Supporting Information

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SI Materials and Methods

Mouse Strains. Animal work was carried out under Institutional Animal Care and Use Committee-approved protocols at Emory University. Mouse lines used were as follows: Ptch1^{tm1Bjw} (referred to as *Ptc$^{flox/flox}$*) (MGI: 2675356), Tg(Atoh1-cre/Esr1*)14Fsh (referred to as *Math1-CreER*) (MGI: 3615691), Arl13b^{tm1Tc} (referred to as *Arl13b$^{flox/flox}$*) (MGI: 4948239), Tg(CAG-cre/Esr1*)5Amc (referred to as *CAGG-CreER*) (MGI: 2182767), Tg(Neurod2-Smo*A1) 199Jols (referred to as *nD2::SmoA1*) (MGI: 3830738), and Tg(Atoh1-GFP)1Jejo (referred to as *Math1-GFP*) (MGI: 3703598).

Mouse Genotyping.

Reaction	Primers	Products
Arl13b $^{flox/flox}$ conditional	CondF: AGGACGGTTGAGAACCACTG Con200R: CGACCATCACAAGTGTCACC	WT 150 bp, flox 200 bp
Arl13b $^{flox/flox}$ deletion	CondF: AGGACGGTTGAGAACCACTG Con949R: AACTGGGACACCCAAATGAG	WT 809 bp, flox 949 bp, deleted 379 bp
Ptc$^{flox/flox}$ conditional	PtcFloxF: AGTACGAGCCATGCAAGACC PtchFloxR: CCACCAGTGATTTCTGCTCA	WT 307 bp, flox 347 bp
Generic Cre	TomCreF: TGACCCGGCAAAACAGGTAGTTA TomCreR: TTCCCGCAGAACCTGAAGATGTT	Cre 550 bp
WT positive control	WT3: CTGCGGCAAGTTTTTGGTTG WT4: AGGGCTTCTCGTTGGCTACAAG	WT 217 bp
nD2::SmoA1	E67Fwd: AATCTCTGCTCCTGCGTTGGG SmoRev: CTCGGTCAGTCTCACACTTG	Transgene 650 bp
Math1-GFP	Math1-GFP F: CTGACCCTGAAGTTCATCTGCACC Math-GFP R: TCCCTGTTGTAGTTGTACTCCAGC	Transgene 320 bp

Deletion Analysis. For PCR, mouse genomic DNA was isolated from ear punch biopsy before deletion (epidermis pretamoxifen) or from ear punch biopsy or liver biopsy at death (epidermis posttamoxifen, liver posttamoxifen) following overnight digestion in DirectPCR (Tail) lysis buffer (Viagen Biotech) containing 300 ug/mL proteinase K, followed by heat inactivation. PCR was performed using primer pairs to distinguish the Arl13b wild-type and floxed alleles (Arl13b conditional) or deleted (Arl13b deletion) allele. Semiquantitative PCR conditions were as follows: 25 or 30 cycles at 94 °C for 30 s, 59 °C for 30 s, and 72 °C for 45 s. Products were run out on 2% agarose TBE gels.

Tamoxifen Injections. Tamoxifen was prepared as previously described (28). For *Ptch1* and *Arl13b* deletion, pregnant dams were IP injected with 4 mg of tamoxifen on E14.5, and live pups were harvested via C-section on E19/P0 and cross-fostered to nursing CD-1 dams. For postnatal *Arl13b* deletion, pups were injected s.c. at P4, P6, and P8 or intraperitoneally at P14, P16, and P18 with 3 mg/40 g body weight tamoxifen.

Primary MB Culture. Mice were followed and killed by isoflurane inhalation after they developed symptoms of MB, which included head doming, hunched posture, preferential turning to one side, lethargy, and/or more than 15% weight loss. Tumors were isolated by gross dissection and minced in sterile HBSS to achieve a single-cell suspension. The suspension was passed through 100 μm and 40 μm cell strainers to remove extraneous tissue and cell aggregates before being spun down. The cell pellet was resuspended in Neurobasal medium (21103049; Thermo Fisher Scientific) supplemented with GIBCO B-27 (17504044; Invitrogen), glutamate, pyruvate, and Pen/Strep and plated at 2×10^6 cells per well in a 24-well plate on Matrigel-coated coverslips or wells (356234; BD Biosciences). Wells were infected with lentivirus at a multilplicity of infection (MOI) of 8 and treated with 3 μg/mL ShhN as appropriate 3 h after plating.

Tissue Handling, H&E, and Arl13b Staining. Adult mice were anesthetized via isoflurane inhalation, cleared via cardiac perfusion with ice-cold PBS, and fixed with ice cold 4% PFA. Brain tissues were harvested and postfixed overnight at 4 °C before being stored in 70% ethanol at 4 °C. Tissues were dehydrated, paraffin embedded, and sectioned at 10 μM thickness on a Leica RM2245 microtome. Arl13b staining was performed as previously described (56).

Western Blotting. Western blotting was performed as previously described (41, 57) with antibodies listed below. Gli1 and Gli2 lysates were made using RIPA buffer with Roche protease inhibitors (41) while other lysates were made using modified RIPA buffer and SigmaFast protease inhibitors (58). Values displayed are volume intensity as measured from a chemiluminescent image and normalized to total protein as measured on a stain-free gel.

Proliferation Labeling and Immunofluorescence Quantitation. Cellular proliferation was monitored by staining for either Ki67 or BrdU. For BrdU incorporation, 3 μg/mL BrdU was added to coverslip-containing wells 4 h before harvest. Coverslips were fixed in 4% PFA for 30 min, rinsed with 1× PBS, and treated with 2N HCl for 30 min to expose BrdU antigen, then blocked in 1× PBS + 0.1% Triton-X + 1% heat-inactivated sheep serum. Ki67 coverslips were not HCl-treated. Coverslips were mounted in ProLong Gold antifade reagent (P36934; ThermoFisher Scientific) and imaged using a 40× oil objective on an Olympus Fluoview FV1000 confocal microscope and Olympus Fluoview v4.2 or 20×/40× dry objectives on a Leica CTR6000 microscope with SimplePCI. Five to ten fields per coverslip were captured from two coverslips per condition for each of three biological replicates, except where noted. BrdU images were blinded to remove experimenter bias and quantified by hand. CellProfiler software was used to quantify Ki67 images by identifying nuclei and marker-positive cells; this process was automated to remove experimenter bias (59).

Antibodies Used.

Primary.

mouse anti-BrdU F(ab′)2 (B44), 347580, 1:500 IF; BD Biosciences

rabbit anti-Ki67, ab15580, 1:500 IF; Abcam

mouse anti-Arl13b, N295B/66, 1:1,000 WB (mouse samples), 1:500 IF; Neuromab

rabbit anti-Arl13b, 1711–1-AP, 1:1,000 WB (human samples); Proteintech

mouse anti-Gli1, L42B10; 1:1,000 WB; Cell Signaling

goat anti-Gli2, AF3635; 1:500 WB; R&D

goat anti-Gli3, AF3690, 1:1,000 WB; R&D

Secondary.

HRP-conjugated donkey anti-goat IgG, AP180P, 1:5,000 WB; Millipore

HRP-conjugated donkey anti-mouse IgG, 715–035-150, 1:2,000 WB; Jackson ImmunoResearch

HRP-conjugated donkey anti-rabbit IgG, NA934, 1:5,000 WB; GE Healthcare

goat F(ab')$_2$ anti-mouse 488, 115–546-003, 1:200 IF; Jackson ImmunoResearch

goat F(ab')$_2$ anti-mouse 647, 115–605-003, 1:200 IF; Jackson ImmunoResearch

donkey anti-rabbit IgG, Alexa Fluor 488 conjugate, A-21206 1:200 IF; ThermoFisher Scientific

Hoechst nuclear stain, 33,258, 1:3,000 IF; Acros

For *Arl13b* deletion in MEFs, cells were plated in six-well plates at a density of 3×10^5 cells per well. After 24 h, cells were rinsed and treated with no virus or adenoviral Cre in 0.5% FBS media. After 72 h, cells were treated with 0.5% FBS media or Shh CM and either 100 nM SAG, 5 μM cyclopamine, 100 nM SANT1, or a combination thereof. After 24 h, cells were harvested by trypsinization, spun down, and flash-frozen.

Quantitative Reverse Transcriptase. RNA extraction, reverse transcription, and qPCR were performed as previously described (57).

Target	Forward	Reverse
Ptch1	TGCTGTGCCTGTGGTCATCCTGATT	CAGAGCGAGCATAGCCCTGTGGTTC
Gli1	CTTCACCCTGCCATGAAACT	TCCAGCTGAGTGTTGTCCAG
Arl13b	CACGTCACTATGATTTCTATGGG	CCATCCGACTTGGTTATGAG
Pold3	ACGCTTGACAGGAGGGGGCT	AGGAGAAAAGCAGGGGCAAGCG
PTCH1	ACAAACTCCTGGTGCAAACC	CTTTGTCGTGGACCCATTCT
GLI1	GAACCTTCCTACCAGAGTCC	GTGCTGCTGCCCTATGTG
ARL13B	GAATCCAAGGAGAATACCCTG	CCAACACCAATATAGGCTTTCC
GAPDH	TCCTCTGACTTCAACAGCGACA	ATGGTACATGACAAGGTGCGG

Lentivirus Production and Infection. Lentivirus was produced using the Sigma MISSION Lentiviral Packaging Mix (SHP001) and Promega FuGENE 6 (E2691) according to the manufacturer's instructions. Lentiviral supernatants were concentrated 100× using the Clontech Lenti-X Concentrator (631231) before use for primary MB culture. All infections were done in 8 μg/mL polybrene.

Lentiviral Constructs.

Mouse.

scramble: Sigma MISSION pLKO.1-puro Non-Mammalian shRNA (SHC 002)

sh*Arl13b* 442: TGATGGTCGGCCTTGATAATG

sh*Arl13b* 968: GCTCAGGACACGATCTCATAA

Human.

scramble: Sigma MISSION TRC2 pLKO.5-puro Non-Mammalian shRNA (SHC 202)

sh*ARL13B*: CCTATATTGGTGTTGGCAAAT

Statistical Analysis. All statistical analysis was done using GraphPad Prism 7 software. A log-rank test was used to evaluate significance of the survival data. A two-way ANOVA followed by Fisher's LSD test and Bonferroni correction for multiple comparisons was used in all analyses that varied both genotype (Arl13b status) and treatment condition. Student's *t* test was used for proliferation comparison of human MB cell lines.

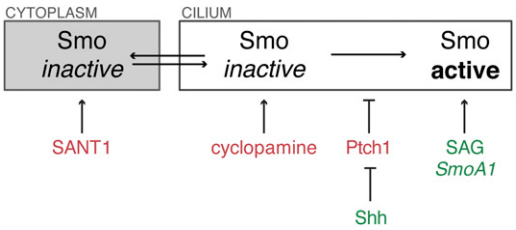

Fig. S1. Small-molecule drugs modulate Smo activation and localization. Pharmacological manipulation of Smo by small-molecule drugs produces three different Smo activation states: outside the cilium and inactive (SANT1), inside the cilium and inactive (cyclopamine), and in the cilium and activated (SAG). The *SmoA1* mutation directly activates Smo, as does SAG, which is distinct from the endogenous Smo activation that occurs through derepression by Ptch1 after Shh ligand binding. Figure adapted from ref. 12.

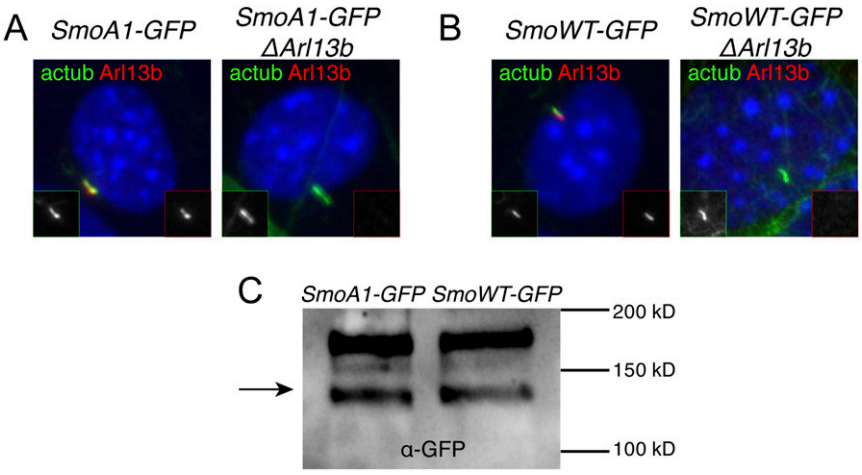

Fig. S2. Treatment with adenoviral Cre produces robust deletion of *Arl13b* in culture. (*A* and *B*) Treatment with adenoviral Cre in *Arl13b*$^{flox/flox}$ *SmoA1-GFP* (*A*) and *SmoWT-GFP* (*B*) stable lines results in robust loss of Arl13b after 72 h to allow for protein turnover. (Magnification, 40×.) (*C*) Western blot against GFP shows that, in *Arl13b*$^{flox/flox}$ MEF lines expressing *SmoA1-GFP* and *SmoWT-GFP*, the tagged Smo proteins express at comparable levels.

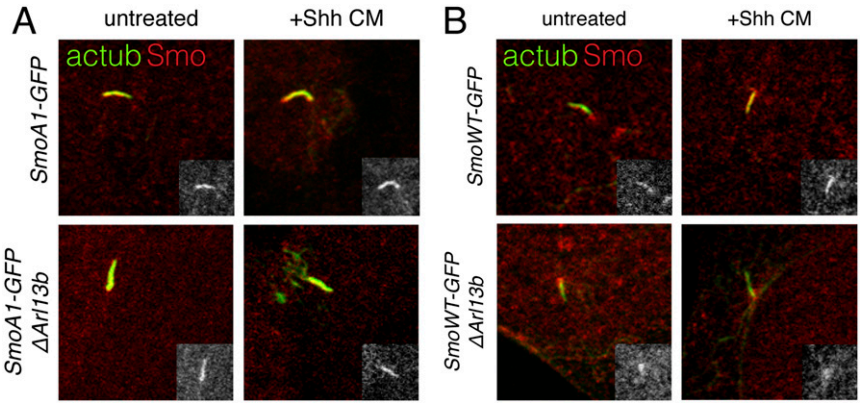

Fig. S3. Loss of Arl13b affects localization of SmoWT-GFP but not SmoA1-GFP. (*A*) SmoA1-GFP (as detected by an antibody against Smo) is present in the cilium regardless of Arl13b presence or Shh stimulation. (*B*) SmoWT-GFP is enriched in cilia upon Shh simulation. *Arl13b* deletion results in abnormally distributed ciliary SmoWT-GFP similar to endogenous Smo in *Arl13b*^{hnn} cilia. Actub, acetylated α-tubulin. (Magnification, 60×.)

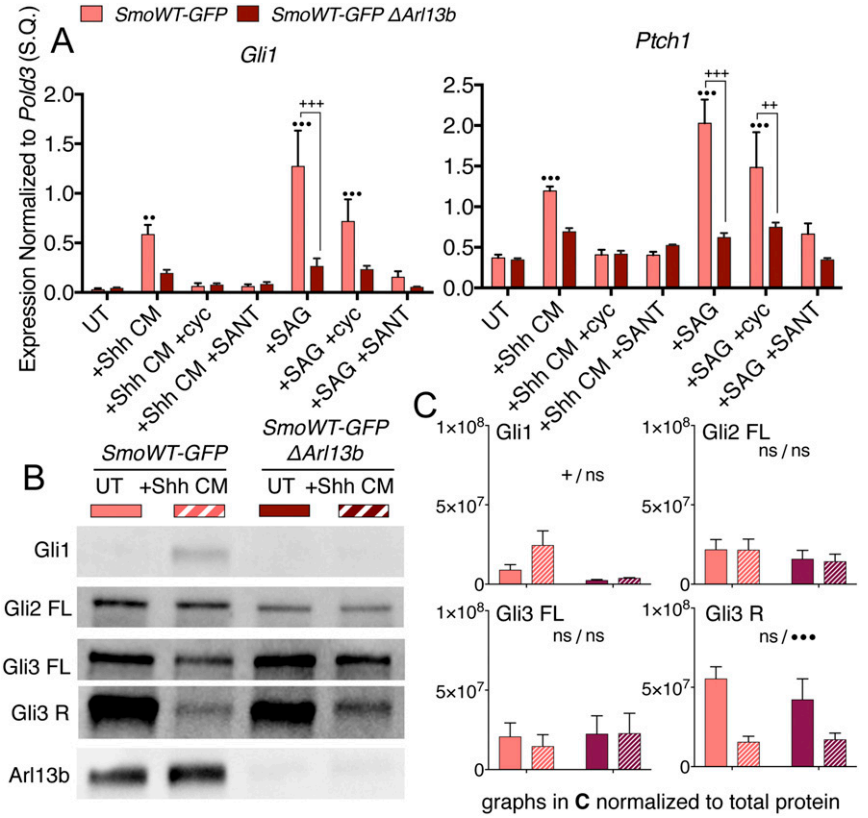

Fig. S4. *Arl13b* deletion in *SmoWT-GFP* stable lines decreases signaling output. (*A*) qRT-PCR for Shh targets *Gli1* and *Ptch1* shows that pathway activation is lowered when Arl13b is deleted. All data are mean ± SEM; $^{++}P < 0.005$ and $^{+++}P < 0.0005$ between genotypes within a given treatment; $^{\bullet\bullet}P < 0.005$ and $^{\bullet\bullet\bullet}P < 0.0005$ compared with untreated within a given genotype; two-way ANOVA with corrections for multiple comparisons. (*B*) Western analysis of Gli3 processing in untreated and Shh-stimulated *SmoWT-GFP* MEFs with and without Arl13b. Significant reduction in Gli3R levels occurs upon Shh stimulation with and without Arl13b. (*C*) Bar graphs show quantification of the Western blots represented in *B*. Data are mean ± SEM of at least three biological replicates; $^{+}P < 0.05$ represents genotype significance, $^{\bullet\bullet\bullet}P < 0.001$ represents treatment significance; S.Q., starting quantity.

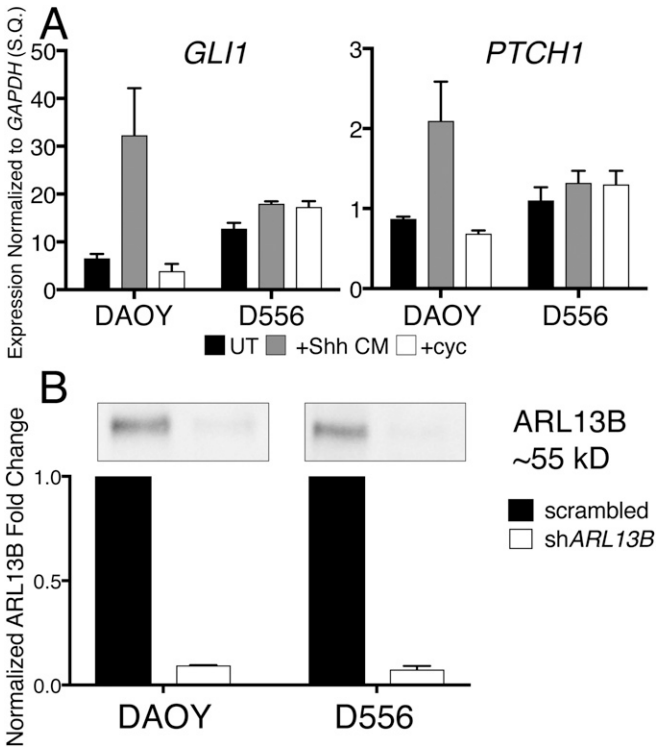

Fig. S5. DAOY and D556 cells respond differently to SHH modulation but effectively lose ARL13B after knockdown. (*A*) qRT-PCR shows expression of SHH targets *GLI1* and *PTCH1*. DAOY cells are responsive to Shh CM and cyclopamine, while D556 cells are not. Data are mean ± SEM of three biological replicates. S.Q., starting quantity. (*B*) Western blot for ARL13B shows that protein levels are reduced after knockdown of *ARL13B* by shRNA. Data are mean ± SEM of two biological replicates.

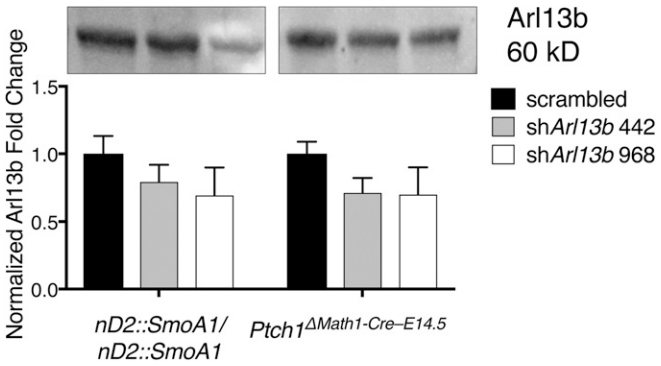

Fig. S6. shRNA against *Arl13b* reduces Arl13b protein level. Western blot for Arl13b shows that protein levels are reduced after knockdown of *Arl13b* by shRNA. Data are mean ± SEM of two biological replicates.

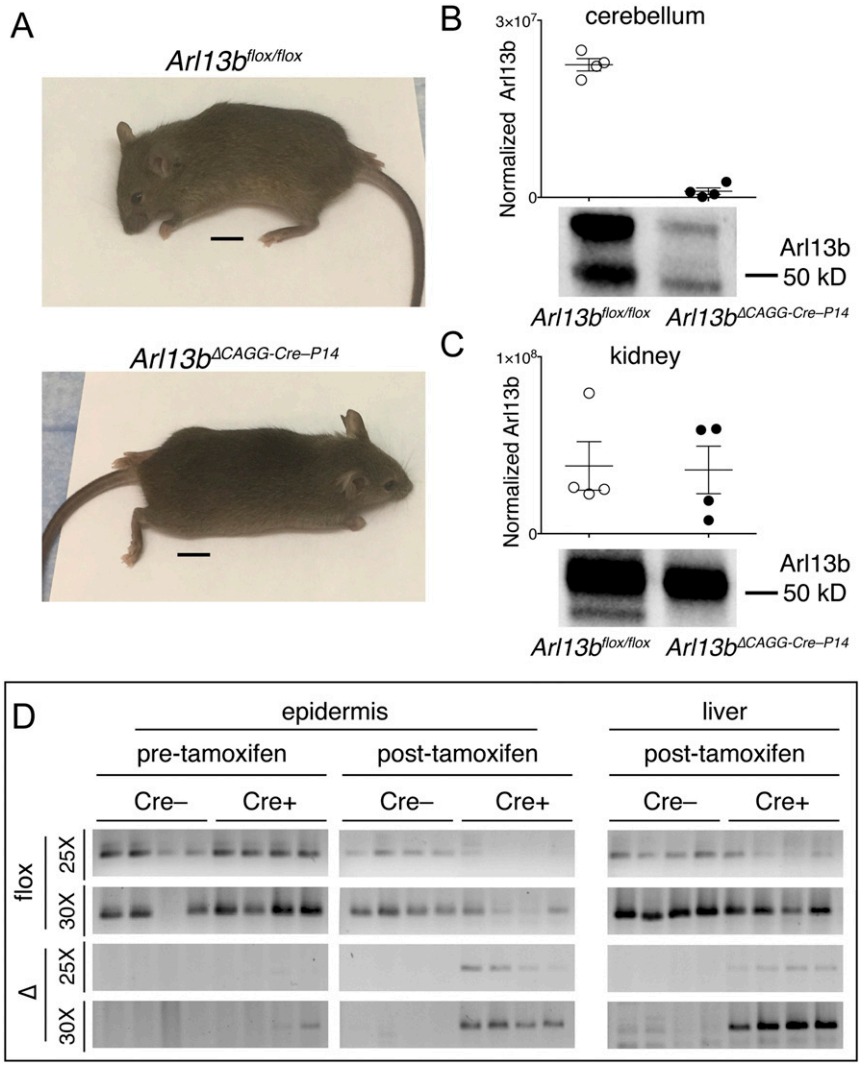

Fig. S7. Postnatal depletion of Arl13b at P14 results in grossly normal skin, liver, cerebellum, and overall appearance. (*A*) Cre-negative (Cre−) (*Top*) and *Arl13b*-depleted (*Bottom*) littermates are roughly the same size and do not show outward phenotypic differences. (*B* and *C*) Western blots for Arl13b in the cerebellum (*B*) and kidney (*C*) of Cre− and Cre+ littermates show level of Arl13b protein loss after tamoxifen treatment. Quantification shows four individual animals and mean ± SEM. (*D*) Semiquantitative PCR analysis of both the floxed allele (top two rows) and deleted allele (bottom two rows) at both 25 (linear amplification range) and 30 (exponential amplification range) cycles shows deletion in the epidermis and liver of Cre+ animals post-tamoxifen treatment but not in Cre− littermates.