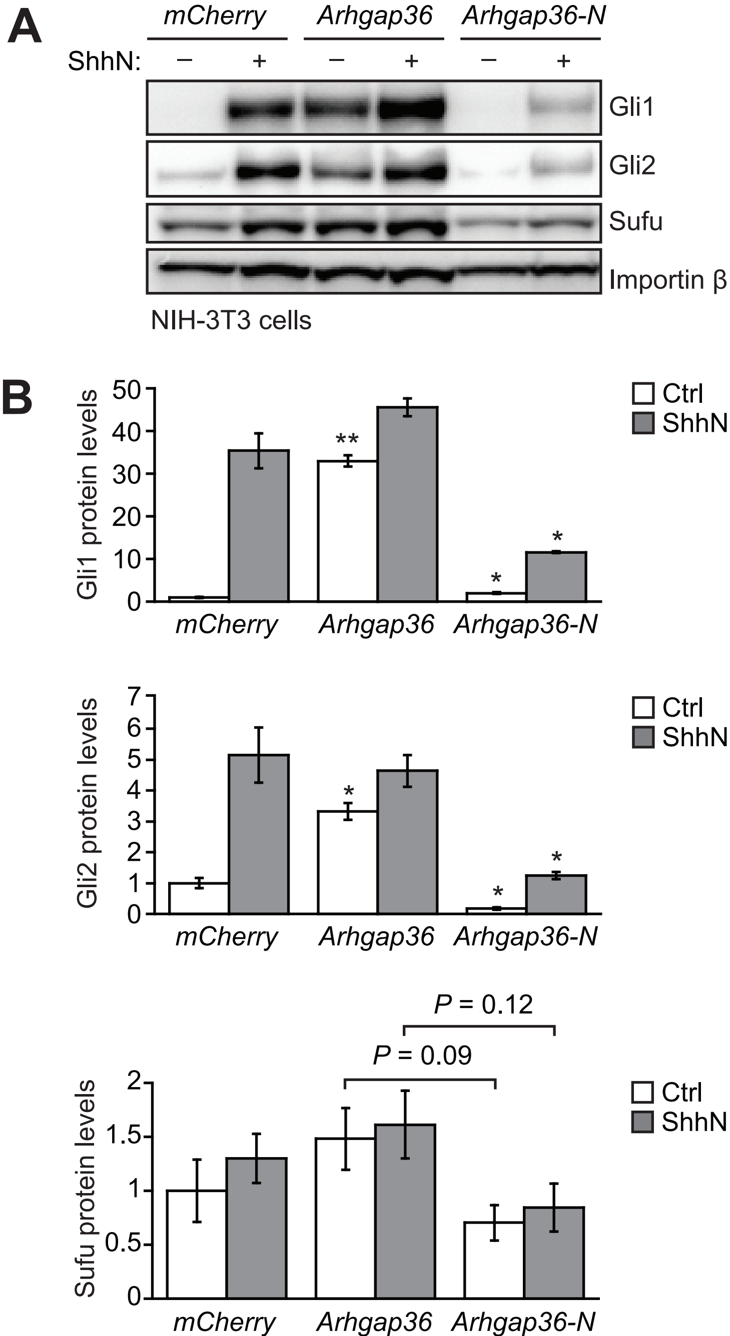

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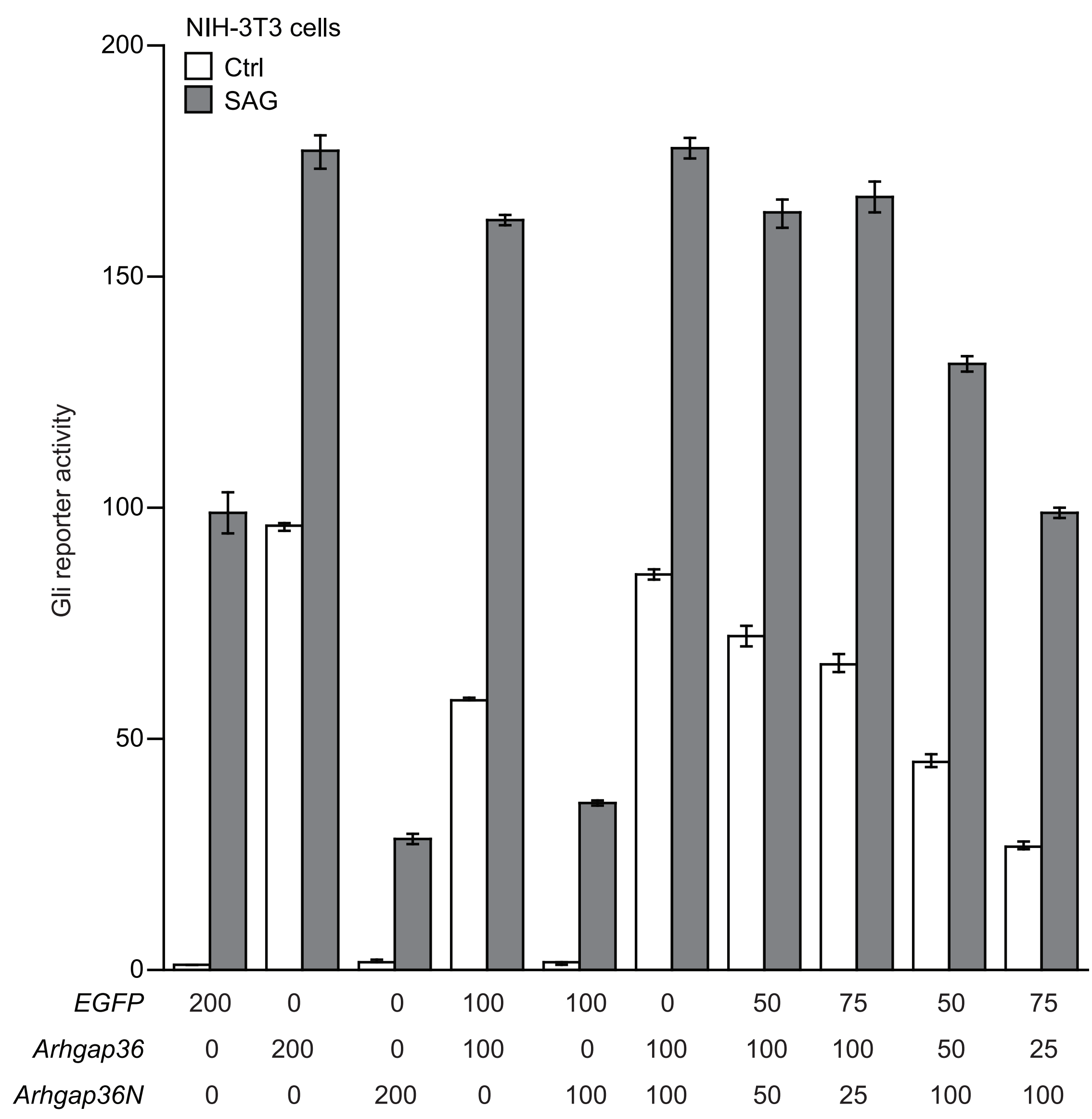

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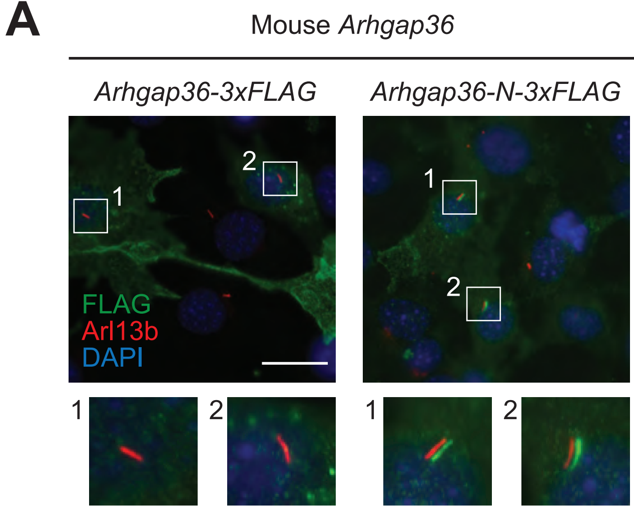


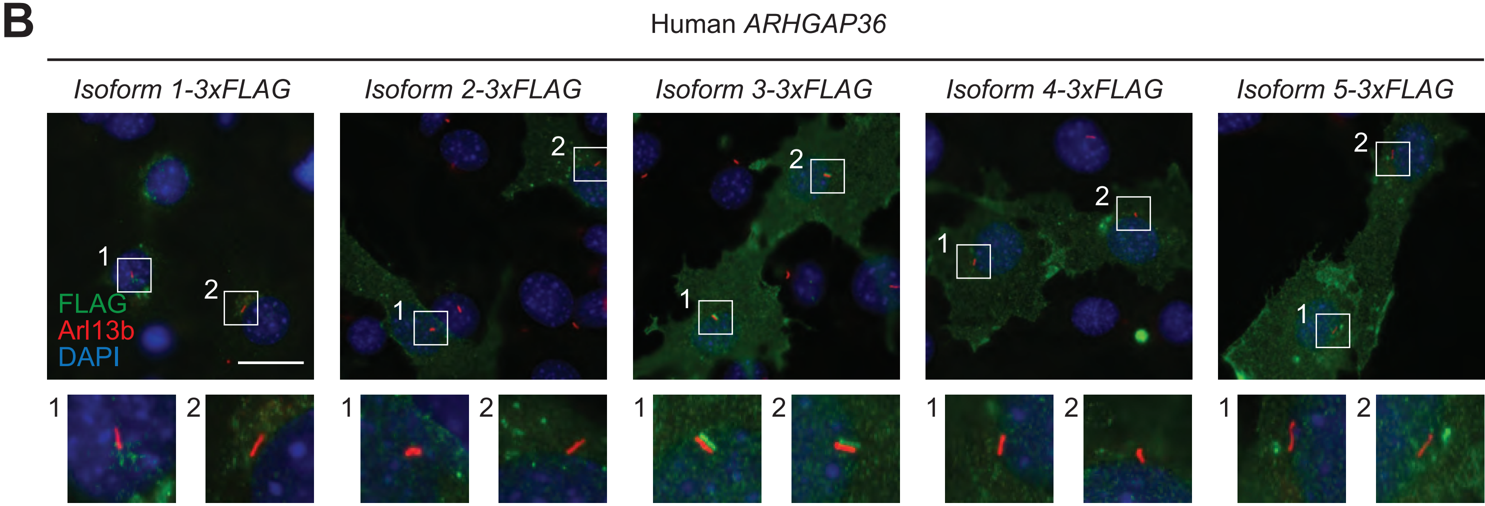

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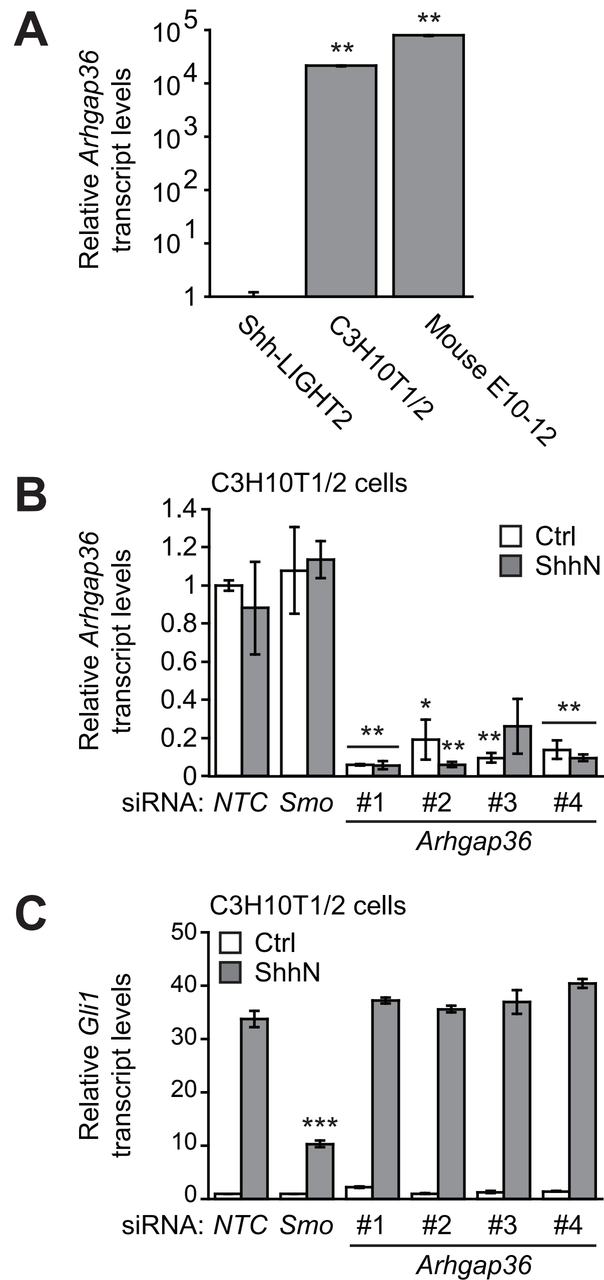

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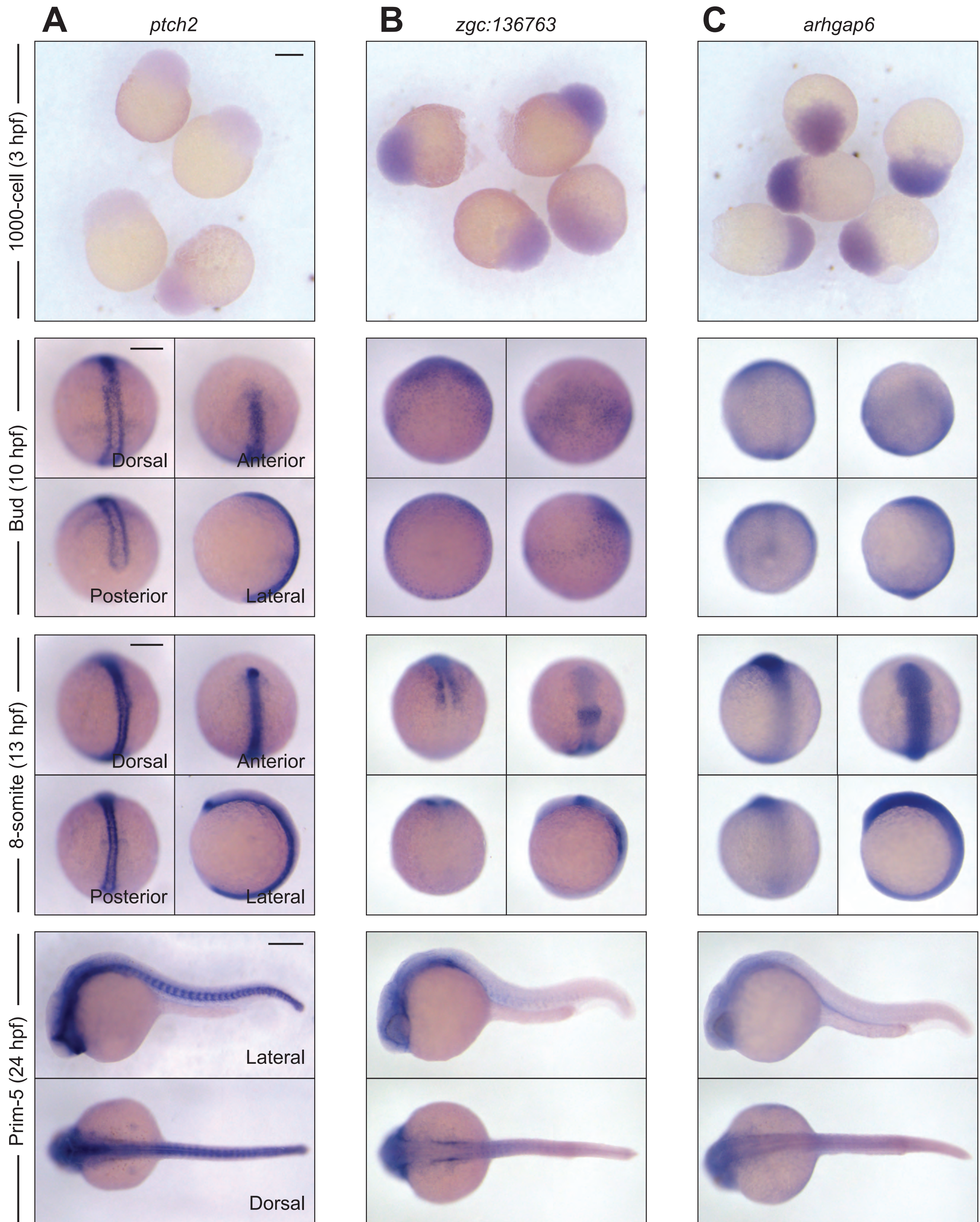

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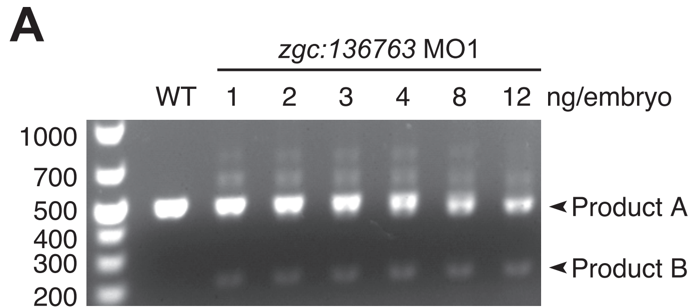


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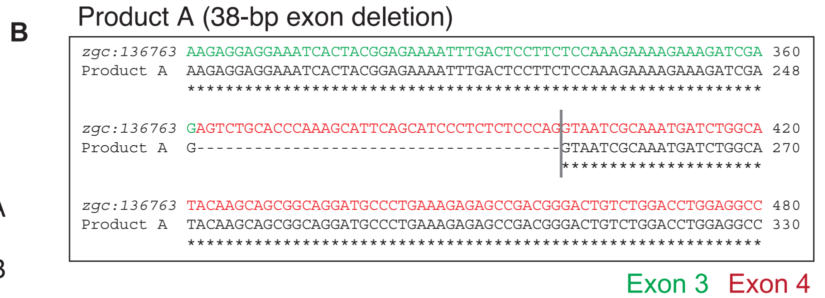


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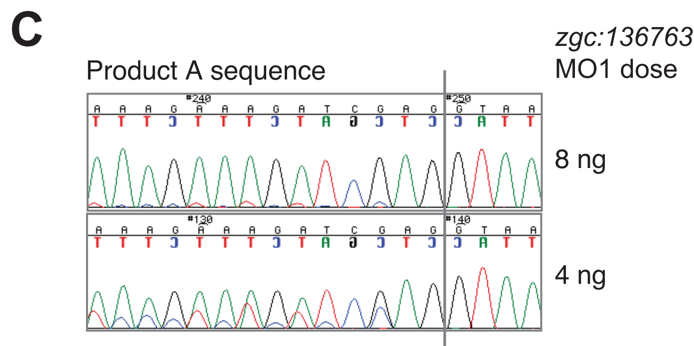


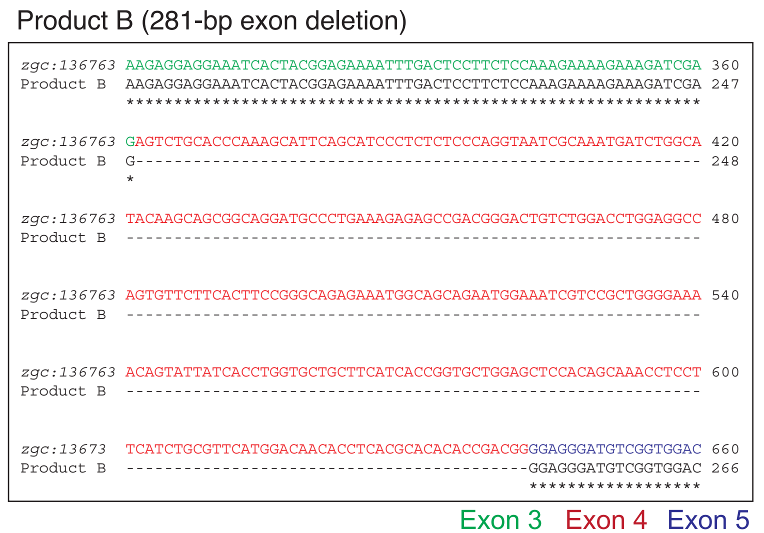


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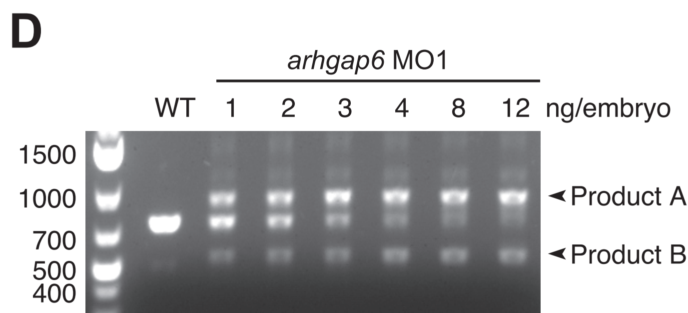


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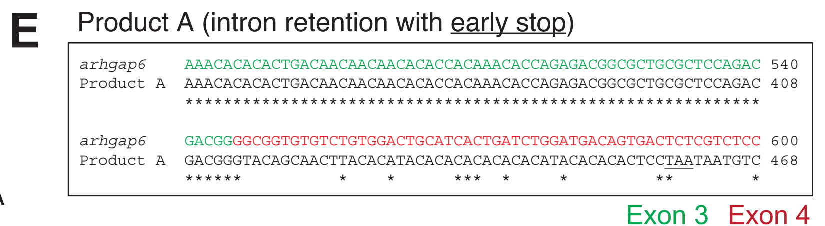

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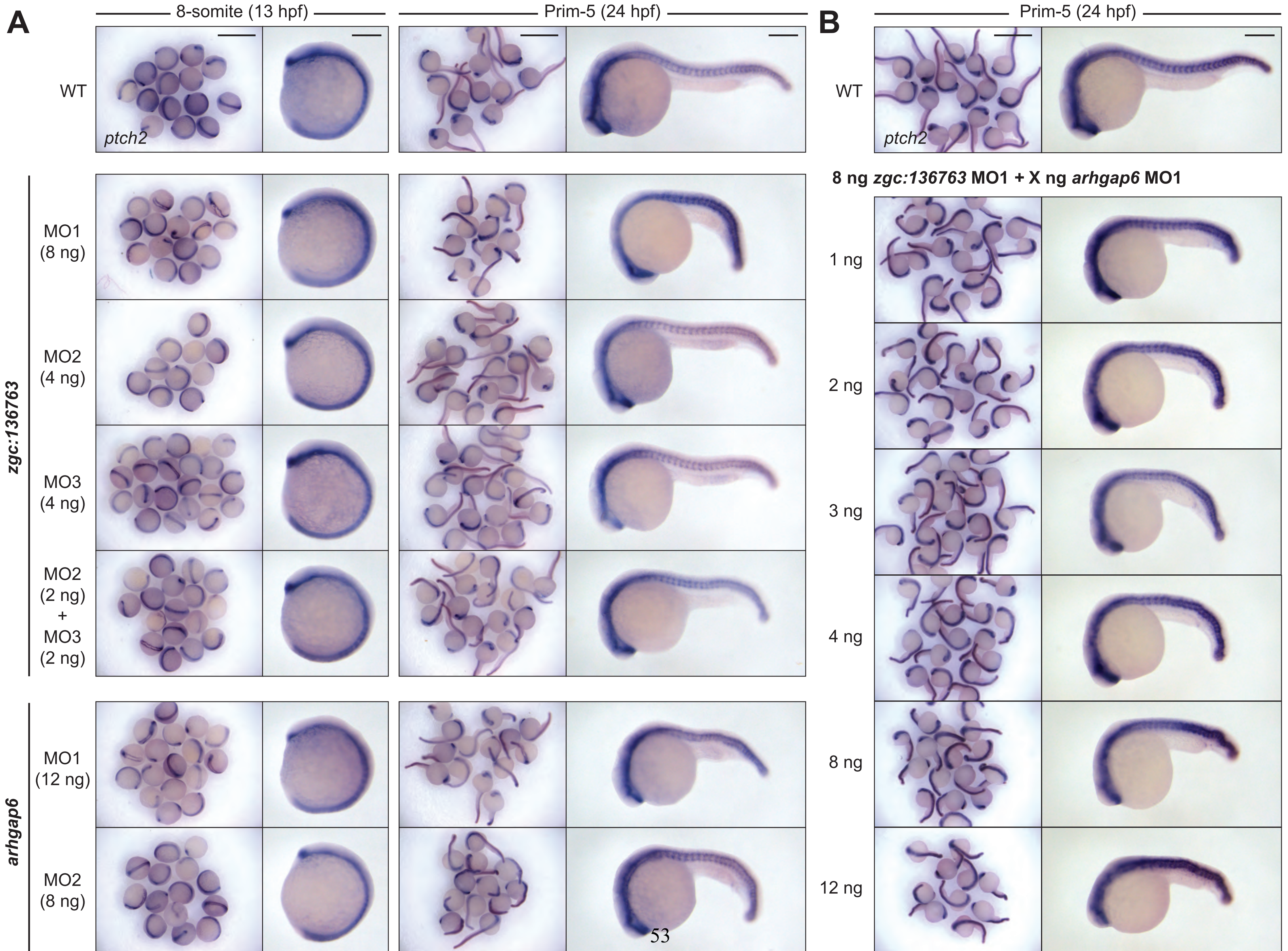

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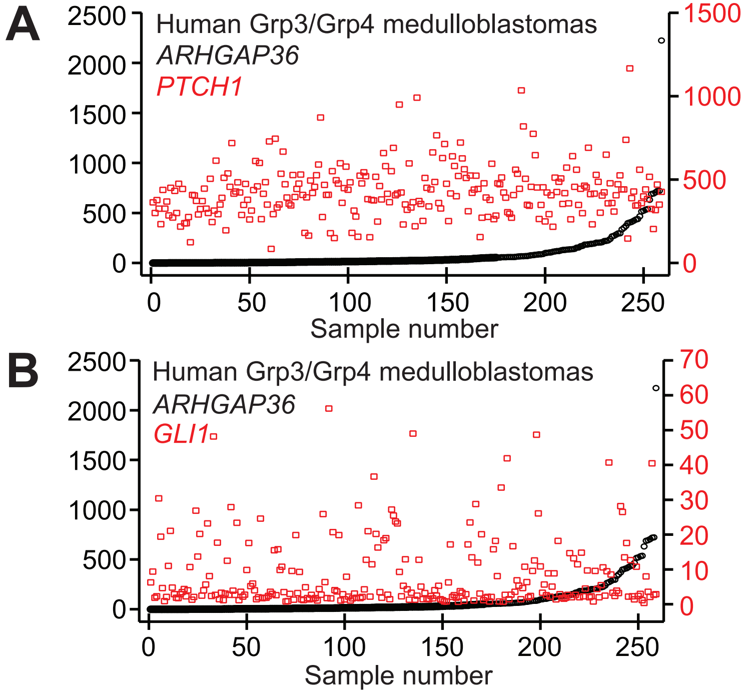

# Figure S17

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ARHGAP36 isoform 3    1 ------------------------------------------------------------   0
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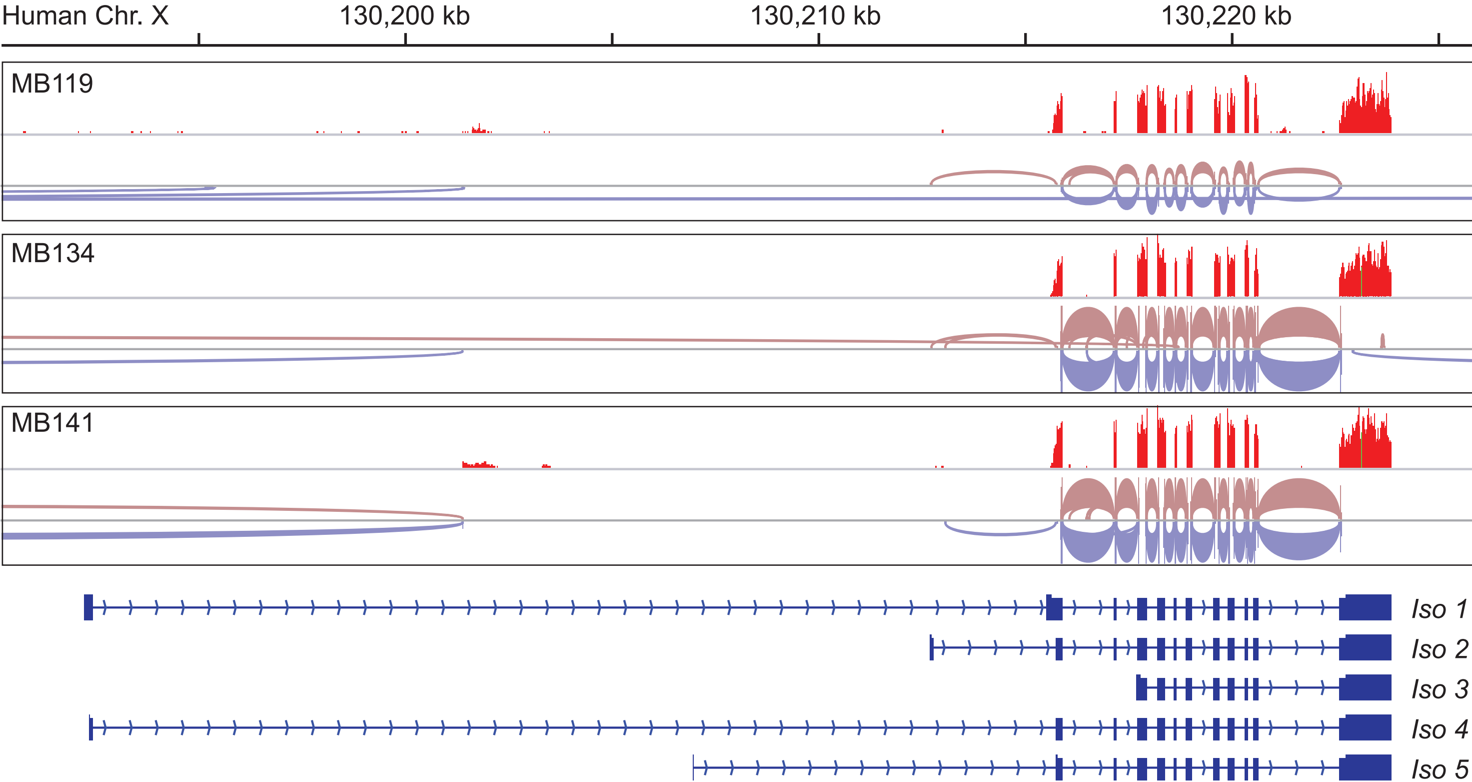

# Figure S19