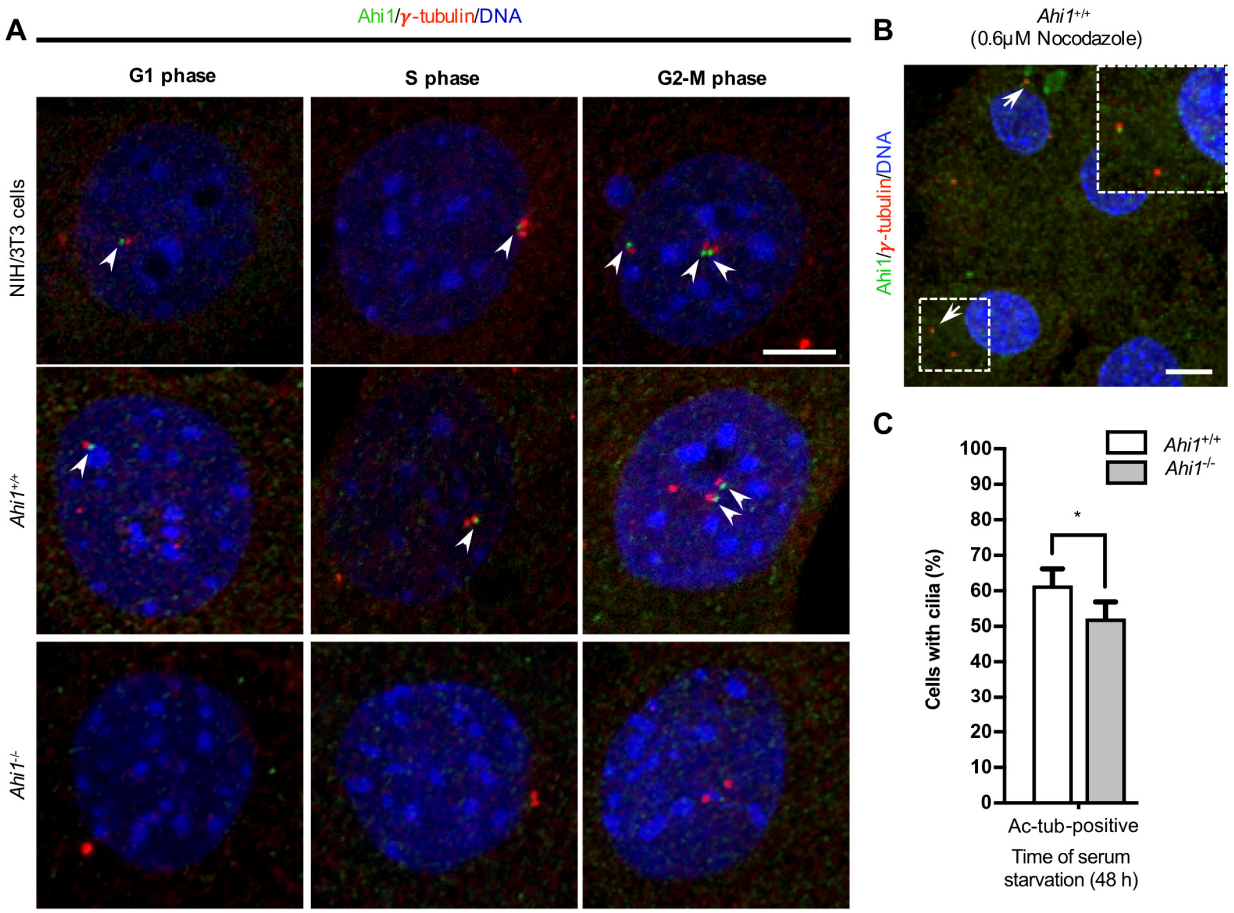

Figure S1. Ahi1 localizes adjacent to the centrosome during interphase in fibroblasts and is required for cilia formation. (**A**) Representative images of NIH/3T3 cells, and *Ahi1*$^{+/+}$ and *Ahi1*$^{-/-}$ mouse embryonic fibroblasts (MEFs) grown in 10% FBS for 24 h, fixed and immunolabeled with Ahi1 (green) and the centrosome marker, γ-tubulin (red). DNA was labeled with Hoechst (blue). Arrows indicate Ahi1 localization in cells at different stages of the cell cycle. No immunoreactivity for Ahi1 was observed in *Ahi1*$^{-/-}$ MEFs (third row). Scale bar, 5 μm. (**B**) *Ahi1*$^{+/+}$ MEFs treated with 0.6 μM nocodazole for 16 h showing immunolabeling of Ahi1 localization (green; arrow) only to one of the centrioles labeled with γ-tubulin (red) indicating Ahi1 localization independent of

microtubule polymerization. Scale bar, 10 µm. **(C)** Number of cilia expressed as a percentage in serum-starved (48 h) *Ahi1*$^{+/+}$ and *Ahi1*$^{-/-}$ MEFs. Ciliation was assessed by immunofluorescence using acetylated α-tubulin (Ac-tub). Error bars represent the s.e.m. Significance was determined by chi-square test (*, P<0.05).

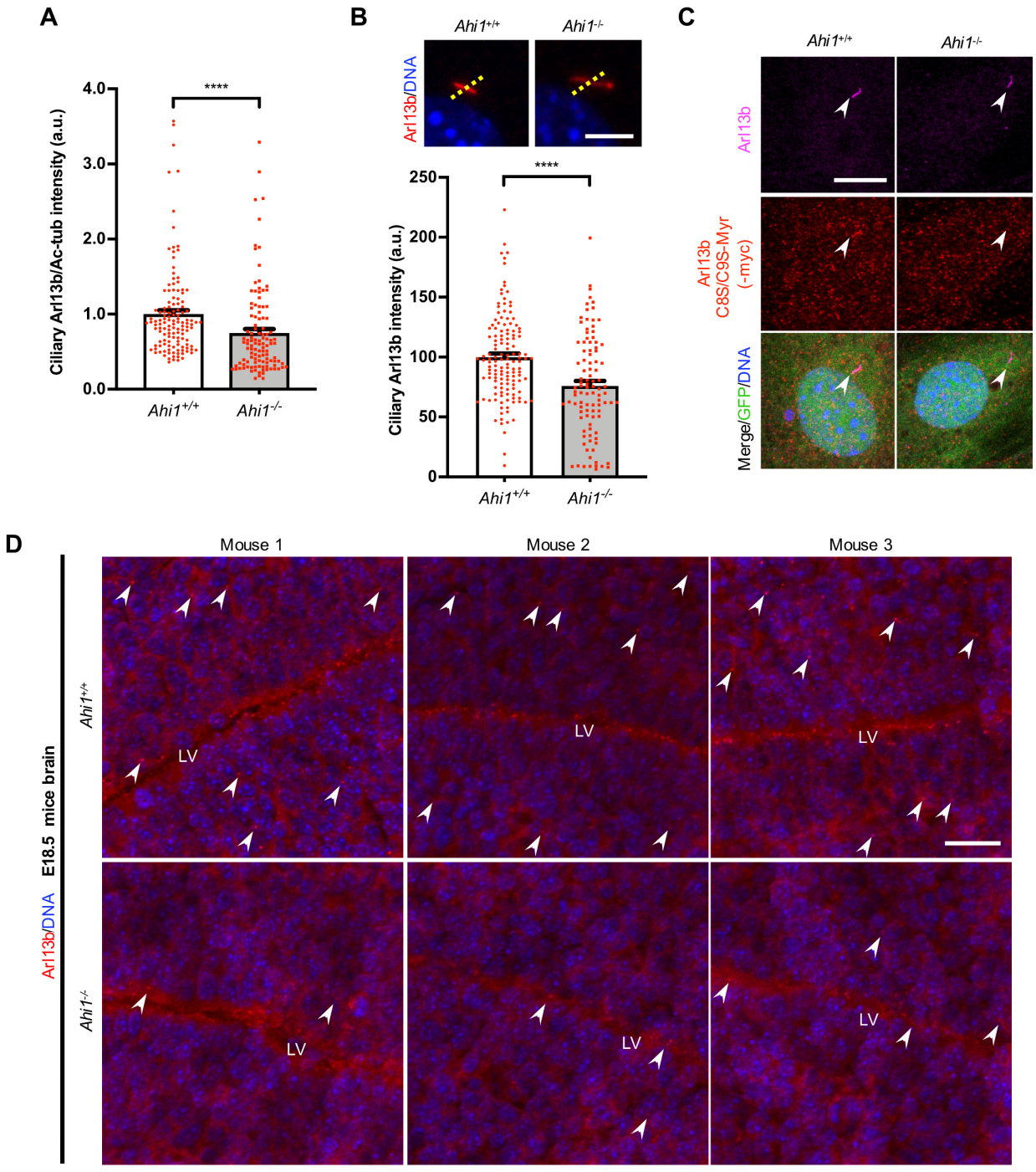

Figure S2. Arl13b immunolabeling is reduced in *Ahi1*$^{-/-}$ fibroblasts and mouse brain.

(**A**) Arl13b and acetylated α-tubulin (Ac-tub) ratios obtained for cilia in *Ahi1*$^{-/-}$ and wild-type mouse embryonic fibroblasts (MEFs). Each red point represents a measurement for

a single cilium. (**B**) Sum of pixel intensities of Arl13b immunofluorescence along the yellow dotted line over a threshold background and expressed as the relative intensity of Arl13b in *Ahi1*$^{-/-}$ and wild-type cilia. Each red point represents a measurement for a single cilium. Scale bar, 5 μm. The graph represents data obtained n>100 cilia/group (n=4/genotype). Error bars represent the s.e.m. Significance was determined by the Mann-Whitney test (****, P<0.0001). (**C**) *Ahi1*$^{+/+}$ and *Ahi1*$^{-/-}$ MEFs were co-transfected with GFP and mutant Arl13b C8S/C9S-Myr (-myc tagged) plasmids. Twenty-four hours after plasmid transfection, cells were serum-starved for 48 h and immunolabeled for total Arl13b (magenta) and exogenous myc-Arl13b (red). DNA was labeled with Hoechst (blue). Ciliary co-localization of Arl13b with the Arl13b-myc-tagged mutant was observed only in *Ahi1*$^{+/+}$ cells, and not in *Ahi1*$^{-/-}$ cells. Arrowheads point to cilia. Scale bar, 10 μm. (**D**) Immunolabeling of cilia was performed in embryonic mouse brain (E18.5) from *Ahi1*-null and wild-type mice (n=3/genotype) using Arl13b as a ciliary marker (red). DNA was labeled with Hoechst (blue). Reductions in Arl13b-positive cilia were consistently noted in *Ahi1*$^{-/-}$ mice. Arrowheads denote cilia labeling. Scale bar, 20 μm. LV, lateral ventricle.

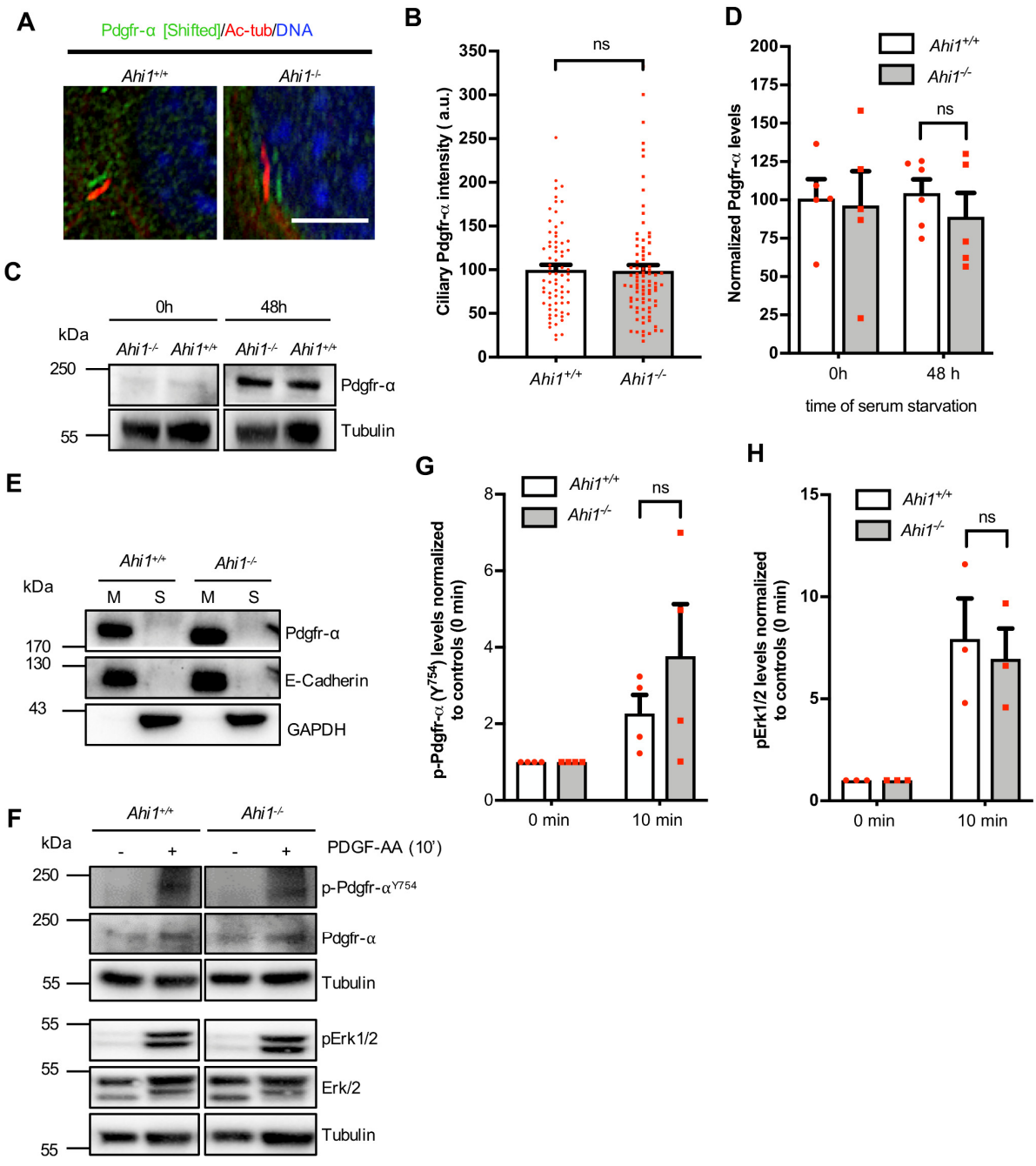

Figure S3. Ciliary recruitment, expression levels, and phosphorylation (Y754) of the platelet-derived growth factor receptor alpha (Pdgfr-α) after PDGF-AA stimulation are unaffected in *Ahi1*-null fibroblasts. (**A**) After 48 h of serum-starvation, *Ahi1*$^{+/+}$ and *Ahi1*$^{-/-}$ mouse embryonic fibroblasts (MEFs) were fixed and co-labeled for Pdgfr-α (green)

and acetylated α-tubulin (Ac-tub; red). DNA was labeled with Hoechst (blue). The green channel is shifted for a better visualization of Pdgfr-α distribution at the primary cilium. Scale bar, 5 µm. (**B**) Quantification of Pdgfr-α labeling intensity at primary cilia. n>100 cilia/group (n=4/genotype). Each red point represents a measurement for a single cilium. (**C**) Western blot analysis of Pdgfr-α in *Ahi1*$^{+/+}$ and *Ahi1*$^{-/-}$ MEFs grown in 10% FBS (0 h) and serum-starved for 48 h. Tubulin was used as a loading control. (**D**) Quantification of Pdgfr-α levels normalized to *Ahi1*$^{+/+}$ values. n≥5/genotype, experiments were performed in duplicate. (**E**) Cell fractionation analysis of Pdgfr-α by western blot analysis in *Ahi1*$^{+/+}$ and *Ahi1*$^{-/-}$ MEF cultures that were serum-starved for 48 h. M, membrane fraction and S, soluble fraction. E-cadherin was used as a control for membrane bound proteins and Gapdh for soluble proteins. Results indicate that Pdgfr-α is preferentially localized in MEF cell membranes. (**F**) *Ahi1*$^{+/+}$ and *Ahi1*$^{-/-}$ MEFs were serum-starved for 48 h and then cells stimulated with PDGF-AA for 10 min. Total cell lysates were analyzed by western blot analysis with the indicated antibodies. Tubulin was used as a loading control. n≥3/genotype, experiments were performed in duplicate. (**G** and **H**) Quantification of western blot analyses for p-Pdgfr-α^{Y754} and p-Erk1/2. The displayed values for MEFs stimulated with PDGF-AA were normalized to the values of non-stimulated cells (set to 1). Error bars represent the s.e.m. Significance was determined using the Mann-Whitney test for (**B**) and unpaired two-tailed *t* tests for (**D**, **G** and **H**), ns=not significant.

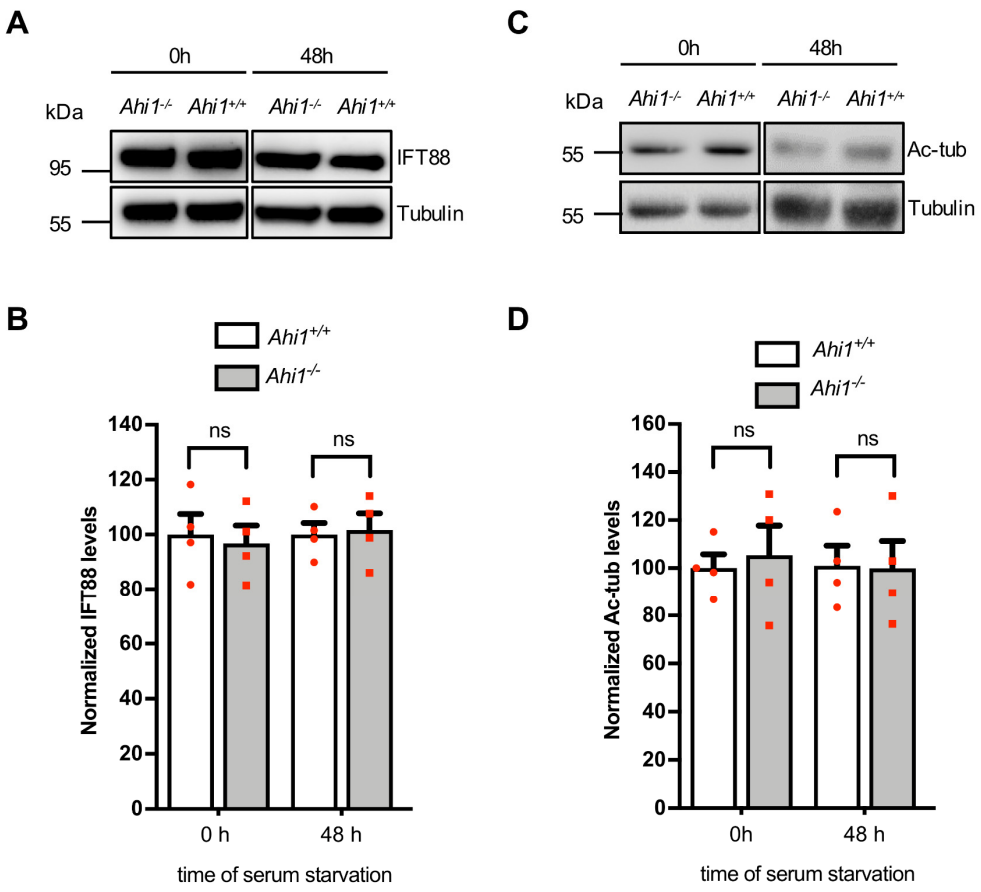

Figure S4. Western blot analysis of IFT88 and acetylated α-tubulin (Ac-tub) levels in fibroblasts. Representative immunoblots of lysates from *Ahi1*$^{+/+}$ and *Ahi1*$^{-/-}$ mouse embryonic fibroblasts (MEFs) seeded under standard culture conditions (0 h) or serum-starved for 48 h. Lysates were probed with anti-IFT88 (**A**) and Ac-tub (**C**) antibodies and detected with chemiluminescence. Quantification of IFT88 (**B**) and Ac-tub (**D**) chemiluminescent signals were normalized to *Ahi1*$^{+/+}$ values. Tubulin was used as a loading control. n=4/genotype, experiments were performed in duplicate. Error bars represent the s.e.m. Significance was determined by unpaired two-tailed *t* tests, ns=not significant.

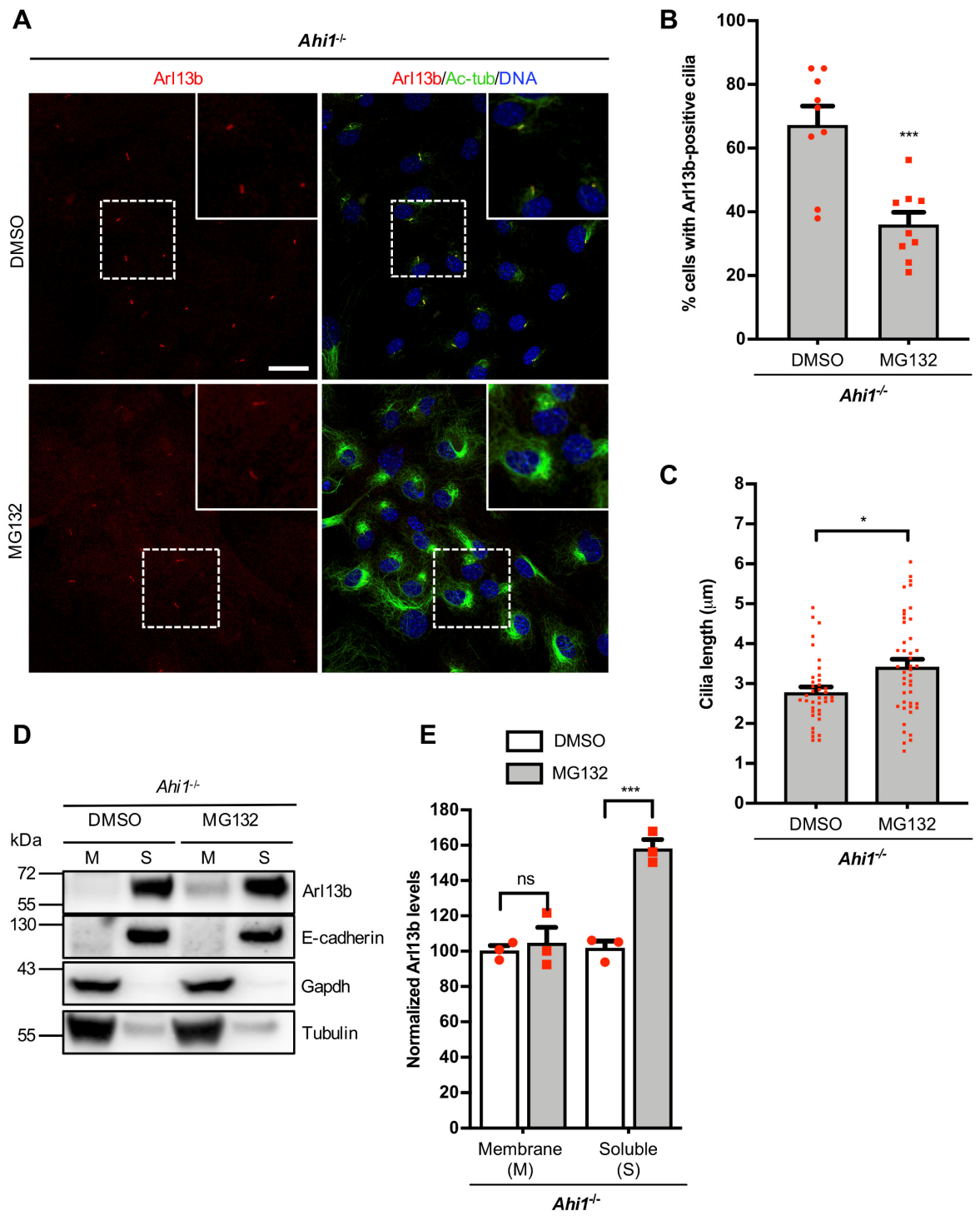

Figure S5. Proteasome inhibition decreases primary cilia formation and increases cilia length in *Ahi1*-null cells. (**A**) *Ahi1*$^{-/-}$ mouse embryonic fibroblasts (MEFs) were treated for 6 h with vehicle (DMSO) or MG132 and immunolabeled for Arl13b (red) and

acetylated α-tubulin (Ac-tub; green). DNA was labeled with Hoechst (blue). Before treatments, cells were serum-starved for 48 h to induce formation of Arl13b-positive primary cilia (red). Insets show higher magnification views of the indicated regions in the boxed areas. The second column shows expected co-localization of the two cilia markers (Arl13b and Ac-tub) in the vehicle group, but abnormal localization of these proteins is observed in cells treated with MG132. Scale bar, 30 µm. (**B**) Quantitative analysis of cells with Arl13b-positive cilia expressed as a percentage and (**C**) in cilia length. In (**B** & **C**) each red point represents a measurement for a single cilium. n=3/genotype. (**D**) Arl13b analysis in membrane (M) and soluble (S) fractions by western blotting in *Ahi1*$^{-/-}$ ciliated MEFs (48 h of serum depletion) after treatment with MG132 or vehicle (DMSO) for 6 h. E-cadherin was used as a control for membrane fractions and Gapdh as a control for soluble fractions. (**E**) Quantification of Arl13b levels (n=3/genotype) in membrane and soluble fractions in the presence or absence of MG132. Tubulin was used as a loading control for the M and S fractions and bars represent normalization to DMSO treatment. Error bars represent the s.e.m. Significance was determined by the chi-square test, Mann-Whitney test, and unpaired two-tailed *t* test for **B**, **C** and **E**, respectively (*, $P<0.05$; ***, $P<0.001$).

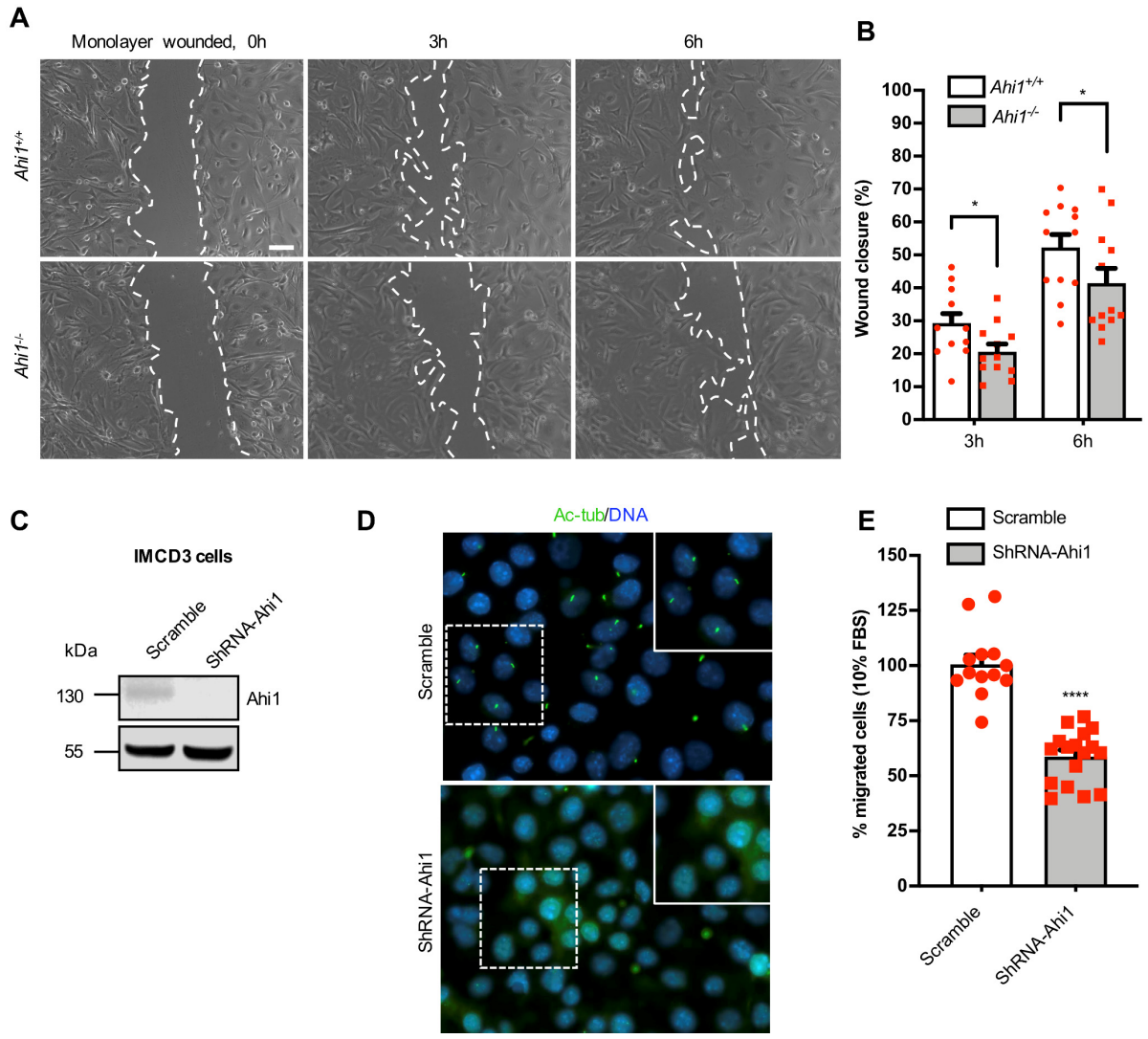

Figure S6. Impaired directional migration in Ahi1-null fibroblasts at sub-confluence and in Ahi1-knockdown inner medullary collecting duct (IMCD3) cells. (**A**) Representative images of *Ahi1*$^{+/+}$ and *Ahi1*$^{-/-}$ mouse embryonic fibroblast (MEF) cultures at 0, 3 and 6 h after monolayers were scratched. Cell cultures were grown to sub-confluence and scratched. Scale bar, 100 μm. (**B**) Quantitative analysis of percentage wound closure after 3 and 6 h. Quantification was performed as described in the material and methods section. n=3/genotype, experiments were carried out in triplicate with at

least three different fields along the wound being analyzed over the time for each experiment. Error bars represent the s.e.m. Significance was determined using unpaired two-tailed *t* tests (*, $P<0.05$). (**C**) Western blot analysis of Ahi1 levels in control cells (scramble ShRNAi) and in Ahi1-knockdown cell lines (ShRNA-Ahi1), as previously described (Hsiao et al., 2009). Tubulin was used as a loading control. (**D**) Scramble and ShRNA-Ahi1 cells were immunostained with the ciliary marker, acetylated α-tubulin (Ac-tub; green) with Ahi1-knockdown cells showing significantly fewer cilia, as previously described (Hsiao et al., 2009). DNA was labeled with Hoechst (blue). (**E**) Migration analysis expressed as a percentage of Scramble and ShRNA-Ahi1 cells towards 10%-FBS using transwell inserts with Ahi1-knockdown cells showing significantly impaired migration. Cell cultures were serum-starved overnight and equal number of cells were seeded on top of the inserts and incubated for 6 h. The number of cells that migrated to the bottom of the well (10%-FBS) were counted using nuclei staining (with Hoechst) and fluorescent microscopy. Experiments were performed in triplicate. Cell counts were obtained in at least ten fields at 20x magnification per experiment. Error bars represent the s.e.m. Significance was determined by unpaired two-tailed *t* test (****, $P<0.0001$).

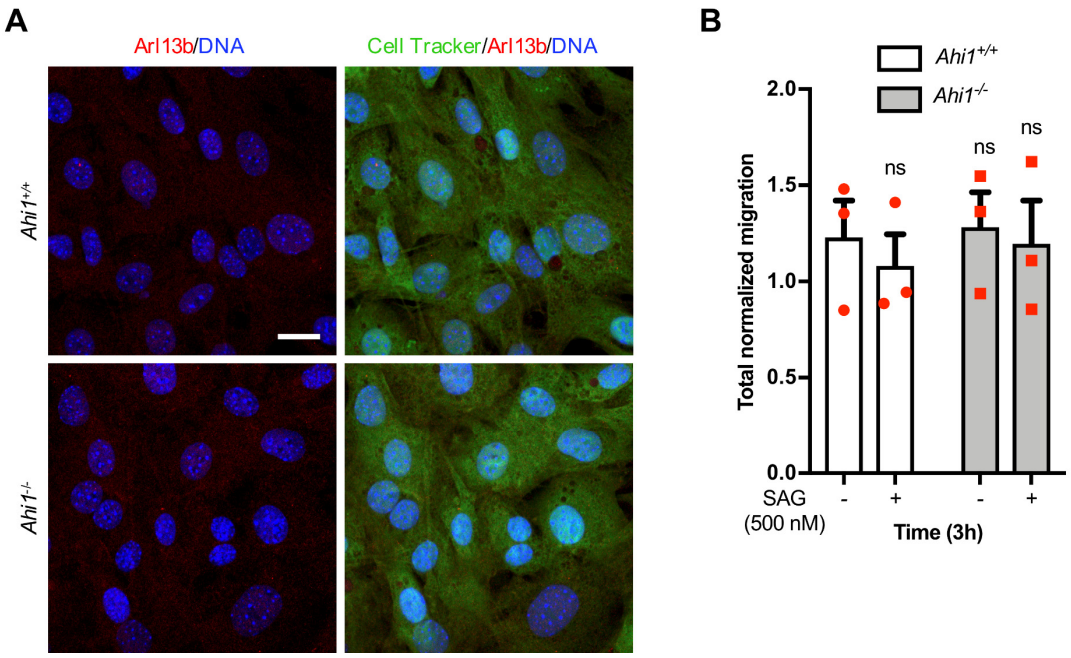

Figure S7. Chemotactic response to a SAG stimulus in non-ciliated Ahi1-depleted fibroblasts. Experiments were performed similarly as in (Mariani et al., 2016). Briefly, after 24 h of plating, *Ahi1*$^{+/+}$ and *Ahi1*$^{-/-}$ mouse embryonic fibroblasts (MEFs) were labeled with 5 μM of fluorescent Cell Tracker Green CMFDA (Thermo Fisher Scientific) and either fixed and immunolabeled for Arl13b (red; DNA was labeled with Hoechst (blue), scale bar, 20 μm) (**A**) or their chemotactic response monitored to SAG or vehicle (DMSO) by using FluoroBlok Transwell inserts for 3 h (8 μm pore size) (BD Falcon) (**B**). Cells were detached with 5 mM EDTA and transferred to the inserts at a density of 2 x 10^4 cells/well. Fluorescence in the bottom compartment was measured in a VICTOR Nivo Microplate Reader. Migration index is reported as Relative Fluorescent Units (RFU) over time. No significant differences in migration to SAG were obtained between wild-type and Ahi1-null cells. Error bars represent the s.e.m. Significance was determined by two-way ANOVA (ns=not significant).

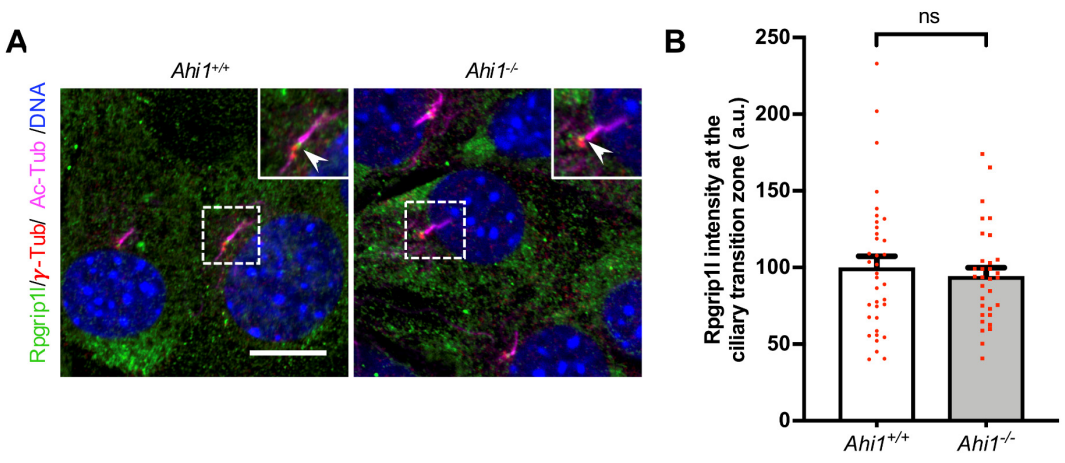

Figure S8. Rpgrip1l levels at the transition zone are normal in *Ahi1*-null cells. (**A**) *Ahi1*$^{+/+}$ and *Ahi1*$^{-/-}$ mouse embryonic fibroblasts (MEFs) were serum-deprived for 48 h and immunolabeled for Rpgrip1l (green), γ-Tubulin (γ-Tub) (red) and acetylated α-tubulin (Ac-Tub) (magenta). DNA was labeled with Hoechst (blue). Arrowheads point to Rpgrip1l localization at the ciliary transition zone. Insets show magnified images of the boxed regions. Scale bar, 30 µm. (**B**) Quantification of Rpgrip1l intensity at the ciliary transition zone in images acquired with a confocal microscope (LSM 880, Zeiss) with Airyscan. Rpgrip1l relative levels (green) at the transition zone was considered as the labeled area between γ-Tub (red) and the proximal part of the axoneme labeled with Ac-Tub (magenta). n>30 cells (n=2/genotype), each red point represents a measurement for a single cilium. Error bars represent the s.e.m. Significance was determined by the Mann-Whitney test, ns=not significant.