

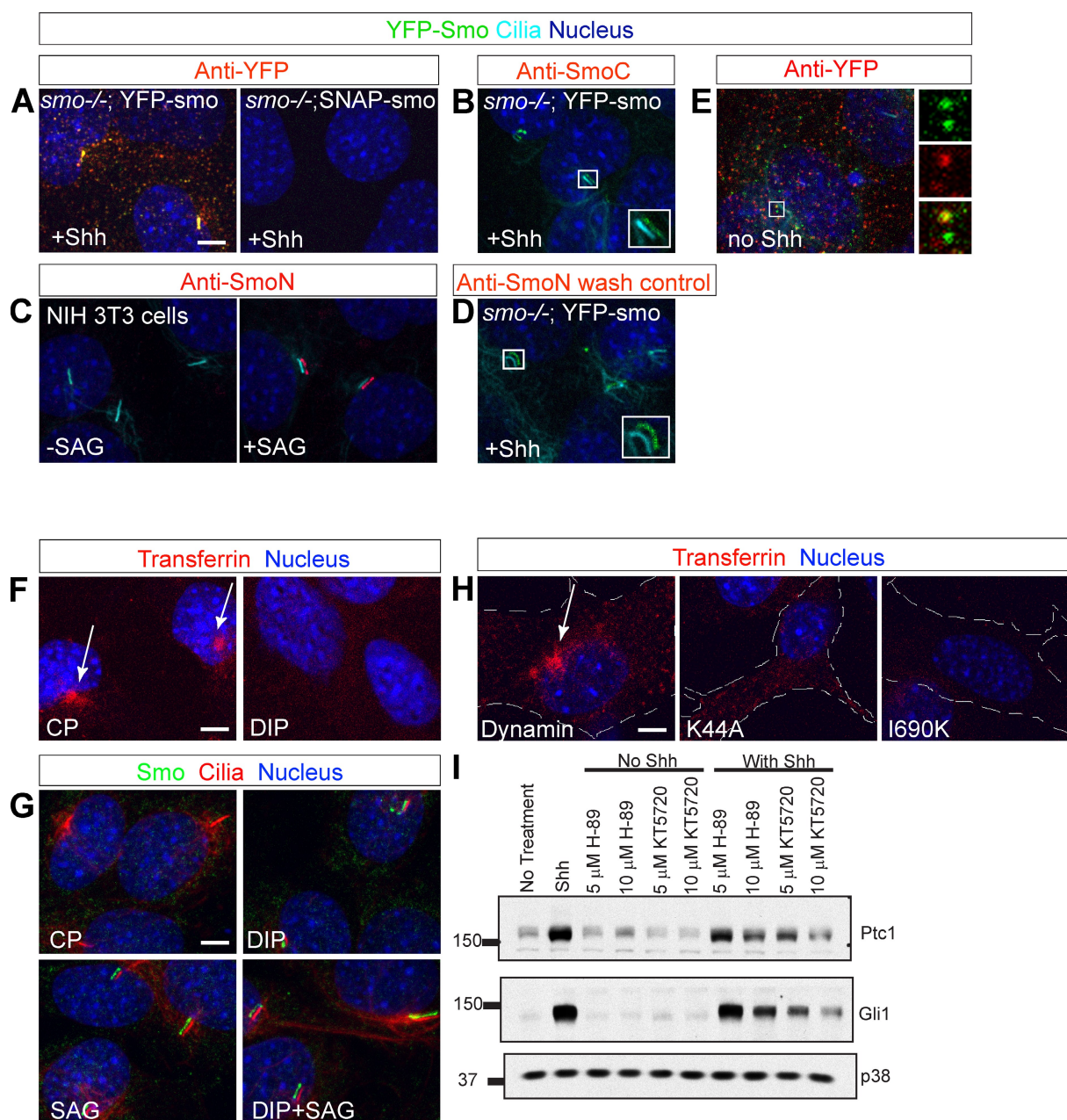
Milenkovic et al., <http://www.jcb.org/cgi/content/full/jcb.200907126/DC1>

Figure S1. Control experiments support the results shown in Figs. 2, 4, and 5. (A–E) Controls for Fig. 2 (A–E), Fig. 4 (F and G), and Fig. 5 I. (A) Anti-YFP is specific because it binds and aggregates Smo only when added to Shh-treated (2 h) *smo*^{-/-} cells producing YFP-Smo but not cells producing SNAP-Smo (which lacks the YFP epitope). (B) The anti-SmoC antibody does not label Shh-treated live, intact YFP-Smo cells because the epitope recognized by the antibody (Fig. 2 A) is intracellular. (C) Anti-SmoN antibody is specific because it only stains cilia when added to intact SAG-treated cells. The cilia of untreated cells are devoid of Smo and should show no staining. (D) Media from cells treated with anti-SmoN and extensively washed does not contain residual anti-SmoN because it does not label Smo present at cilia of cells pretreated with Shh. (E) The YFP-Smo aggregates produced by the anti-YFP can be found either on the surface of the cell, detected by the secondary antibody in nonpermeabilized cells (red), or internalized and therefore nonaccessible to the secondary antibody, showing only YFP fluorescence (green). Blocking dynamin-dependent endocytosis does not prevent Smo accumulation in cilia. Insets show enlarged views of the boxed regions. (F and G) DIP but not a control scrambled peptide (CP) blocks the endocytosis of the transferrin receptor (F) but does not affect the SAG-induced accumulation of Smo in primary cilia (G). DIP treatment alone induces Smo movement into cilia probably because it causes an increase in the surface-localized pool of Smo, which is analogous to how Smo overexpression causes constitutive Smo accumulation in cilia (Rohatgi et al., 2009). (H) GFP-tagged dominant-negative mutants of dynamin (K44A and I690K) block the endocytosis of transferrin but wild-type dynamin has no effect. Transfected cells are outlined with broken lines. Arrows in F and H indicate transferrin that has been internalized into a perinuclear compartment. (I) PKA inhibitors block Shh pathway activation. Whole-cell lysates made from NIH3T3 cells treated with the PKA inhibitors H-89 or KT-5720 in the presence or absence of Shh for 8 h were analyzed by immunoblotting with antibodies against Gli1 and Ptc1 (proteins encoded by two Hh target genes) and p38 (a loading control). H-89 and KT-5720 blocked the Shh-induced increase in Ptc1 and Gli1 protein levels in a dose-dependent manner. Bars, 5 μ m.

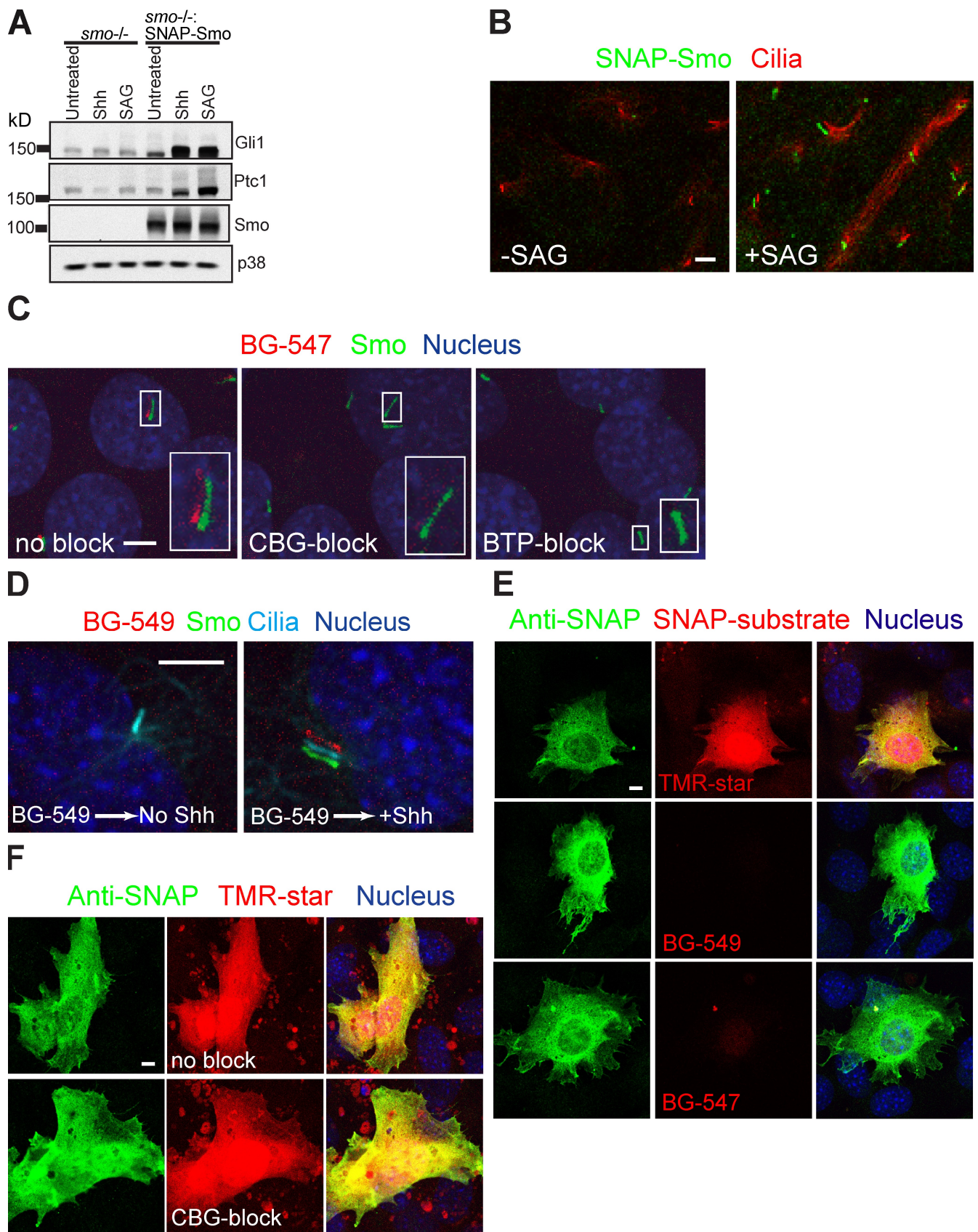


Figure S2. **Characterization of SNAP-Smo cells and SNAP substrates.** *smo^{-/-}* cells producing SNAP-Smo (SNAP-Smo cells) respond appropriately to Shh and SAG by several measures. (A) Western blot analysis showed that Shh exposure (24 h) can induce the Hh target genes *ptc1* and *Gli1* in SNAP-Smo cells but not in the parental *smo^{-/-}* cells. (B) SNAP-Smo translocates to primary cilia in response to SAG. SNAP-Smo cells were treated with SAG (24 h), fixed, and then stained with antibodies to show Smo (green) and cilia (red). Images are shifted overlays of the color channels. (C) Non-cell-permeable fluo-

rescent SNAP substrates can label SNAP-Smo at primary cilia. Images are shown as shifted overlays of the color channels. SNAP-Smo cells pretreated with Shh to induce SNAP-Smo accumulation in cilia were labeled with BG-547 (red), a non-cell-permeable fluorescent SNAP substrate, then fixed and stained with anti-SmoC (green) to independently label SNAP-Smo. Pretreatment with either nonpermeable (CBG block) or cell-permeable (bromothienylpteridine [BTP] block) nonfluorescent blocking substrates prevented labeling by BG-547. Insets show enlarged views of the cilia visible in the small boxes. (D) SNAP-Smo cells were pulse-labeled with BG-549 (red) and chased with a blocking substrate (BTP block) in the presence of Shh (2 h). (E) Non-cell-permeable SNAP substrates do not leak into cells. NIH3T3 cells transfected with a construct encoding free SNAP protein, which becomes distributed throughout the cytoplasm, were labeled (1 h) with TMR-Star (a cell-permeable SNAP substrate) or with BG-547 and BG-549, two non-cell-permeable SNAP substrates. The signals for all substrates are shown in red, and staining with an anti-SNAP antibody (green) was used to identify transfected cells. (F) Cells transfected with the same SNAP construct as in E were treated with a non-cell-permeable CBG block followed by labeling with TMR-star. Pretreatment with CBG block does not decrease the amount of TMR-Star staining, showing that CBG block does not enter cells. Bars, 5 μ m.

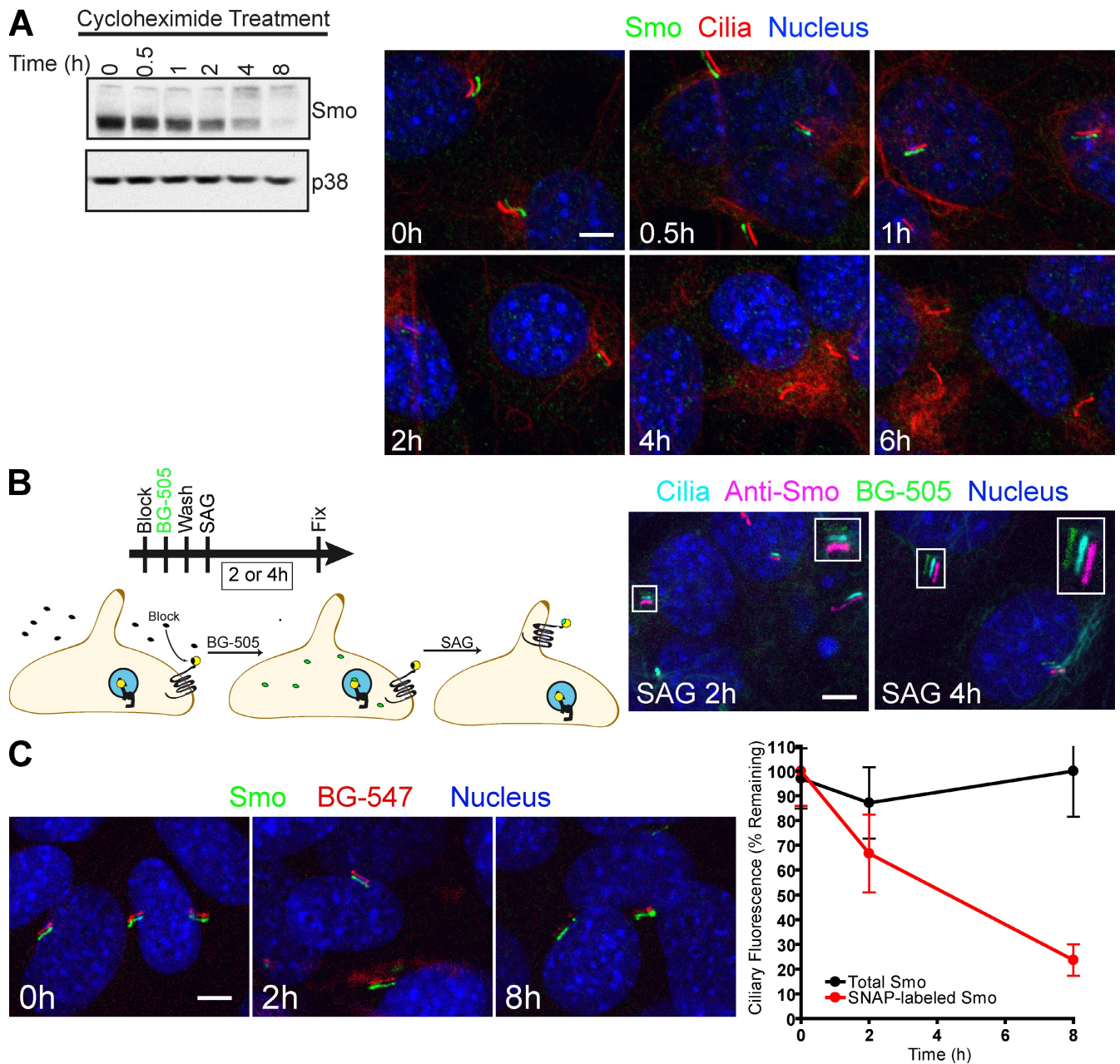


Figure S3. Smo protein undergoes constant turnover. (A) Cycloheximide chase experiments to measure the half-life of total Smo protein and the pool of Smo protein localized in primary cilia. NIH3T3 cells were induced with Shh for 16 h before the addition of cycloheximide (in the continued presence of Shh) to block protein synthesis for the indicated periods of time. The level of total Smo was measured by immunoblotting, and the pool of Smo localized in cilia was detected by immunostaining. (B) Kinetics of intracellular Smo movement to primary cilia in response to Shh. The experiment's outline is diagrammed. The intracellular pool of Smo was labeled with the cell-permeable substrate BG-505 before the addition of 100 nM of the Smo agonist SAG for 2 or 4 h. Total ciliary Smo was labeled at the end of the experiment with anti-SmoC (fuchsia). Although total ciliary Smo was unchanged between 2 and 4 h after induction, intracellular Smo was present in cilia in higher levels at 4 h. Insets display enlarged views of the boxed regions. (C) Pulse-chase analysis shows that Smo at cilia undergoes constant turnover under steady-state conditions. After 24 h of Shh treatment, SNAP-Smo cells were labeled with BG-547 (red) and chased with a cell-permeable blocking substrate (BTP block), in the presence of Shh, for an additional 8 h. Steady-state conditions are measured because total Smo in cilia (green), detected by anti-SmoC antibody, does not change. The mean (\pm SEM) level of BG-547 or total Smo fluorescence at cilia shows that the level of total Smo is stable, whereas pulse-labeled BG-547 Smo undergoes turnover. Bars, 5 μ m.