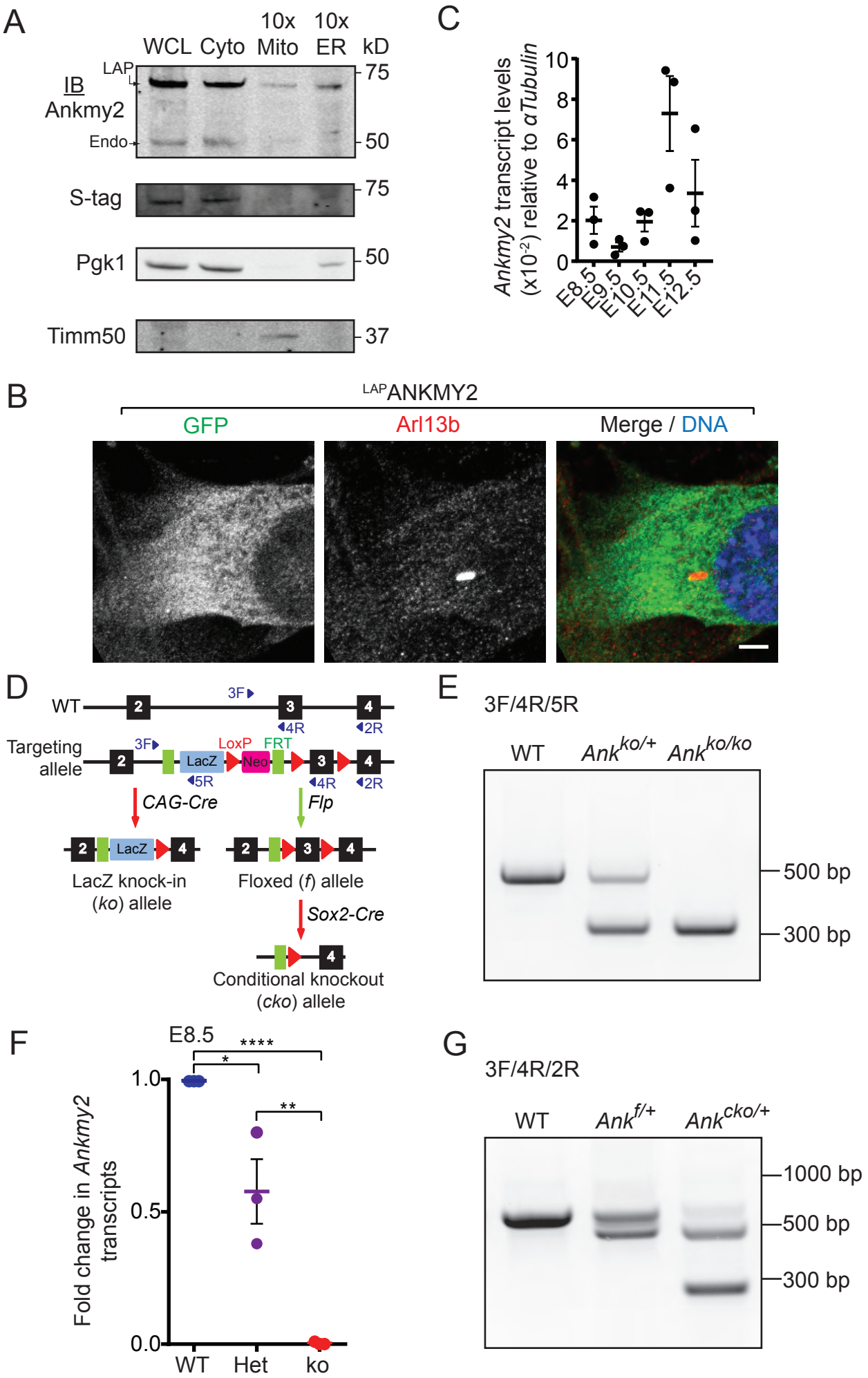
A
WCL
Cyto
10x Mito
10x ER
kD
LAP
IB Ankmy2
Endo
75
50
S-tag
75
Pgk1
50
Timm50
37
C
Ankmy2 transcript levels (x10-2) relative to αTubulin
10
8
6
4
2
0
E8.5
E9.5
E10.5
E11.5
E12.5
B
LAPANKMY2
GFP
Arl13b
Merge / DNA
D
WT
3F
4R
2R
Targeting allele
LoxP
FRT
LacZ
Neo
5R
CAG-Cre
Flp
LacZ knock-in (ko) allele
Floxed (f) allele
Sox2-Cre
Conditional knockout (cko) allele
E
3F/4R/5R
WT
Ankko/+
Ankko/ko
500 bp
300 bp
F
E8.5
****
*
**
Fold change in Ankmy2 transcripts
1.0
0.5
0.0
WT
Het
ko
G
3F/4R/2R
WT
Ankf/+
Ankcko/+
1000 bp
500 bp
300 bp

Figure S1, related to Figure 1. *Ankmy2* knockout mice are embryonic lethal at 10-12 somite stage and exhibit completely open neural tubes.

(A) $^{GFP\text{-}TEV\text{-}Stag}$ANKMY2 stably expressed in NIH 3T3 cells was predominantly found in the cytoplasmic fraction (Methods). (Abbreviations: WCL, whole cell lysate; Cyto, cytoplasmic fraction; Mito, mitochondrial fraction; ER, endoplasmic reticulum fraction). LAP, $^{GFP\text{-}TEV\text{-}Stag}$ANKMY2; endo, endogenous Ankmy2.

(B) Immunofluorescence image showing subcellular localization of ^{LAP}ANKMY2 in NIH 3T3 cells.

(C) qRT-PCR of *Ankmy2* transcript normalized to *αTubulin* in whole embryo extracts of the developing wild type mouse embryo from E8.5 to E12.5. N=3 embryos per time point.

(D) Cartoon depicting the knockout strategy of *Ankmy2*. Blue arrowheads mark primer sites.

(E) Genotyping for wild type, *Ankmy2* knockout and heterozygous alleles using designated primers shown in (D).

(F) qRT-PCR of *Ankmy2* transcript normalized to *αTubulin* in whole embryo extracts of the designated embryos at E8.5. N=3 embryos each.

(G) Genotyping for *Ankmy2* conditional knockout allele using designated primers shown in (D).

Abbreviations: Ank, Ankmy2.

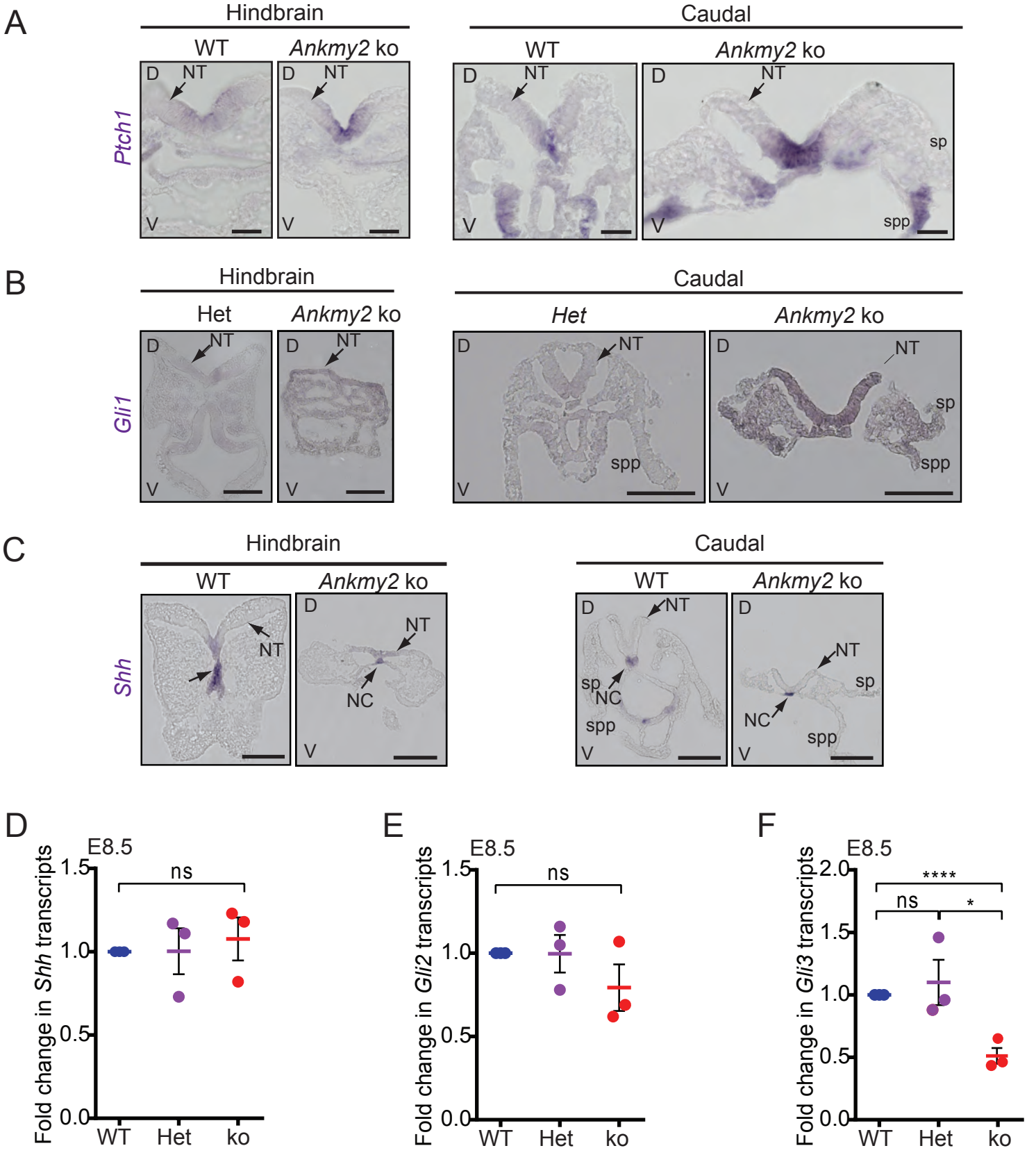
A
Hindbrain
Caudal
WT
Ankmy2 ko
WT
Ankmy2 ko
Ptch1
D
NT
V
sp
spp
B
Hindbrain
Caudal
Het
Ankmy2 ko
Het
Ankmy2 ko
Gli1
NT
spp
sp
C
Hindbrain
Caudal
WT
Ankmy2 ko
WT
Ankmy2 ko
Shh
NT
NC
sp
spp
D
E8.5
ns
Fold change in Shh transcripts
1.5
1.0
0.5
0.0
WT
Het
ko
E
E8.5
ns
Fold change in Gli2 transcripts
F
E8.5
****
ns
*
Fold change in Gli3 transcripts
2.0

Figure S2, related to Figure 2. *Ankmy2* knockout embryos exhibit increased Hh signaling.

(A) RNA *in situ* hybridization for *Ptch1* showed increased expression in the *Ankmy2* knockout embryo at E8.5 in transverse sections of hindbrain and caudal regions. N=5 (control), 4 (ko) embryos.

(B) RNA *in situ* hybridization for *Gli1* showed increased expression in the *Ankmy2* knockout at E8.5 in the caudal region, but not in hindbrain region as shown in the transverse sections. N=3 (control), 2 (ko) embryos.

(C) RNA *in situ* hybridization for *Shh* at E8.5 showed occasionally higher levels in hindbrain, but no difference in caudal region compared to wild type as indicated by the transverse sections of the designated regions. N=4 (control), 5 (ko) embryos.

(D – F) qRT-PCR data of *Shh* (D)*, Gli2* (E) and *Gli3* (F) transcripts normalized to *αTubulin* in whole embryo extracts at E8.5. *Shh* and *Gli2* indicated no difference in expression of mRNA in the *Ankmy2* knockout (ko) embryos while *Gli3* transcript levels are significantly reduced in ko compared to the wild type (WT) and heterozygote (Het) embryos. N=3 embryos/genotype. Data shown as mean ± SEM.

Scale: A-C, 50 μm. Abbreviations: D, dorsal; V, ventral; sp, somatopleure; spp, splanchnopleure; NT, neural tube; NC, notochord. *, $p<0.05$; ****, $p<0.0001$, as determined by unpaired t-test.

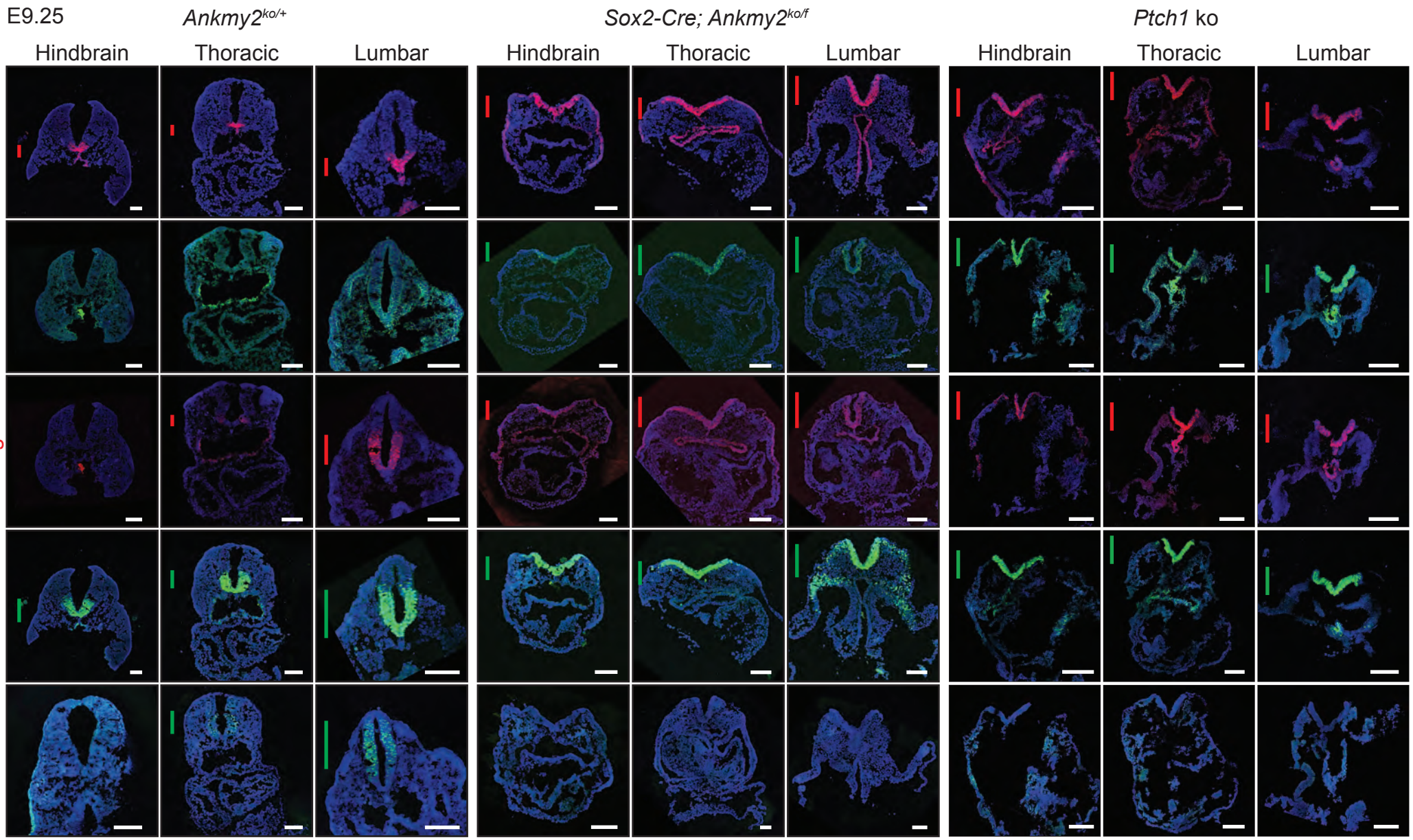
E9.25
Ankmy2ko/+
Sox2-Cre; Ankmy2ko/f
Ptch1 ko
Hindbrain
Thoracic
Lumbar
Hindbrain
Thoracic
Lumbar
Hindbrain
Thoracic
Lumbar
FoxA2
Nkx2.2
Olig2
Nkx6.1
Pax6

Figure S3, related to Figure 3. *Ankmy2* knockout mice exhibit a ventralized neural tube.

Horizontal sections from *Ankmy2*$^{ko/WT}$ (16 somites), *Sox2-Cre; Ankmy2*$^{ko/f}$ (12 somites) and *Ptch1* knockout (15 somites) dissected at E9.25 at hindbrain, thoracic and lumbar regions were stained for the indicated neural tube markers. N=2 embryos for control and *Sox2-Cre; Ankmy2*$^{ko/f}$, N=4 embryos for *Ptch1* knockout immunostained for all markers. All images are counterstained with DAPI. Scale: 100 μm.

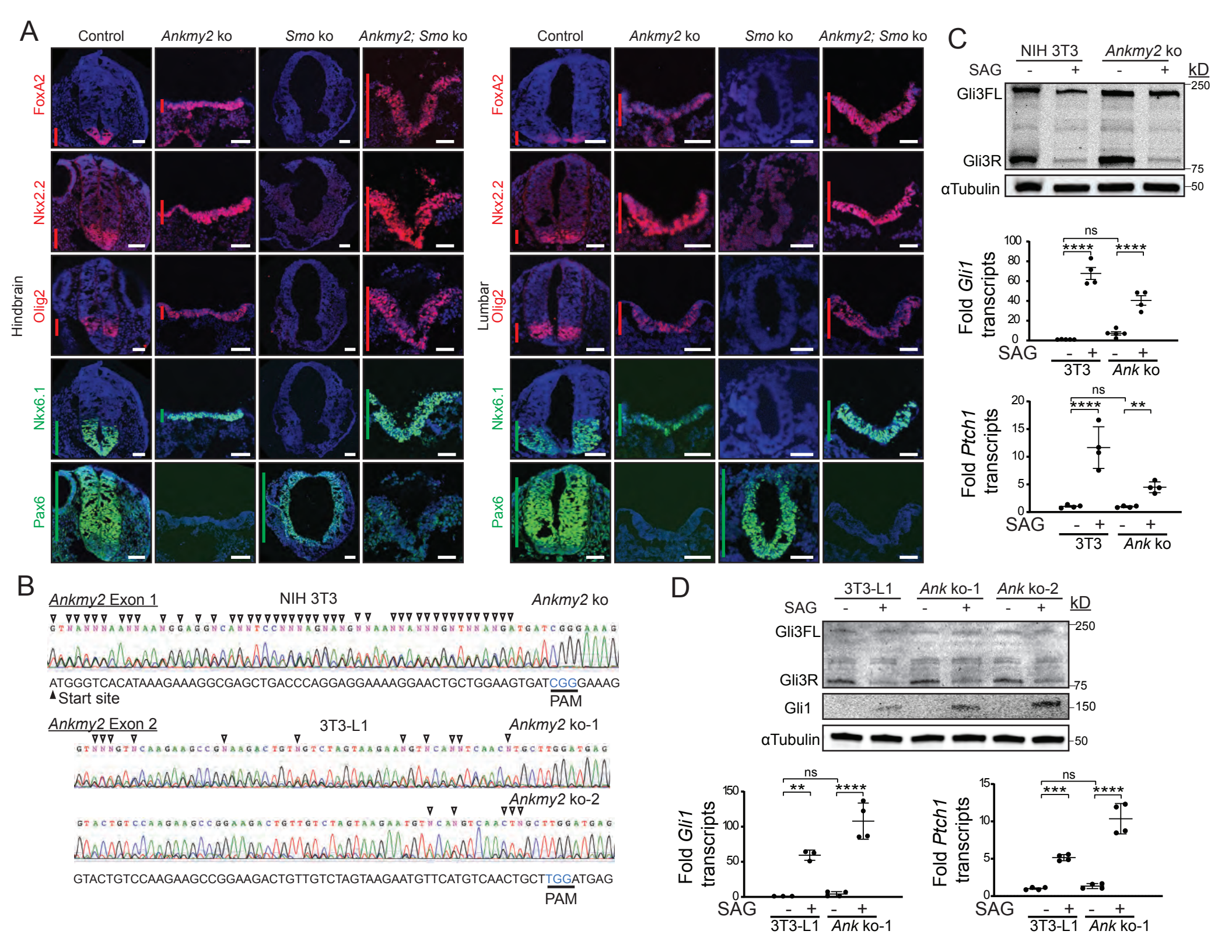
A
Control
Ankmy2 ko
Smo ko
Ankmy2; Smo ko
Hindbrain
Lumbar
FoxA2
Nkx2.2
Olig2
Nkx6.1
Pax6
B
Ankmy2 Exon 1
NIH 3T3
Ankmy2 ko
ATGGGTCACATAAAGAAAGGCGAGCTGACCCAGGAGGAAAAGGAACTGCTGGAAGTGATCGGGAAAG
Start site
PAM
Ankmy2 Exon 2
3T3-L1
Ankmy2 ko-1
Ankmy2 ko-2
GTACTGTCCAAGAAGCCGGAAGACTGTTGTCTAGTAAGAATGTTCATGTCAACTGCTTGGATGAG
PAM
C
NIH 3T3
Ankmy2 ko
SAG
kD
Gli3FL
Gli3R
αTubulin
250
75
50
Fold Gli1 transcripts
Fold Ptch1 transcripts
ns
****
**
3T3
Ank ko
D
3T3-L1
Ank ko-1
Ank ko-2
Gli1
***

Figure S4, related to Figure 4. Hh pathway activation in *Ankmy2* knockouts is independent of *Smo*.

(A) Neural tube sections of hindbrain and lumbar regions from control (16-18 somites), *Ankmy2* knockout (10-12 somites), *Smo* knockout (10 somites) and *Smo; Ankmy2* double knockout (10-12 somites) embryos dissected at E9.25 stained for ventral neural tube markers FoxA2, Nkx2.2, Olig2 and Nkx6.1, and dorsal marker Pax6. All images are counterstained with DAPI. Vertical bars show the extent of expression of the transcription factors. N=3 embryos for *Smo* ko and N=5 embryos for double ko. Scale: 50 μm.

(B) Sequence of *Ankmy2* exon1 and 2 targeted for CRISPR-Cas9 mediated knockout in NIH 3T3 cells and 3T3-L1 cells, respectively. Open arrowheads point to mutations. N, non-specific nucleotide.

(C) Immunoblotting in *Ankmy2* knockout lines in 3T3 cells as treated in Figure 4D show normal Gli3 processing. qRT-PCR shows a lack of significant basal upregulation of *Gli1* and *Ptch1* levels.

(D) Immunoblotting in *Ankmy2* knockout lines in 3T3-L1 cells as treated in Figure 4E show normal Gli3 processing and Gli1 protein levels, while qRT-PCR confirms lack of basal upregulation of *Gli1/Ptch1* transcripts.

Abbreviations: Ank, Ankmy2

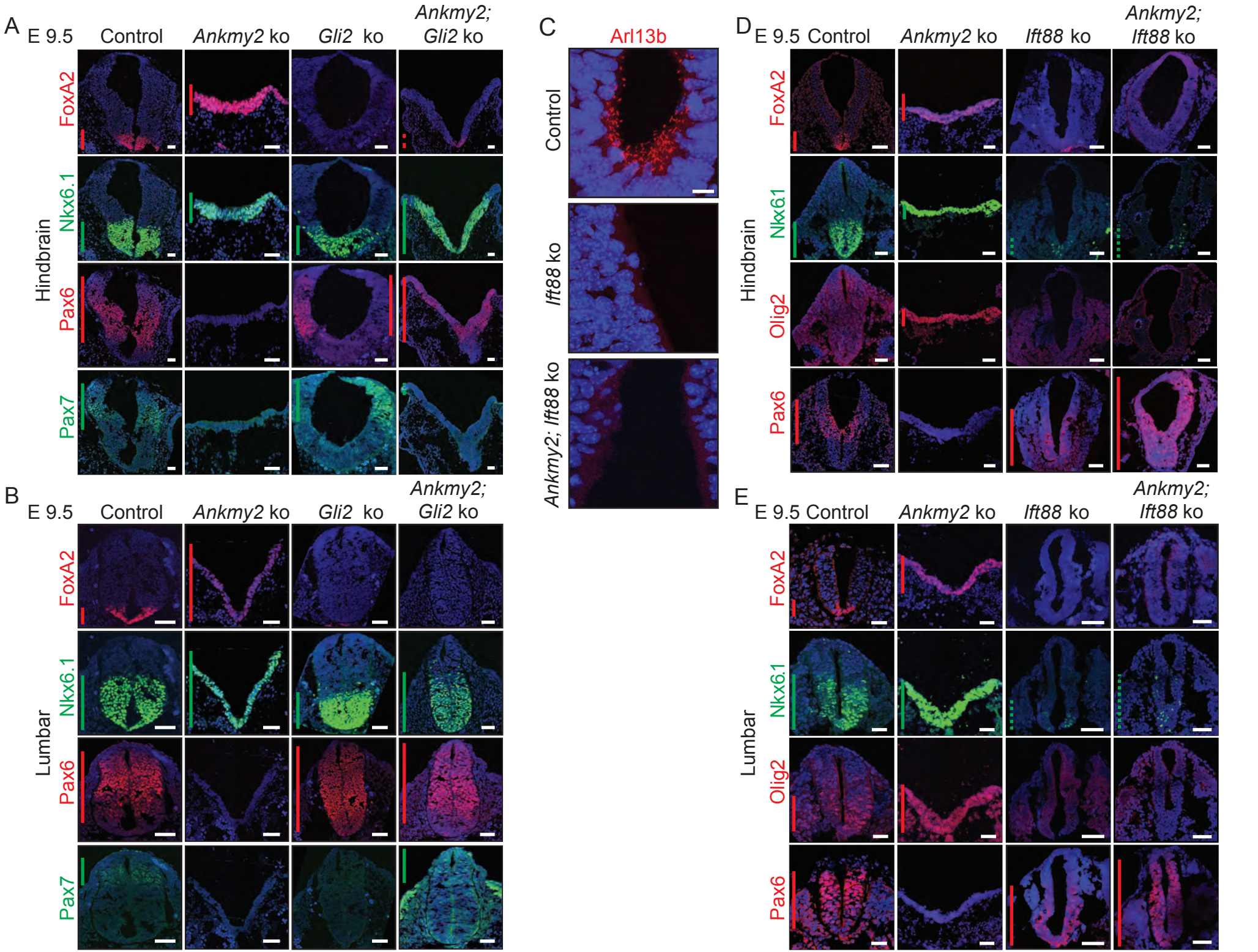
A
E 9.5
Control
Ankmy2 ko
Gli2 ko
Ankmy2; Gli2 ko
Hindbrain
FoxA2
Nkx6.1
Pax6
Pax7
B
Lumbar
C
Arl13b
Control
Ift88 ko
Ankmy2; Ift88 ko
D
Ift88 ko
Ankmy2; Ift88 ko
Nkx61
Olig2
E

Figure S5, related to Figure 5. Hh pathway activation in *Ankmy2* knockouts requires *Gli2* and *Ift88.*

(A – B) Neural tube sections of hindbrain (A) and lumbar (B) regions from control (*Ankmy2*$^{ko/+}$*; Gli2*$^{ko/+}$, 24-26 somites), *Ankmy2* knockout (12 somites), *Gli2* knockout (24-26 somites) and *Gli2; Ankmy2* double knockout (26-28 somites) embryos dissected at E9.5 stained for ventral neural tube markers FoxA2 and Nkx6.1, and dorsal markers Pax6 and Pax7. N=3 embryos each for *Gli2* ko and *Ankmy2; Gli2* double ko stained for each marker.

(C) E9.5 lumbar neural tube sections showing loss of cilia in *Ift88* single and *Ift88*; *Ankmy2* double knockout.

(D – E) E9.5 neural tube sections of hindbrain (D) and lumbar (E) regions from control (26-28 somites) *Ankmy2* knockout (12 somites), *Ift88* knockout (20-22 somites) and *Ift88* and *Ankmy2* double knockout (14-16 somites, 20-22 somites) embryos dissected at E9.5 stained for ventral neural tube marker FoxA2, mid-ventral markers Nkx6.1, Olig2 and dorsal marker Pax6. N=4 embryos for *Ift88* knockout and N=5 embryos for *Ankmy2; Ift88* double knockout stained for all markers except N=2 each for Olig2 immunostaining.

All images are counterstained with DAPI. Vertical bars show the extent of expression of the transcription factor. Dotted bars depict weak expression. Scale: 50 μm.

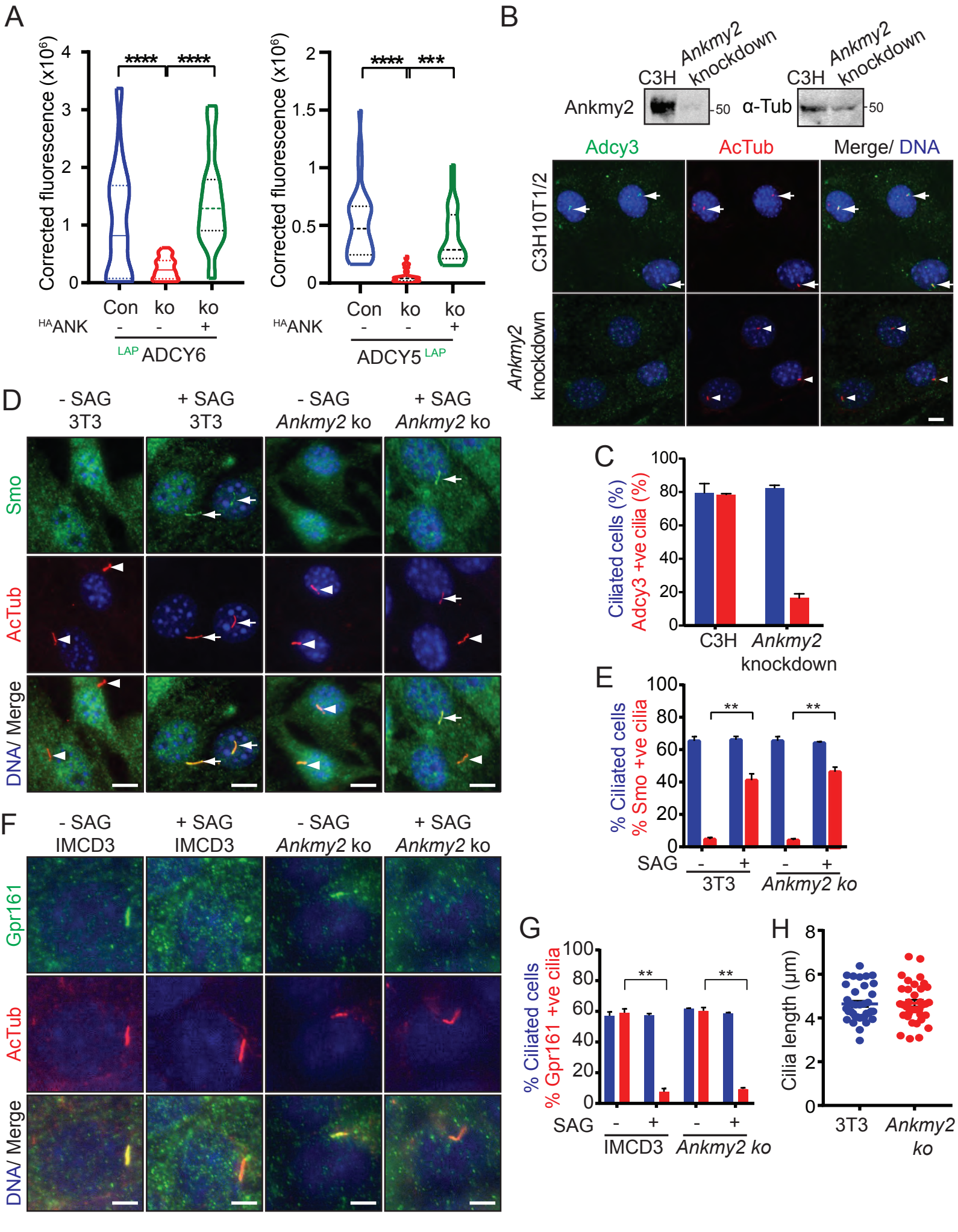
A
Corrected fluorescence (x10^6)
****
****
Con
ko
ko
HA ANK
-
-
+
LAP ADCY6
***
LAP ADCY5
B
C3H
Ankmy2 knockdown
Ankmy2
α-Tub
50
Adcy3
AcTub
Merge/ DNA
C3H10T1/2
Ankmy2 knockdown
C
Ciliated cells (%)
Adcy3 +ve cilia (%)
C3H
Ankmy2 knockdown
D
- SAG 3T3
+ SAG 3T3
- SAG Ankmy2 ko
+ SAG Ankmy2 ko
Smo
AcTub
DNA/ Merge
E
% Ciliated cells
% Smo +ve cilia
**
SAG
3T3
Ankmy2 ko
F
- SAG IMCD3
+ SAG IMCD3
- SAG Ankmy2 ko
+ SAG Ankmy2 ko
Gpr161
AcTub
DNA/ Merge
G
% Ciliated cells
% Gpr161 +ve cilia
IMCD3
Ankmy2 ko
H
Cilia length (μm)
3T3
Ankmy2 ko

Figure S6, related to Figure 6. ACs traffic to cilia in an Ankmy2-dependent manner.

(A) Violin plots showing fluorescence intensities of the ACs in **Figure 6A-B**. N=40-50 cilia each cell line.

(B) – (C) Endogenous levels of Adcy3 was reduced in tetraploid C3H10T1/2 cells upon CRISPR-mediated partial knockdown. Immunoblots in (B) show partial knockdown of Ankmy2. Scale: 5 μm. N>260 cells counted/condition from 3-4 different fields. Data represent mean ± SD.

(D) – (E) Ciliary localization of endogenous Smo was unaffected in *Ankmy2* knockout cells compared to control NIH 3T3 cells treated with ± SAG (500 nM) for 24 h upon starvation. N=150-300 cells counted/ cell line. Scale: 10 μm. Data represent mean ± SEM.

(F) – (G) Ciliary localization of endogenous Gpr161 was unaffected in *Ankmy2* knockout cells compared to control IMCD3 cells treated with ± SAG (500 nM) for 24 h upon starvation. N=250-400 cells counted/cell line. Scale: 10 μm. Data represent mean ± SEM.

(H) Cilia lengths of *Ankmy2* knockout (ko) cells were not significantly different from control NIH 3T3 cells. N=35 cilia/cell line counted. Data represent mean ± SEM.

**, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$ unpaired t-test.

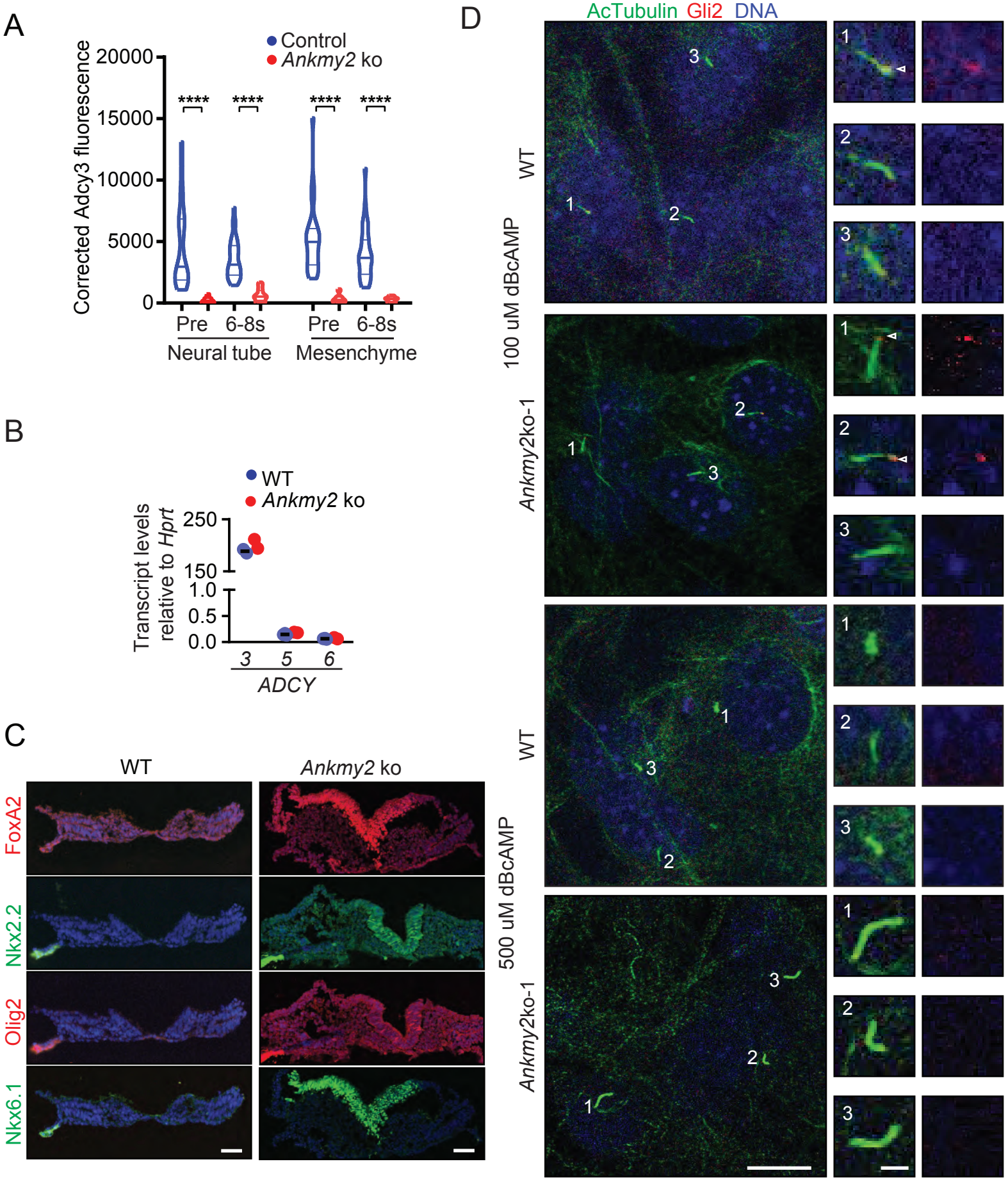
A
Control
Ankmy2 ko
Corrected Adcy3 fluorescence
20000
15000
10000
5000
0
****
****
****
****
Pre
6-8s
Pre
6-8s
Neural tube
Mesenchyme
B
WT
Ankmy2 ko
Transcript levels relative to Hprt
250
150
1.0
0.5
0.0
3
5
6
ADCY
C
WT
Ankmy2 ko
FoxA2
Nkx2.2
Olig2
Nkx6.1
D
AcTubulin Gli2 DNA
WT
100 uM dBcAMP
Ankmy2ko-1
WT
500 uM dBcAMP
Ankmy2ko-1

Figure S7, related to Figure 7. Adcy3 trafficking during neural tube development and role of cAMP signaling in Gli2 activation.

(A) Violin plots showing fluorescence intensities of Adcy3 in **Figure 7D**. N=30 cilia each time point and region.

(B) Whole embryo extracts at E8.5 indicated no difference in expression of *Adcy 3,5,6* transcripts in the *Ankmy2* knockout (ko) compared to the wildtype and normalized to *Hprt*.

(C) Individual immunostained images for neuroprogenitor markers shown in Figure 7C depict ventralization in the neural fold and predominantly overlapping ventral progenitor marker expression in the *Ankmy2* knockout at E8.25. Olig2 is the least ventralized marker at this stage.

(D) Immunofluorescence images showing Gli2 in wildtype (WT) and *Ankmy2* ko (ko-1) 3T3-L1 cells after treatment with 100 μM and 500 μM dBcAMP.

All images are counterstained with DAPI. Scale: C, 50 μm; D, 10 μm inset, 2 μm.