Supplemental Online Materials

Materials and Methods

Immunoflourescence – Cells were plated in Lab-Tek II 8-well Chambered Coverglass dishes (Nalge Nunc International - 155409) that had been coated with fibronectin (Sigma – F1141). Media was aspirated and cell gently rinsed with .2 mL 1XPBS without calcium. Cells were fixed in 1 ml 4% paraformaldehyde in PBS for 10-15 min. Samples were rinsed twice with 0.2 mL 1X PBS, and washed 5 min in 0.2 mL 1X PBS on rocker. Samples were then incubated with 0.2 mL 0.2% PBT (1X PBS + 200 µl Triton-X-100) for 5 min, rinsed once with 0.2 mL 1X PBS and washed for 3 min in 0.2 mL 1X PBS on rocker. Samples were blocked in 0.2 mL 0.1% PBTN (1ml 0.1% PBT + 50µl heat inactivated goat serum for final concentration of 5%) for15-20 min in a humidified chamber. Each sample was incubated in 0.2 mL primary antibody diluted in PBTN overnight at 4°C overnight. Samples were washed 3 times for 5 min in 0.2 mL 0.1% PBT on rocker. Samples were then incubated in 0.2 mL of secondary antibody diluted in PBTN for 15 min. Samples were again washed in 0.2 mL 0.1% PBT on rocker. Anti-fade mounting solution (Southern Biotchnologies Fluoromount-G, 0100-01) was added to wells to wells, and allow to dry overnight. For quantitative assays, after transfection, samples were blinded and plated in duplicate.

Antibodies – *Immunoflouresence* – Monoclonal acetylated tubulin (Sigma – T6793) was diluted 1:500. Monoclonal β-arrestin 1 / 2 (BD Biosciences – 610551) was diluted 1:300. Polyclonal β-arrestin 1 / 2 has been previously described (*S1*) and was diluted 1:500. Monoclonal pericentrin (AbCam – ab4448) was diluted 1:500. Polyclonal rabbit Kif3A (AbCam – ab11259) was diluted 1:500. Polyclonal rabbit FLAG (Sigma – F7425) was

diluted 1:1000. Anti-mouse AlexaFlour 488, anti-rabbit AlexaFlour 488, anti-mouse Texas Red, and anti-Rabbit Texas Red were purchased from Invitrogen (A21202, A21206, T862, and T2767 respectively). *Immunoprecipitation and immunoblotting* – All antibodies were diluted in 5% BSA TBST. Polyclonal rabbit β-arrestin antibody has been used previously (*S1*) and was diluted 1:3000 for western blots. Monoclonal Kif3A antibody (BD Biosciences-611509) was used for immunoprecipitation, while polyclonal Kif3A antibody was diluted 1:1000 for western blotting. Polyclonal rabbit Smoothened antibody (AbCam – ab38686) was diluted 1:500). Polyclonal rabbit GFP antibody (CytoStore-PAB-002) was used for immunoprecipitation and monoclonal GFP antibody (Covance – MMS-118P) was diluted 1:1000 for western blotting.

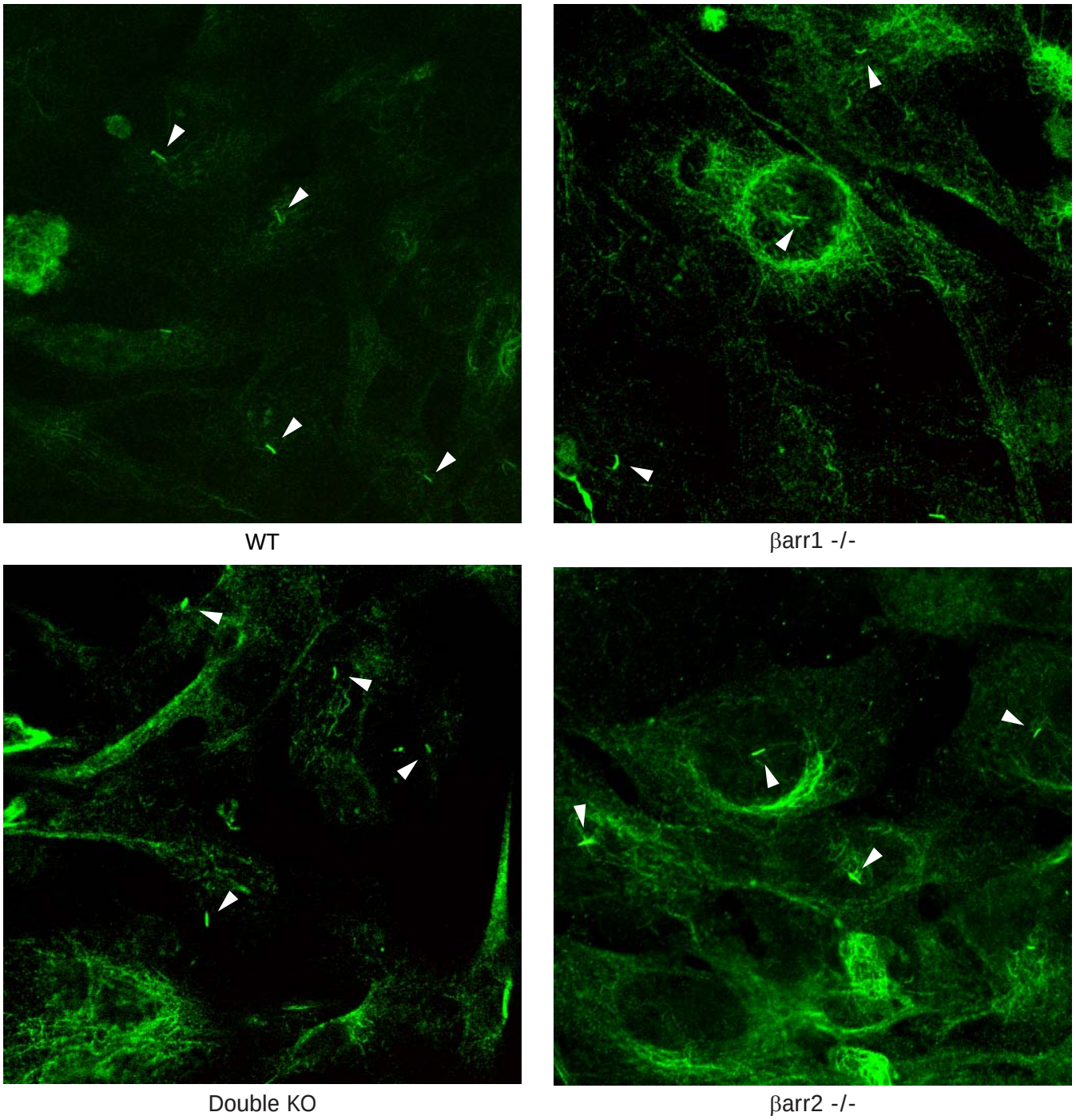
RNA interference – A series of mouse β-arrestin 1 siRNA oligonucleotides were generated with the sequences: 5'-aaagccuucugugcugagaac-3' (NM_177231) previously (*S2*), 5'-acctgcgccttccgctatg-3', 5'-acctgcgccttccggtatg-3', and 5'-aaagaaaggcgagtctacgtg-3', while mouse β-arrestin 2 siRNA oligonucleotides were generated with the sequences: 5'-aaaccugugccuuccgcuaug-3' (NM_145429), 5'-aaggaccggaaagtgttcgtga-3', and 5'-ccaacctcatcgaattcga-3' using Dharmacon custom RNAi services. Kif3A mouse siRNAs were purchased from Dharmacon ON-TARGETplus Duplex library (J-942111-09-0050, J-942111-11-0050, J-942111-12-0050, NM_008443). Cells were transfected as described previously using GeneSilencer transfection reagent (*S3*), with a slight modification where serum-free media containing transfection solution was left on cells overnight before dilution with media + serum.

Cell lines – *FLAG-Smo line* -NIH 3T3 cells (ATCC) were transfected with pcDNA3 Smo-Flag plasmid using Fugene 6 (Roche – 11814443001) and split into new plates at

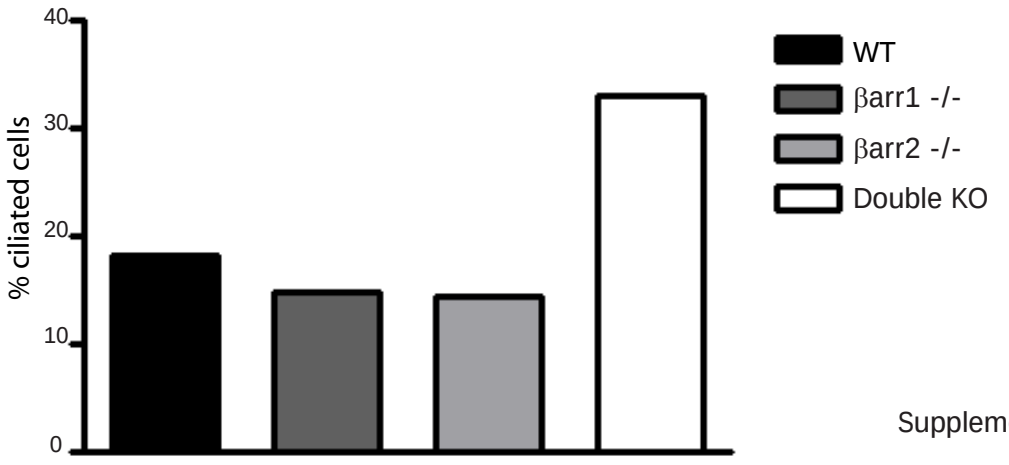
the dilution of 1:100 in the selection medium (DMEM +10% new born calf serum + 0.8mg/ml G418) the next day. The cells were maintained in the selection medium and the medium was changed every 3 days until colonies formed. Colonies were picked and expanded. Expression of FLAG-Smo was detected western blotting with monoclonal FLAG antibody (Sigma). *GFP-Smo line* - NIH-3T3 cells were transfected with peGFP-Smo plasmid using Fugene 6. Cells were selected (G418) for 5 days and then sort by FACS analysis for flouresence. The homogeneous population was then replated and allowed to grow under selection for an additional 10 days.

Luciferase Assay – NIH-3T3 cells were plated at a density of $1X10^5$ per well in 12 well plates 24 hours prior to transfection. After overnight attachment, media was replaced with 400 μL of serum-free media, and cells were transfected with GeneSilencer reagent according to the standard parameters described above with the following mixture of DNA and siRNA for each sample: 640 ng Gli-luciferase reporter plasmid, 160 ng Renilla reporter plasmid (pRLTK), and 4 μg β-arrestin siRNA 1 or 2 or control siRNA oligo. Cells were incubated for 18 hours, and then supplemented with 400 μL of 20% bovine calf serum-DMEM. JHU-64 cells (ATCC) were stimulated for 48 hours with 1 uM muristerone A (Alexis – ALX-370-010) in DMEM + 2% delipidated FBS + 1X ITS (insulin-transferrin-selenium) supplement (Sigma – I2521) + 5 mM HEPES (Sigma – H0887) in order to produce Shh conditioned media. Conditioned media and control media were collected and used to treat transfected cells for 24 hours. Cells were lysed, collected and assayed using the Promega Dual Luciferase Reporter Assay System Kit (E1910) according to the included protocol.

A.

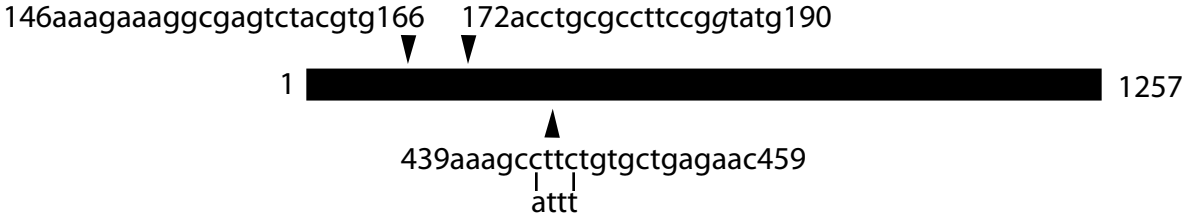


B.



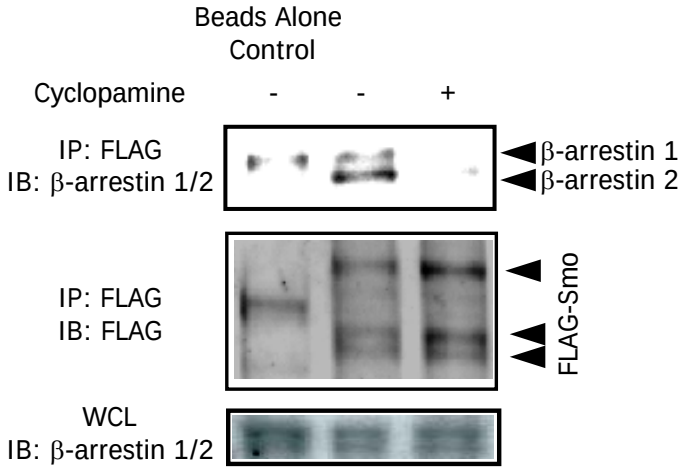
Supplemental Figure 1 - Kovacs, et. al

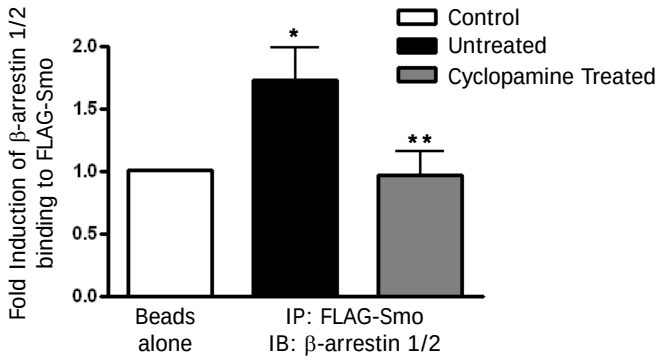
β-arrestin1



β-arrestin2

Supplemental Figure 2 - Kovacs, et. al





Supplemental Figure 3 - Kovacs, et al.

Figure Legends

Fig. S1 – β-arrestins are not required for ciliogenesis. To test whether β-arrestins function in ciliogenesis, we examined wild-type mouse embryonic fibroblasts (MEFs) or MEFs with either β-arrestin 1, β-arrestin 2, or double β-arrestin 1/2 knockout genotypes (*S4*). **(A)** MEFs from control, β-arrestin $1^{-/-}$, β-arrestin $2^{-/-}$, and β-arrestin $1/2^{-/-}$ mouse embryos immunostained with anti-acetylated tubulin antibody to check for primary cilia formation (arrowheads). Note that the presence of β-arrestin 1 or 2 is not required for ciliogenesis. MEFs from wild type animals formed cilia when grown in culture and cilia were visualized by staining for acetylated tubulin. Knockout of β-arrestin 1, β-arrestin 2, or both proteins in tandem had no effect on cilia formation. **(B)** The total number of ciliated cells was counted in the various MEF lines described in (A). Results from 2 separate experiments were combined for graphing purposes. Thus, although β-arrestins are present in the cilia, they appear not to have a role in ciliogenesis.

Fig. S2 – Multiple siRNAs used for knockdown of endogenous β-arrestin1 and β-arrestin2. Oligonucleotides targeting the indicated regions of β-arrestin1 and β-arrestin2 were synthesized as described. These sequences are denoted in the manuscript as βarr1-A (439aaagccttctgtgctgagaac459), βarr1-B/C (172acctgcgccttccg*g*tatg190), βarr1-D (146aaagaaaggcgagtctacgtg166), βarr2-A (175acctgtgccttcgctatg), βarr2-B (148 aaggaccggaaagtgttcgtga168), and βarr2-C (1115ccaacctcatcgaattcga1133). As indicated for βarr1-A and βarr2-A, silent mutations were induced at the indicated residues in order to establish rescue constructs. Note that the βarr1-B/C oligos differ only at residue 186 (the italicized "g"). In a previous paper (*S5*) we characterized βarr1-B as an siRNA with

the capability to knockodown both β-arrestin 1 and 2 in human cells. However, this target sequence is not conserved in the mouse β-arrestin2 sequence and varies at position 186 of the mouse sequence (human – c, mouse – g). Even though βarr1-B was able to knockdown β-arrestin1 in mouse cells, we synthesized βarr1-C with a guanine at the 186 position to verify the knockdown.

Fig S3 – Cyclopamine treatment abolishes the interaction between endogenous β-arrestin 1, β-arrestin 2 and Smo NIH-3T3 cells stably overexpressing FLAG-Smo left untreated or treated with cyclopamine (6μM) for 1 hour. Cells were lysed and immunoprecipitated with anti-FLAG antibody. Samples were subjected to SDS-PAGE analysis and immunoblotted for endogenous β-arrestins (top panel). Samples were checked to ensure FLAG-Smo was immunoprecipitated in a specific manner (middle panel). A significant increase of coimmunoprecipitated β-arrestins 1 and 2 was seen in untreated samples, while cyclopamine treatment abolished the interaction between endogenous β-arrestin 1 and 2 and Smo. *$p<0.05$ compared to beads alone control, and **$p<0.05$ compared to untreated cells. Quantified bands were analyzed by one-way ANOVA with Bonferoni correction, n=3.

Supplemental References

S1. H. Attramadal *et al.*, *J Biol Chem* **267**, 17882 (Sep 5, 1992).
S2. L. Girnita *et al.*, *J Biol Chem* **282**, 11329 (Apr 13, 2007).

S3. S. Ahn, C. D. Nelson, T. R. Garrison, W. E. Miller, R. J. Lefkowitz, *Proc Natl Acad Sci U S A* **100**, 1740 (Feb 18, 2003).
S4. T. A. Kohout, F. S. Lin, S. J. Perry, D. A. Conner, R. J. Lefkowitz, *Proc Natl Acad Sci U S A* **98**, 1601 (Feb 13, 2001).
S5. D. Gesty-Palmer *et al.*, *J Biol Chem* **281**, 10856 (Apr 21, 2006).