Fig. S3

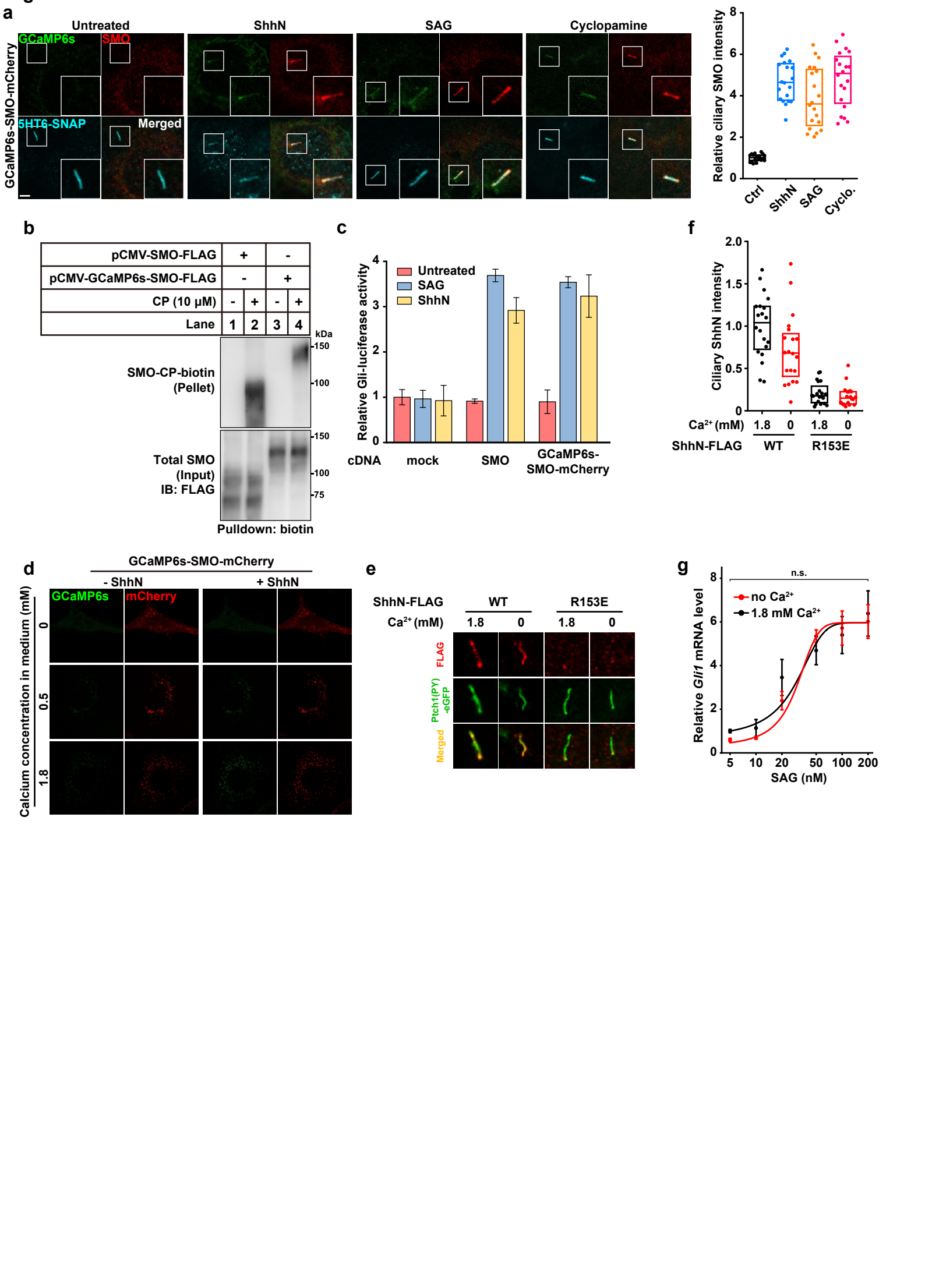
a
Untreated
ShhN
SAG
Cyclopamine
GCaMP6s
SMO
5HT6-SNAP
Merged
GCaMP6s-SMO-mCherry
Relative ciliary SMO intensity
Ctrl
ShhN
SAG
Cyclo.
b
pCMV-SMO-FLAG
pCMV-GCaMP6s-SMO-FLAG
CP (10 μM)
Lane
kDa
SMO-CP-biotin (Pellet)
Total SMO (Input) IB: FLAG
Pulldown: biotin
c
Relative Gli-luciferase activity
Untreated
SAG
ShhN
cDNA
mock
SMO
GCaMP6s-SMO-mCherry
f
Ciliary ShhN intensity
Ca2+ (mM)
ShhN-FLAG
WT
R153E
d
GCaMP6s-SMO-mCherry
- ShhN
+ ShhN
GCaMP6s
mCherry
Calcium concentration in medium (mM)
e
ShhN-FLAG
WT
R153E
Ca2+ (mM)
FLAG
Ptch1(PY)-eGFP
Merged
g
n.s.
no Ca2+
1.8 mM Ca2+
Relative Gli1 mRNA level
SAG (nM)

Fig. S3. Validation of GCaMP6s-SMO-mCherry-FLAG reporter and the effect of extracellular Ca^{2+}.

a. Left panel: confocal microscopy images of ciliary localization of GCaMP6s-SMO-mCherry-FLAG upon treatment with ShhN, SAG or cyclopamine. NIH3T3 cells transfected with GCaMP6s-SMO-mCherry-FLAG and 5-hydroxytryptamine subtype 6 receptor (5HT6)-SNAP (a marker for primary cilia) plasmids grown in round-bottom dish were allowed to reach confluent and serum starved in 0.5% FCS overnight. Then cells were treated with ShhN-conditioned medium (1:6), SAG (100 μM) or cyclopamine (5 μM) for 4 hours. 1 hour before imaging, SNAP-Cell 647-SiR (1 μM) substrate was used to label cells for 30 min and was washed out. Images were taken after 30 min. Bar = 5 μm. Right panel: Quantification of ciliary SMO intensities upon indicated treatment. n = 20.

b. Analysis of GCaMP6s-SMO-FLAG cholesterylation in HEK-293T cells.

c. Gli-luciferase activity of SMO(WT) and GCaMP6s-SMO-mCherry in *Smo*-null NIH3T3 cells.

d. Representative confocal images of GCaMP6s intensities in NIH3T3 cells in different concentrations of Ca^{2+}-containing media. Related to Fig. 2e. Bar=10 μm.

e. Effect of extracellular Ca^{2+} on ShhN-PTCH1 interaction. ShhN-FLAG were produced in HEK-293T cells transfected with pCMV-ShhN(1-198)-FLAG as a secreted protein, concentrated 50 folds, and dialyzed against PBS. NIH3T3 cells were transfected with the cilium-retention mouse PTCH1(PY)-eGFP (harboring Y631F/Y1320F mutations) cDNA and let grown to sub-confluent. Cells were serum-starved in 0.5% FCS overnight and treated with or without 1:50 diluted ShhN-FLAG supernatant in Ca^{2+}-free or Ca^{2+}-containing media. Cells were then briefly rinsed twice with cold PBS, fixed with 4% PFA, and stained with anti-FLAG antibody. Bar=2 μm.

f. Quantification of ciliary ShhN in **e**. n=20 per condition.

g. SAG-induced *Gli1* expression is independent of extracellular Ca^{2+} concentration. Sub-confluent NIH3T3 cells were serum-starved in 0.5% FCS overnight and switched to normal DMEM or Ca^{2+}-free DMEM containing indicated concentration of SAG.

Cells were harvested for RNA extraction after 6 hours. Error bars represent s.d..

p=0.2944, Two-way ANOVA with Tukey’s test.