**SUPPLEMENTARY MATERIALS**

# Msi2 maintains quiescent state of hair follicle stem cells by directly repressing the Hh signaling pathway

Xianghui Ma[1¶], Yuhua Tian[1¶], Yongli Song[1], Jianyun Shi[1], Jiuzhi Xu[1], Kai Xiong[2], Jia Li[1], Wenjie Xu[1], Yiqiang Zhao[1], Jianwei Shuai[3], Lei Chen[4], Maksim V. Plikus[5]，Christopher Lengner[6], Fazheng Ren[1]*, Lixiang Xue[2]* and Zhengquan Yu[1]*

**Supplementary Figures**



**Supplementary Materials and Methods**

**Supplementary References**

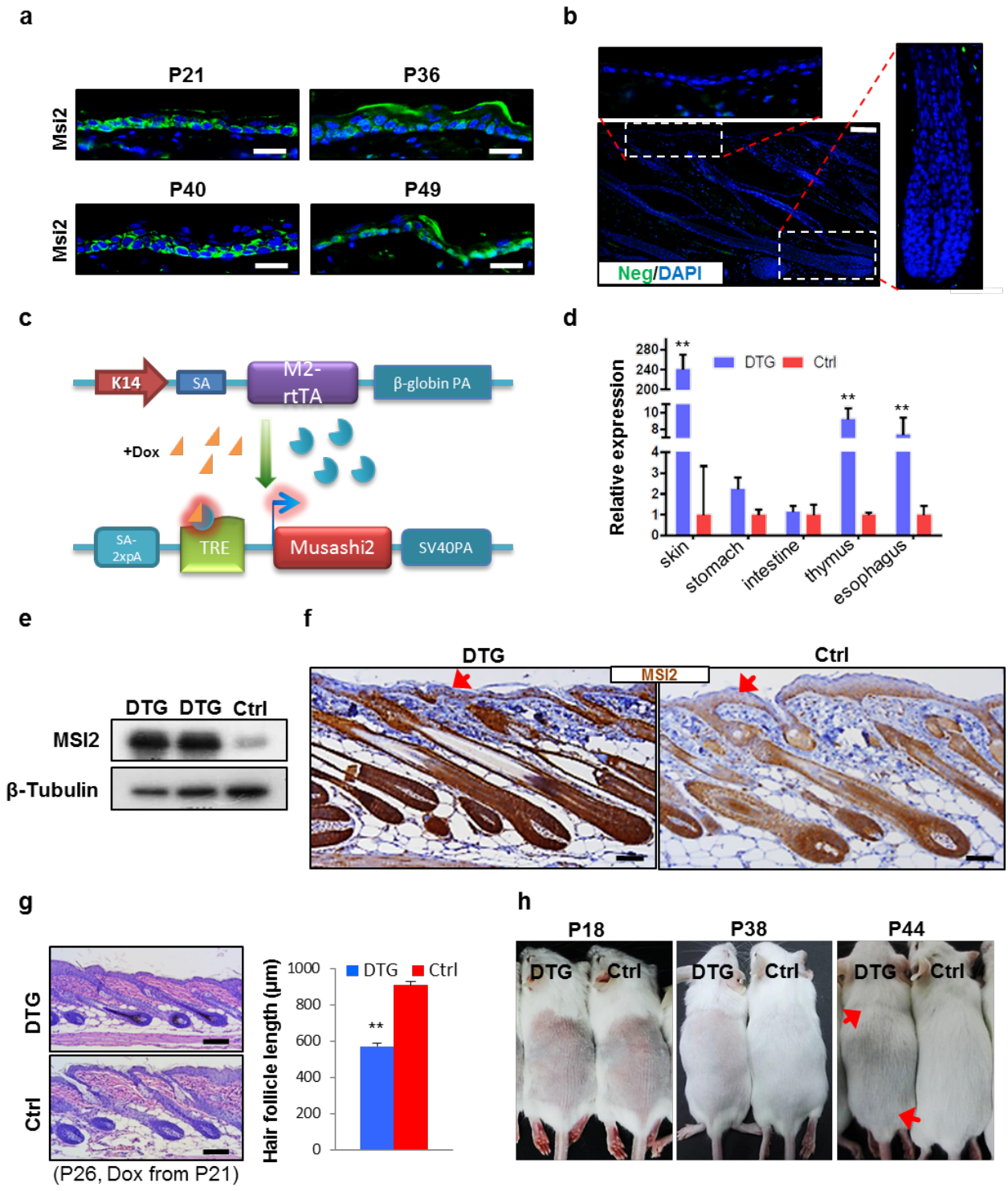



**Figure S1 Forced MSI2 expression causes delayed anagen.** (**a**) IF for Msi2 in WT mouse epidermis at indicated time points. Scale bar, 50μm. (**b**) Negative control for Msi2 in WT mouse skin. Scale bar, 50μm. (**c**) Schematic maps of constructs used to generate *K14-rtTA*::*TRE-MSI2* double transgenic mice (DTG). (**d**) qRT-PCR for *Msi2* in the skin,

stomach, intestine, thymus and esophagus of control and DTG mice at P25 following Dox treatment for 3 days. (**e**, **f**) Western blotting (**e**) and IHC (**f**) for MSI2 in skin of Dox-treated DTG and control mice. **p<0.01. Scale bar, 50μm. Arrows point to positive signal of Msi2 in epidermis. (**g**) Representative histological images of dorsal skin from control and DTG mice at P26 following Dox treatment from P21. Quantification of HF length in gender-matched littermates between control (n=3) and DTG mice (n=3) at P26. **p< 0.01. Scale bar: 50μm. (**h**) Gross images of control and DTG mice, Dox-treated and shaved at P18. Animals were photographed at P18, P38 and P44. (n=7 for DTG and n=5 for control). Arrows point to the boundaries of hair regrowth region.

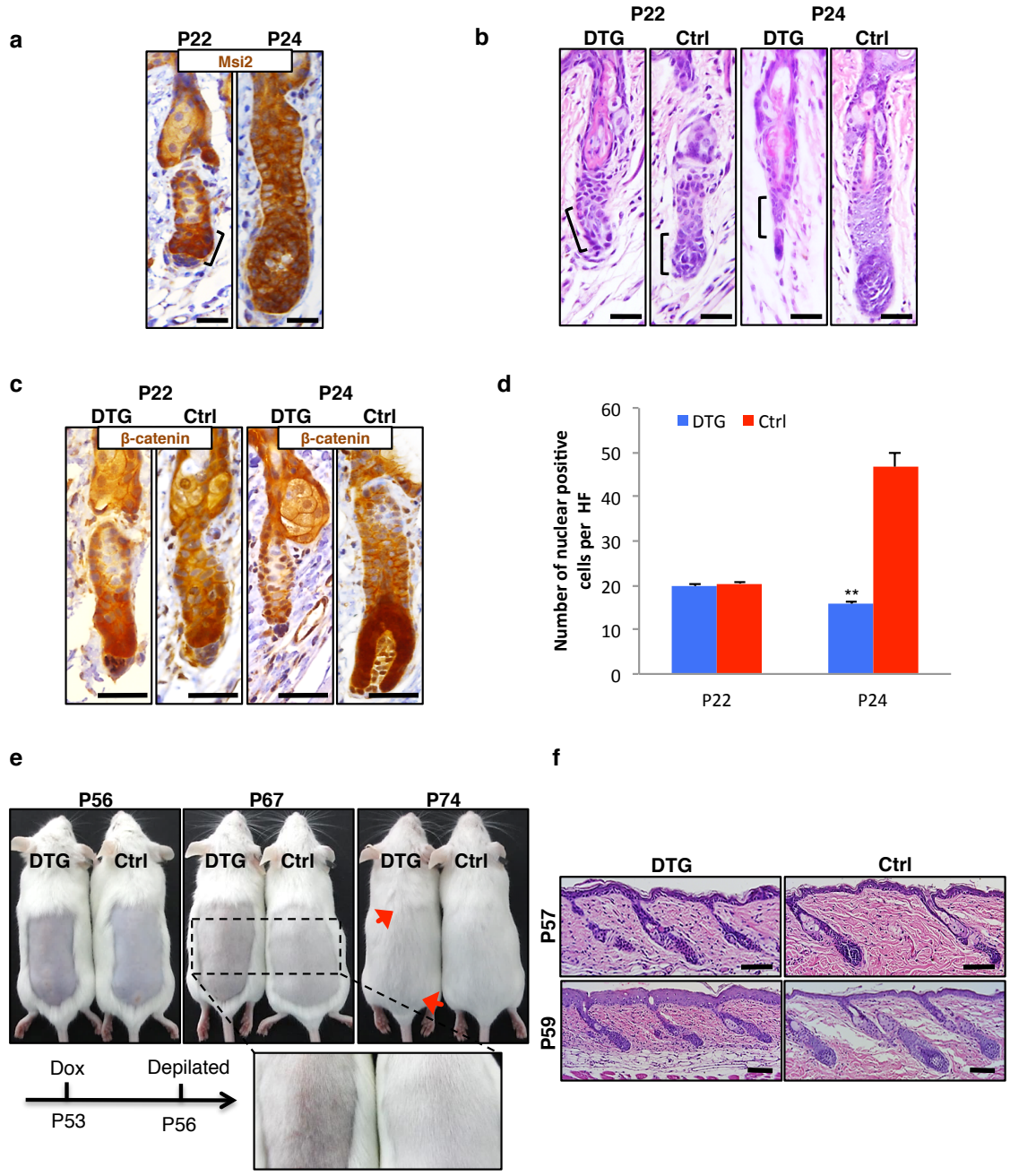



**Figure S2. Msi2 induction represses anagen entry after hair depilation.** (**a**) Staining for Msi2 shows that it is expressed in sHG and TACs in control HFs after 36 hours (P22) and 72 hours (P24) post-depilation. Brackets mark extended sHG and TACs. Scale bar: 50μm. (**b**) Representative histological images show extended sHG and TACs at 36 hours post-depilation and stalled sHG at 72 hours post-depilation in DTG mice as compared to control. Brackets mark sHG. Scale bar: 50 μm. (**c**) Staining for β-catenin in control and DTG HFs at P22 and

P24. Scale bar: 50 μm. (**d**) Quantification of nuclear β-catenin positive cells per HF (BU+HGs). Dox was administered at P18, and skin was depilated at P21. n=3 for DTG and n=3 for control. ** $p<0.01$. (**e**) Gross images of depilated skin in control and DTG mice at indicated time points. Mice were treated with Dox starting at P53. (n=4 for DTG and n=5 for control). Arrows mark boundaries of depilation. (**f**) Histology of dorsal skin from control and DTG mice treated with Dox starting at P53 and depilated at P56. Scale bar: 50 μm.

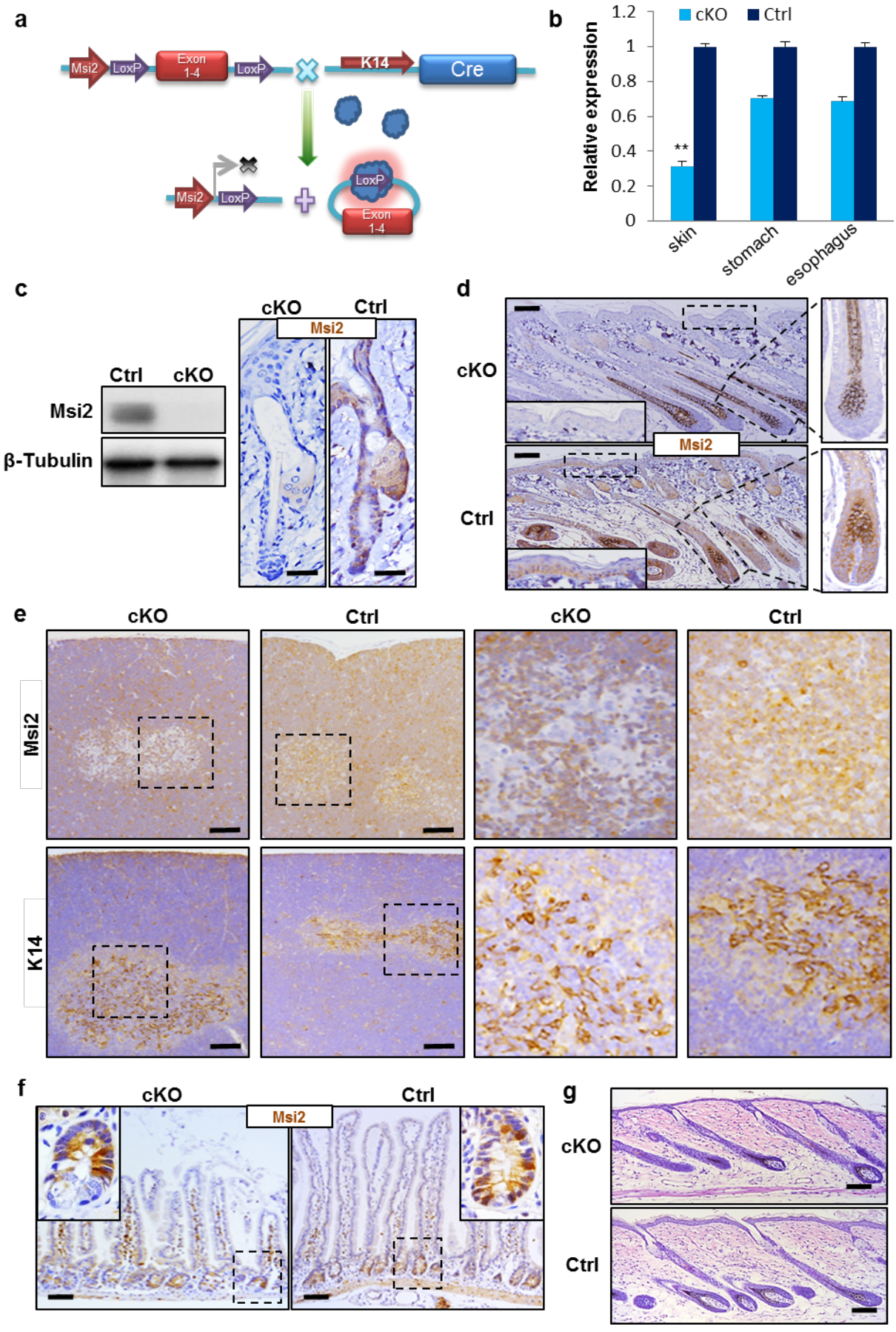



**Figure S3. Generation and identification of *Msi2* conditional knockout mice. (a)**

Schematic of constructs used to generate *K14-Cre*::*Msi2*$^{fl/fl}$ conditional knockout mice (cKO). (**b**) qRT-PCR analysis of *Msi2* showing that it is significantly reduced in cKO skin compared to cotnrol at P24. ** $p < 0.01$. n=3 for cKO and n=3 for control mice. (**c**) Western blotting for Msi2 shows that it is efficiently deleted in the skin of cKO mice at P24. Staining for Msi2 shows that it is absent in HFs of cKO mice at P21. Scale bar, 50μm. (**d**) Staining for Msi2 shows that it is absent from the basal layer of epidermis and HFs of cKO mice at P27. (**e**) Staining for Msi2 and K14 in control and cKO thymus. Dashed boxes mark regions of higher magnification (right two panels). Scale bar: 50μm. (**f**) Staining for Msi2 in control and cKO intestine. Scale bar: 100μm. (**g**) Histology of control and cKO HFs at P26. Scale bar: 50μm.

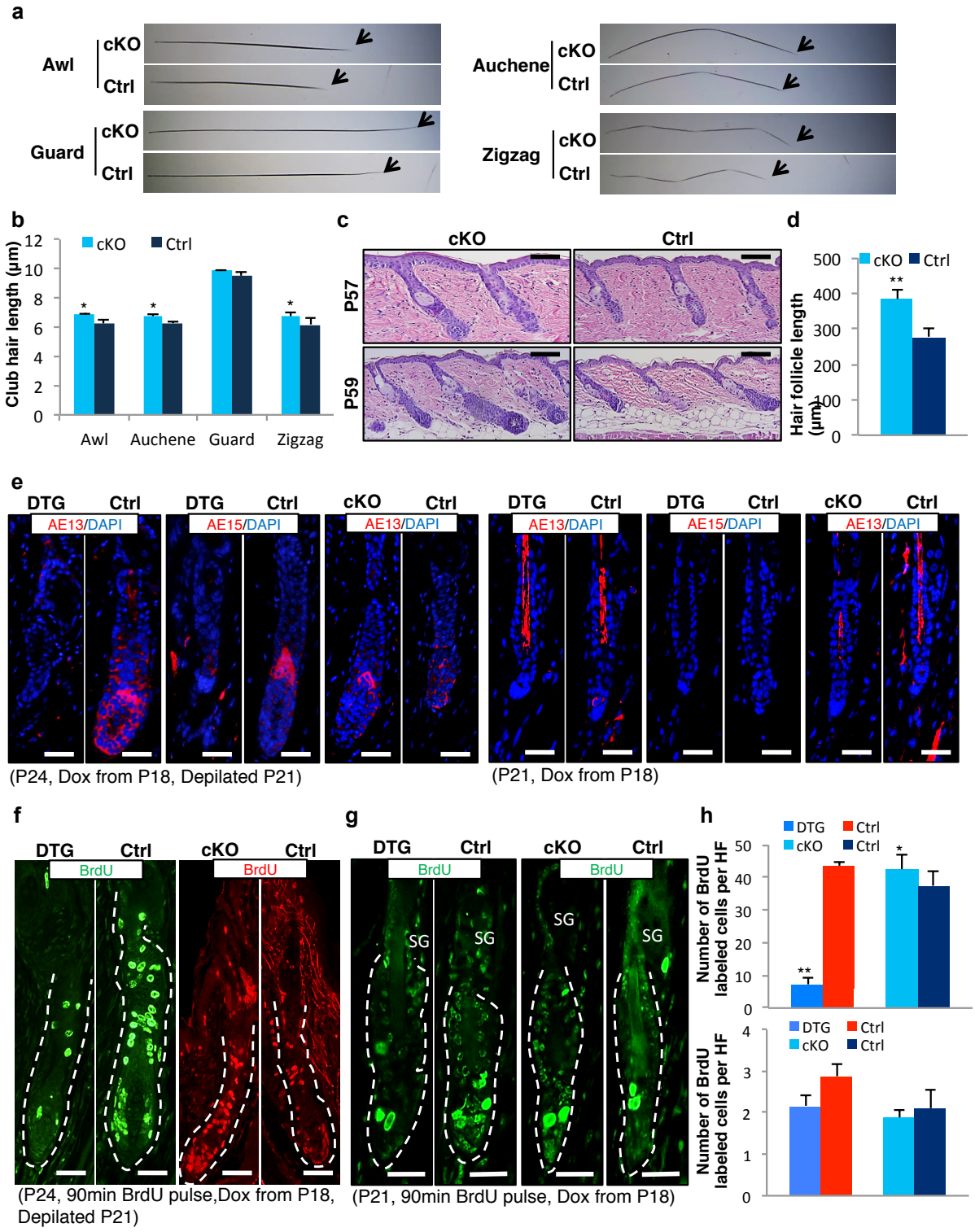



**Figure S4 Msi2 inhibits regrowth and cell proliferation in hair follicles.** (**a**) Representative images of control and cKO hair shafts of four types collected 21 days after hair depilation at P21. Arrows point at the shaft end. (**b**) Quantification of hair shaft lengths in control and cKO mice from panel (**a**). n=20 hairs per type. *p<0.05. (**c**) Histology of dorsal

skin from control and cKO mice at indicated time points. Mice were depilated at P56. Scale bar: 50 μm. (**d**) Quantification of HF length at P59 in Panel (**c**). ** $p<0.01$. (**e**) Left panels: Immunofluorescence for AE13 and AE15 in control and DTG HFs, and AE13 in control and cKO HFs at P24. The control and DTG mice were treated with Dox starting at P18, and depilated at P21. The control and cKO mice were depilated at P21. Right panels: Immunofluorescence for AE13 and AE15 in control and DTG HFs at P21 pretreated with dox at P18, and AE13 in control and cKO follicles at P21. Scale bar, 50μm. (**f**) Immunofluorescence for BrdU in control and DTG follicles or control and cKO follicles. The control and DTG mice were treated with Dox at P18, depilated at P21. 90 min pulse of BrdU incorporation was performed at 72 hours post-depilation (P24). Scale bar, 50 μm. (**g**) Immunofluorescence for BrdU in control and DTG follicles or control and cKO follicles. The mice were treated with Dox starting at P18 and 90 min pulse of BrdU incorporation was performed pre-depilation at P21. Scale bar, 50 μm. (**h**) Quantification of BrdU labeled cells per HF (BU+HGs) in Panel (**f**) (top panel), and in Panel (**g**) (lower panel). * $p<0.05$, ** $p<0.01$. n=3 for all mice.

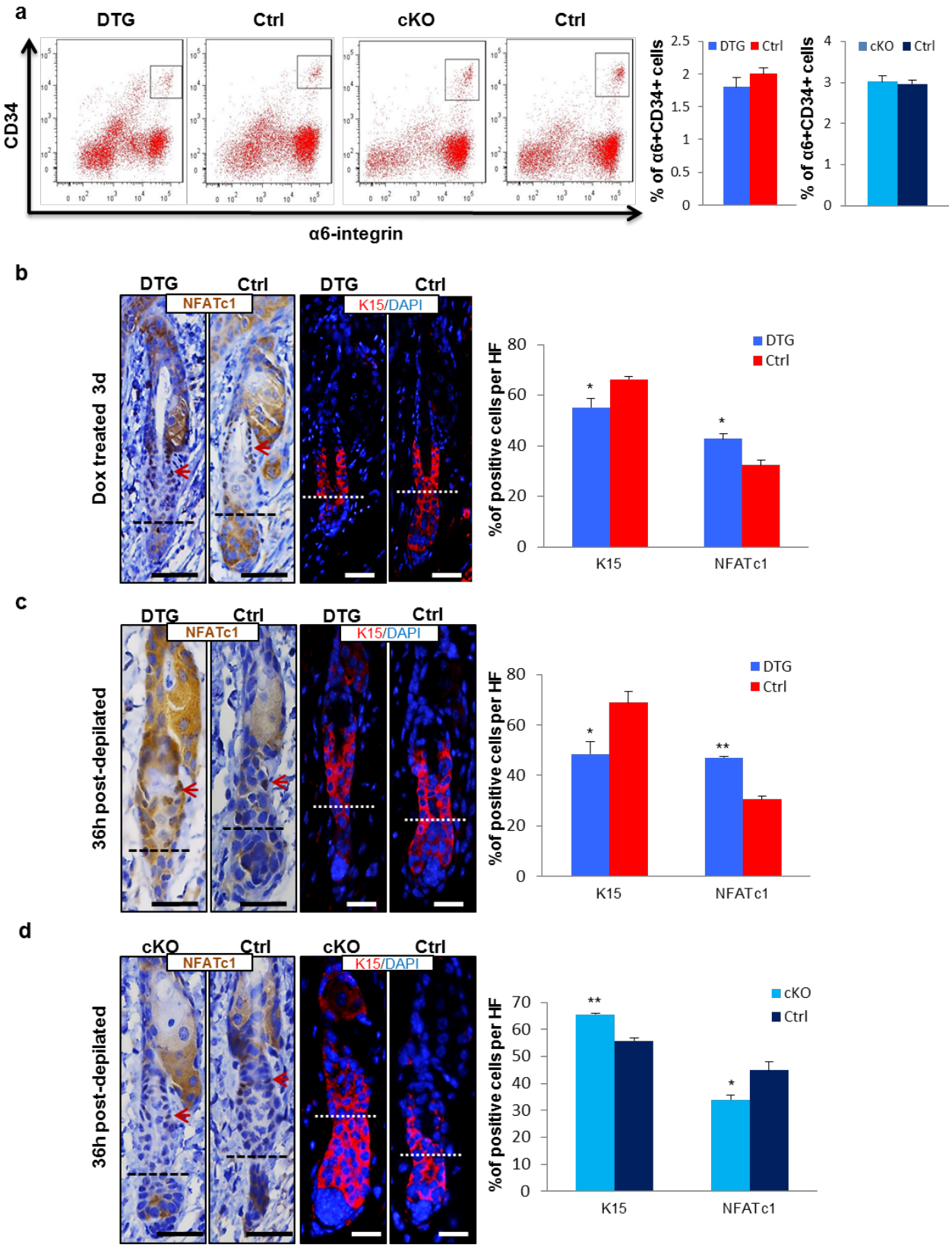



**Figure S5 Msi2 influences state of HFSCs.** (**a**) FACS profiles and quantification of CD34$^{+}$α6-integrin$^{+}$ cells in control and DTG, or control and cKO skin at P24. The DTG mice

were Dox-treated at P21. (**b**) Immunostaining for NFATc1 and K15 in control and DTG HFs Dox treated for 3 days starting at P21. Quantification of NFATc1 or K15 positive cells in control and DTG follicles. * $p<0.05$; ** $p<0.01$. DTG, n=3; Control, n=3. Scale bar, 50 μm. (**c**) Immunostaining for NFATc1 and K15 in control and DTG hair follicles HFs at P21 36 hours post-depilation. Quantification of NFATc1 or K15 positive cells in control and DTG follicles. * $p<0.05$. The control and DTG mice were treated with Dox starting at P18, and depilated at P21. DTG, n=3; Control, n=3. Scale bar, 50 μm. (**d**) Immunostaining for NFATc1 and K15 in control and cKO hair follicles 36 hours post-depilation at P21. Quantification of NFATc1 or K15 positive cells in control and cKO HFs. * $p<0.05$, ** $p<0.01$. n=3 for cKO and n=3 for control mice. Scale bar: 50 μm. Dashed lines denote boundaries of bulge (BU) and secondary hair germ (HG). Arrows point to positive nuclear NFATc1 signal.

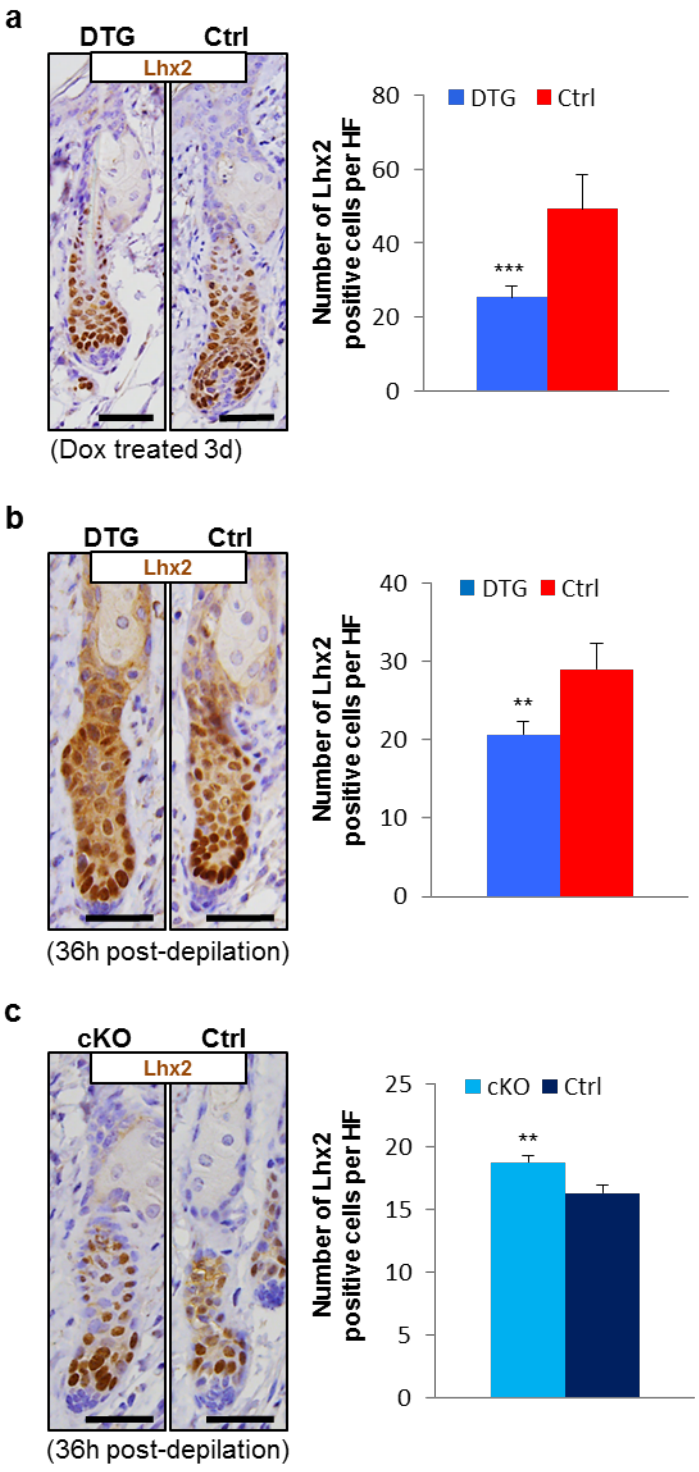



**Figure S6 Lhx2 positive cells in DTG and cKO follicles.** (**a**) IHC for Lhx2 in control and DTG hair follicles pre-treated with Dox for 3 days at P21. Quantification of Lhx2 positive cells in control and DTG follicles. *** $p<0.001$. DTG, n=3; Control, n=3. Scale bar, 50 μm. (**b**) IHC for Lhx2 in control and DTG hair follicles 36 hours post-depilation at P21. The

control and DTG mice were pre-treated with Dox at P18, and depilated at P21. Quantification of Lhx2 positive cells in control and DTG follicles. ** $p<0.01$. DTG, n=3; Control, n=3. Scale bar, 50 μm. (**c**) IHC for Lhx2 in control and cKO hair follicles 36 hours post-depilation at P21. Quantification of Lhx2 positive cells in control and cKO follicles. ** $p<0.01$. cKO, n=3; Control, n=3. Scale bar, 50 μm. The dashed line denotes the boundary of bulge (BU) and secondary hair germ (HG).

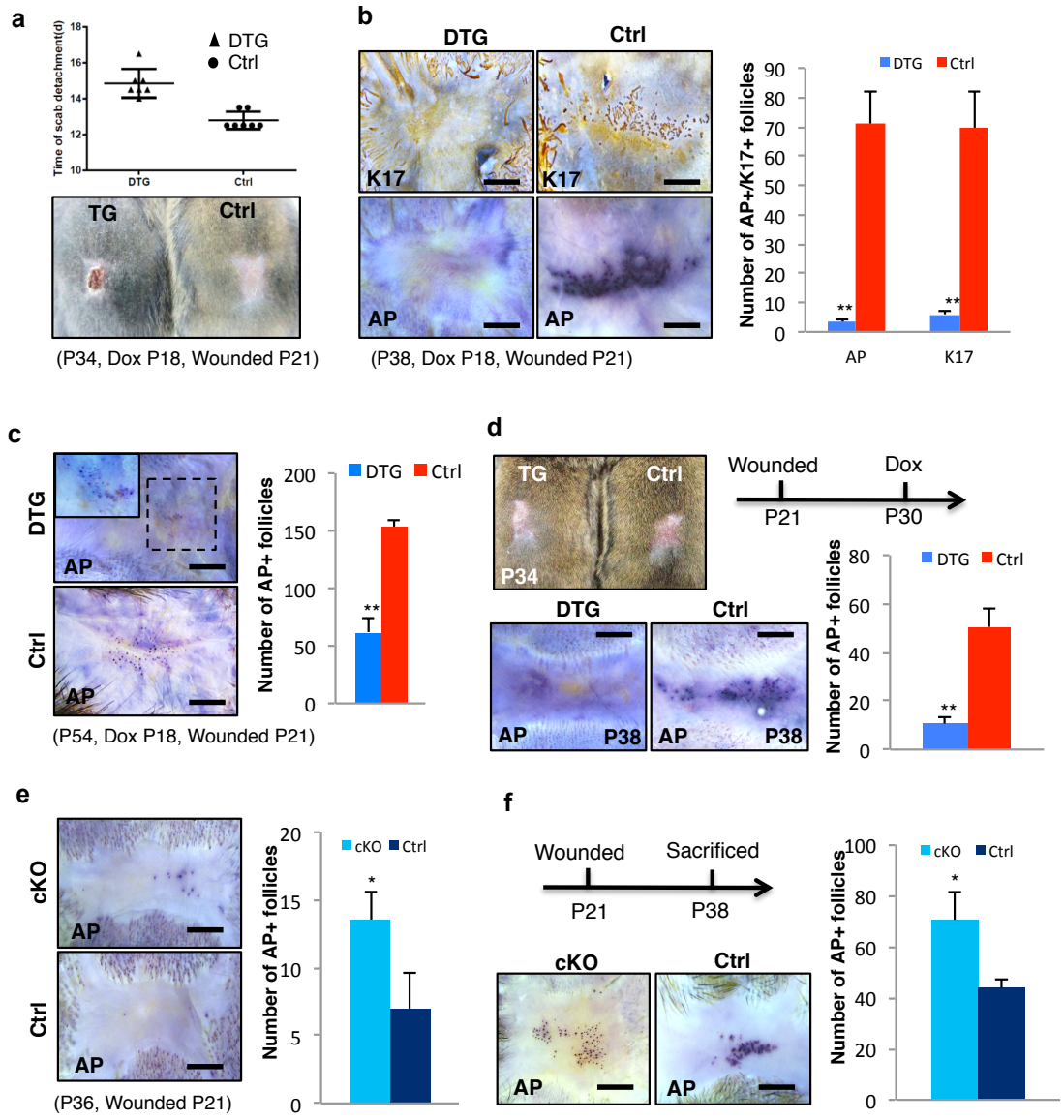



**Figure S7. Forced MSI2 expression impairs hair neogenesis.** (**a**) Gross images of wounds from control and DTG mice at 14 days post-wounding. Mice were Dox-induced at P18 and wounded at P21. Quantification of scab detachment time in control and DTG mice is shown. (**b, c**) K17 or AP staining and quantification of *de novo* regenerated HFs in the center of wounds from control and DTG mice at P38 (**b**) and P54 (**c**). Mice were Dox-induced at P18 and wounded at P21. (**d**) Gross images of wounds from control and DTG mice at P34. AP staining and quantification for *de novo* regenerated HFs in the center of wounds from control and DTG mice. Mice were wounded at P21 and treated with Dox at P30. (**e, f**) AP staining

and quantification for *de novo* regenerated HFs in control and cKO mice at 15 (**e**) and 17 (**f**) days post-wounding. n = 3 for all experiments shown. * $p<0.05$; ** $p<0.01$; *** $p<0.001$. Scale bar: 400 μm.

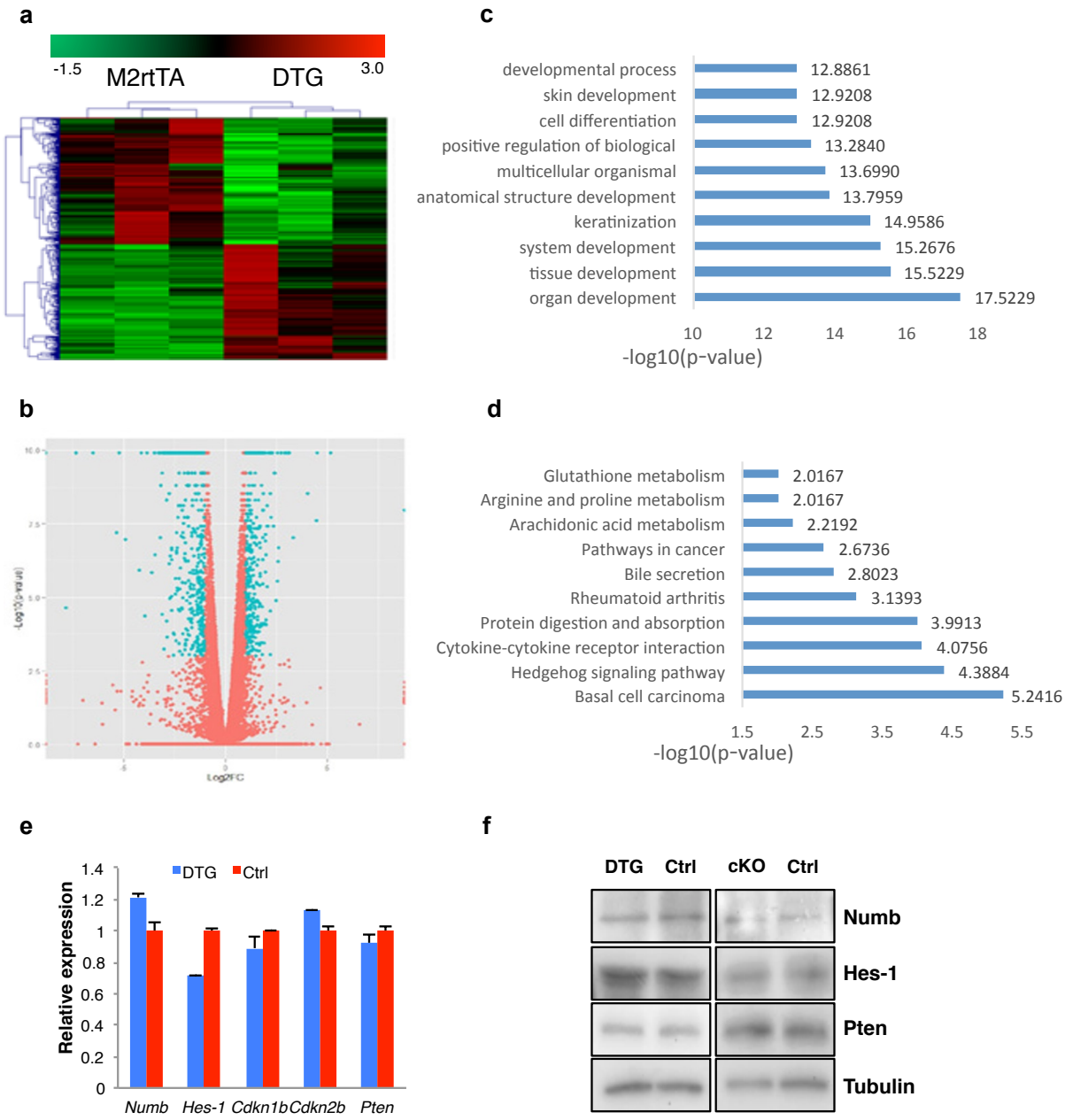
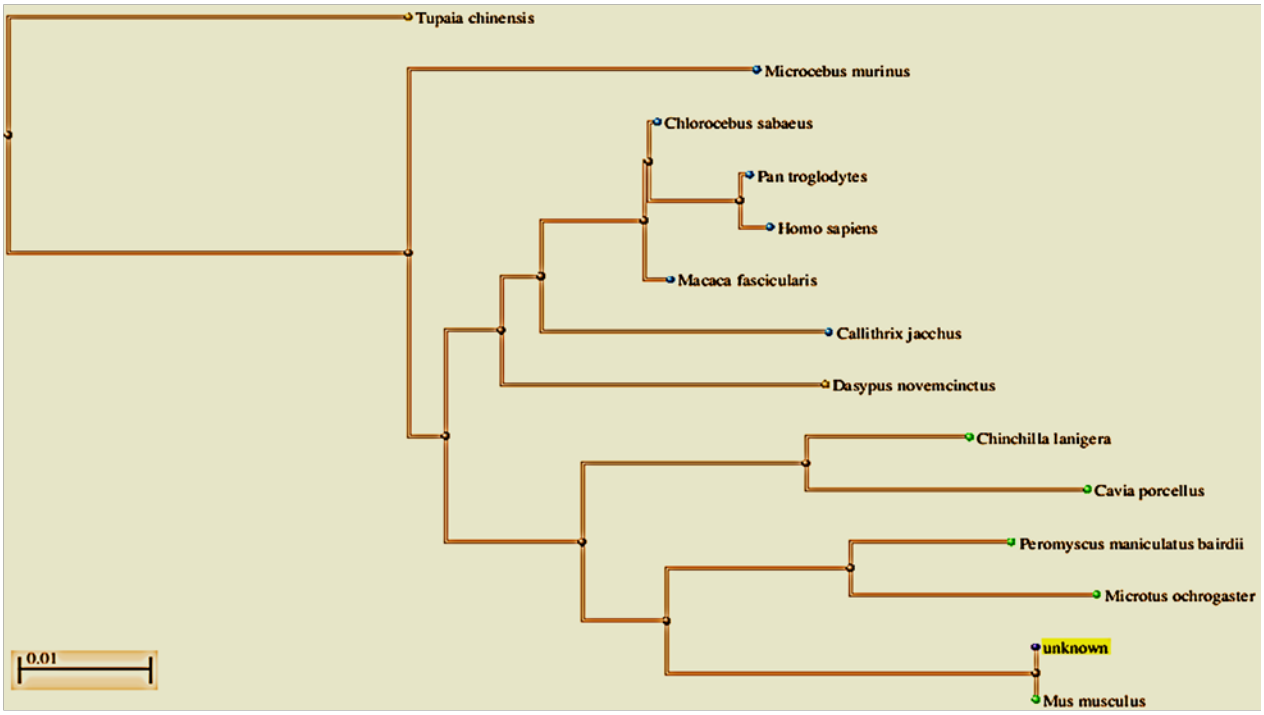



**Figure S8. Transcriptome profiling and verification in MSI2 overexpressing skin.** (**a**) Heatmaps and hierarchical clustering of skin transcriptome profiles from control (n=3) and DTG mice (n=3) treated with Dox for 3 days (P21-P24). (**b**) Volcano plot of skin transcriptome profiles in control vs. DTG mice. (**c**) GO analysis of biological process on differentially expressed genes in DTG skin. (**d**) KEGG pathway analysis of differentially expressed genes in DTG skin. (**e**) qRT-PCR analysis on mouse skin for *Numb*, Notch signaling pathway (*Hes-1*), TGFβ signaling pathway (*Cdkn1b*, *Cdkn2b*), and *Pten* at 36 hours

post-depilation. Control and DTG mice were treated with Dox at P18 and depilated at P21. n=3 for DTG and n=3 for control mice. (**f**) Western blotting on skin for Numb, Hes-1 and Pten in 36 hours post-depilation. Control and DTG mice were treated with Dox at P18 and depilated at P21. Control and cKO mice were depilated at P21.

**a**




**b**

| Description | Total score | Query cover | E value | Ident | Accession | motif matched |
|---|---|---|---|---|---|---|
| **Mus musculus SHH** | 719 | 100% | 0 | 100% | XM_006535649.2 | completely |
| **Peromyscus maniculatus bairdii SHH** | 512 | 100% | 2.00E-141 | 91% | XM_006994537.1 | 1stG:T mismatch |
| **Microtus ochrogaster SHH** | 449 | 88% | 1.00E-122 | 91% | XM_005367458.1 | completely |
| **Dasypus novemcinctus SHH** | 427 | 88% | 6.00E-116 | 89% | XM_004458603.1 | completely |
| **Chlorocebus sabaeus SHH** | 416 | 87% | 1.00E-112 | 89% | XM_007983598.1 | completely |
| **Pan troglodytes SHH** | 411 | 86% | 7.00E-111 | 89% | XM_009444290.1 | completely |
| **Homo sapiens SHH** | 399 | 84% | 1.00E-107 | 89% | NM_000193.3 | 2ndT:C mismatch |
| **Macaca fascicularis SHH** | 396 | 84% | 2.00E-106 | 89% | XM_005551269.1 | completely |
| **Callithrix jacchus SHH** | 383 | 82% | 1.00E-102 | 89% | XM_002807047.3 | completely |
| **Microcebus murinus SHH** | 350 | 84% | 1.00E-92 | 86% | XM_012786262.1 | completely |
| **Chinchilla lanigera SHH** | 313 | 84% | 2.00E-81 | 85% | XM_013504572.1 | completely |
| **Cavia porcellus SHH** | 298 | 82% | 5.00E-77 | 85% | XM_003469458.3 | completely |
| **Tupaia chinensis SHH** | 292 | 84% | 2.00E-75 | 83% | XM_006143824.1 | 3rdT:C mismatch |

**Figure S9. Homologies of the identified Msi2 binding motif in mouse *Shh* 3'-UTR with *Shh* mRNA from other species.** (**a**) Distance tree view of the identified Msi2 binding motif in *Shh* 3'-UTR across species. Unknown indicates Msi2 binding motif sequence of mouse *Shh*. (**b**) List of homologies of the Msi2 binding motif in *Shh* 3'-UTR by BLASTS pairwise alignment.

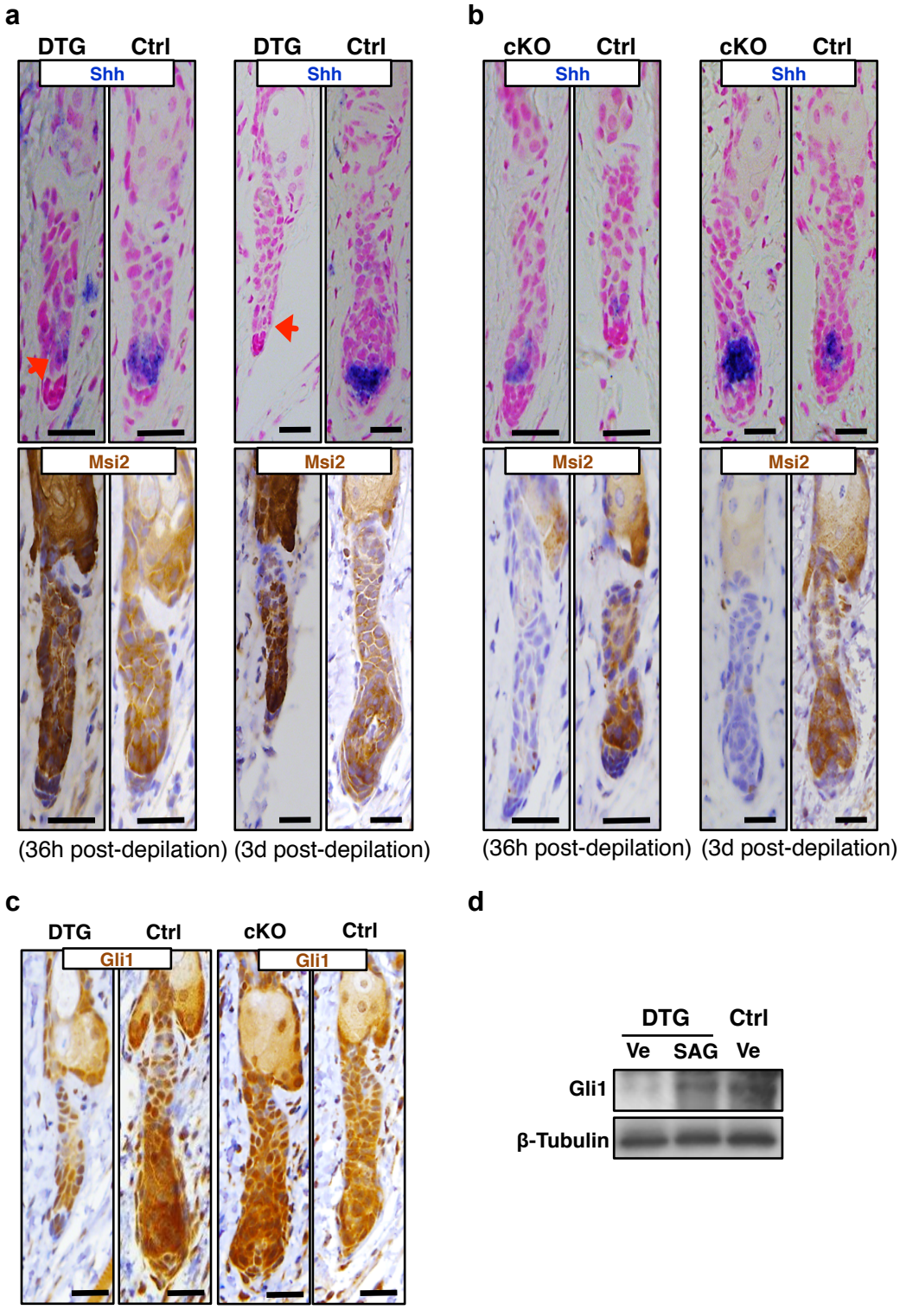



**Figure S10. Msi2 represses the Hh signaling.** (**a**) *In situ* hybridization for *Shh* and IHC for Msi2 in control and DTG HFs 36 hours and 3 days post-depilation. (**b**) *In -situ* hybridization for *Shh* and IHC for Msi2 in control and cKO HFs 36 hours and 3 days post-depilation. (**c**) IHC for Gli1 in control and DTG, or in control and cKO HFs at P24 3 days post-depilation.

Control and DTG mice were treated with Dox at P18 and depilated at P21. Control and cKO mice were depilated at P21. Scale bar: 50 μm. (**d**) Western blotting for Gli1 in control and DTG skin 36 hours post-depilation at P21. Control and DTG mice were treated with Dox from P18 and depilated at P21. Control mice were topically treated with a single 25 μL application of vehicle (95% acetone/5% DMSO), while DTG mice were given a single 25 μL topical application of SAG (0.06 μg per μL in vehicle, 120 mM). Second application was done 24 hours later. Ve: vehicle.

## SUPPLEMENTARY MATERIALS AND METHODS

### Mouse strains

*TRE-MSI2* and *Msi2*$^{flox/flox}$ mice were previously described (Kharas *et al.*, 2010; Wang *et al.*, 2015). *K14-rtTA* and *K14-Cre* mice were obtained from Jackson Laboratory (stock numbers 007678 and 004782).

### Histological analyses and immunostaining

Immunochemistry and immunofluorescence were performed as previously described (Yuan *et al.*, 2015). Skin samples were fixed in 4% PFA, paraffin-embedded, sectioned at 5 μm and stained with hematoxylin and eosin (H&E, Sigma-Aldrich, St. Louis, MO). For immunofluorescence staining, paraffin sections were microwave pretreated. The following primary antibodies and dilutions were used: Msi2 (rabbit, 1:800, Novus Biologicals，Littleton, CO), Msi1 (rat, 1:500, MBL, Woburn, MA), AE13 (mouse, 1:100, Abcam, Cambridge, United Kingdom), AE15 (mouse, 1:100, Abcam, Cambridge, United Kingdom), Ki67 (rabbit, 1:200, Thermo Scientific, Fremont, CA), K15 (mouse, 1:150, Vector Labs, Burlingame, CA), Sox9 (mouse, 1:1200, Abcam, Cambridge, United Kingdom), NFATc1 (mouse, 1:25, Santa Cruz Biotechnology, Dallas, TX), Gli1 (rabbit, 1:1000, Novus Biologicals，Littleton, CO), p-Smad1/5/8 (rabbit, 1:100, Cell Signaling Technology, Danvers, MA), CyclinD1 (mouse, 1:50, Santa Cruz Biotechnology, Dallas, TX), β-Catenin (mouse, 1:1000, Sigma-Aldrich, St. Louis, MO), Lhx2 (goat, 1:100, Santa Cruz Biotechnology, Dallas, TX).

For BrdU staining, BrdU at the concentration of 5 μg/g of body weight was injected I/P. Dorsal skin samples were collected, paraffin embedded and sectioned. Sections were

treated with 2x SSC: Formamide (Amresco, Solon, OH) at 1:1 at 65℃ for 2 hours. Sections were then immersed in 2M HCl at 37℃ for 30 min and rinsed with 0.1M boracic acid and washed in 1% PBST. 5% normal goat serum in 1% PBST with 0.1M Glycine treatment ar RT for 1 hour was performed to block nonspecific epitopes. Anti-BrdU antibody was inclubated overnight at RT (rat, 1:50, Abcam, Cambridge, United Kingdom; mouse, 1:1000, Sigma-Aldrich, St. Louis, MO). All images were visualized with Leica CytoVision® DM5500 electronic fluorescence microscope and assembeled using Adobe PhotoShop CS6 software.

**Western blotting**

Mouse dorsal skin samples were homogenized in ice-cold lysis buffer (Beyotime, Shanghai, China) with 1% Phenylmethylsulfonyl fluoride (PMSF) protease inhibitors (Beyotime, Shanghai, China). After digesting 30 min on ice, the suspension was centrifuged at 12,000g for 5min at 4℃, obtaining the supernatant and quantifying the concentration with a BCA (bicinchoninic acid) protein assay kit (Beyotime, Shanghai, China). With denaturing condition, 40μg total protein was separated with 10% or 12% SDS-PAGE electrophoresis, and transferred to 0.45μm aperture polyvinylidene difluoride (PVDF) membrane (Calbiochem-Merck Millipore; Darmstadt, Germany). The membrane was blocked 1h under room temperature in 5% nonfat dry milk and later then directly incubated with primary antibody at 4℃ overnight. After incubating 1h at room temperature with secondary antibody conjugated with horseradish peroxidase (HRP) (1:10,000; Beyotime, Shanghai, China), the membrane signal was visualized with immobilon chemiluminescent western HRP substrate

(Calbiochem-Merck Millipore; Darmstadt, Germany). Reference gene protein was incubated together with the target protein to normalize of the expression levels. The antibodies were used in this paper as below: Msi2 (rabbit, 1:1,000, Novus Biologicals，Littleton, CO), Msi1 (rat, 1:1,000, MBL, Woburn, MA), Shh (rabbit, 1:400, Boster, Wuhan，China), Gli1 (rabbit, 1:1000, Novus Biologicals，Littleton, CO), p-Smad1/5/8 (goat, 1:300, Santa Cruz Biotechnology, Dallas, TX), CyclinD1 (mouse, 1:100, Santa Cruz Biotechnology, Dallas, TX), β-Catenin (mouse, 1:100, Santa Cruz Biotechnology, Dallas, TX); Tubulin (mouse, 1:4000, Sigma-Aldrich, St. Louis, MO) and GAPDH (mouse, 1:1,000; Beyotime, Shanghai, China) were used as reference gene protein respectively.

**qRT-PCR assay**

Total RNA was isolated from dorsal mouse skin with TRIzol Reagent (Invitrogen, San Diego, CA). 2μg of RNA was reverse transcribed with the M-MLV Reverse Transcriptase (Promega, Madison, WI) using Oligo (dT) primers. Real-time PCR was performed with the LightCycler 480 SYBR Green I master mix on a LightCycler 480 real-time PCR system (Roche, Mannheim, Germany). *GAPDH* was used as an internal control and calculated based on the formula $2^{-\Delta\Delta Cp}$. The primer sequences were as follows:

| Gene name | Forward (5' to 3') | Reverse (5' to 3') |
|---|---|---|
| *Shh* | AAAGCTGACCCCTTTAGCCTA | TGAGTTCCTTAAATCGTTCGGAG |
| *Gli1* | GGCTGTCGGAAGTCCTATT | CACTGGCATTGCTAAAGGC |
| *Gli2* | GCCGATTGACATGAGACACC | CTGAAGGGTGACTCTCCAGG |
| *Gli3* | CACAGCTCTACGGCGACTG | CTGCATAGTGATTGCGTTTCTTC |

| | | |
|---|---|---|
| *Ptch1* | AGGCAGCTAATCTCGAGACC | GCGCCTTCTTCTTTTGGAGT |
| *Ptch2* | CACTGCCAGTAAAGTCCAAGTAT | CGCTCGATCATCCCATTTTCAA |
| *Hhip* | TGAAGATGCTCTCGTTTAAGCTG | CCACCACACAGGATCTCTCC |
| *Msi2* | ACGACTCCCAGCACGACC | GCCAGCTCAGTCCACCGATA |
| *Thbs1* | CATCCAGAGCATCTTCACCAG | CAGCCTTTGTTCCTGAGAATG |
| *Pten* | TGGATTCGACTTAGACTTGACCT | GCGGTGTCATAATGTCTCTCAG |
| *Numb* | AAAGCAGTGAAGGCCGTTCT | GTTTTCTCGTCCACAACTCTGAG |

**FACS assays**

The *TRE-MSI2* mice were treated with Dox for 3 days and skin samples were collected at P24. Surface marker used to isolate HFSCs were CD49f (α-6 integrin) and CD34 (Yuan *et al.*, 2015). Dermal adipose was removed with scalpel and skin was treated with trypsin (GIBCO, Grand Island, NY) at 4℃ overnight. Resulting cell suspension was filtered through 70-μm and then 40-μm strainer (BD Biosciences, Franklin Lakes, NJ) and then incubated with primary antibodies: CD49f-PE (1:500, eBioscience, San Diego, CA), CD34-eFluor660 (1:100, eBioscience, San Diego, CA) for 1h on ice. Cells were washed and re-suspended in 2% FBS in PBS. Cell sorting was performed as described previously (Blanpain *et al.*, 2004) on BD FACS Aria II cell sorter (Beckman Coulter, Brea, CA). Analysis was performed with FlowJo software.

**Wounding and whole-mount hair follicle neogenesis assay**

Wound-induced hair neogenesis assay was performed as previously described (Ito *et*

*al.*, 2007; Yuan *et al.*, 2015). Full-thickness 2.25 $cm^2$ wounds were created on dorsal skin at P21 in mice anaesthetized with sodium pentobarbital. For *de novo* HF analysis, mice were sacrificed at 15 or 17 days post-wounding. Wound tissues were incubated in 20 mM EDTA in PBS at 37℃ overnight. Next, epidermal portion was gently peeled off under a dissecting microscope, and fixed in 4% PFA for 1 hour, rinsed in PBS, blocked with 3% $H_2O_2$ and Immunostained for K17 (Rabbit, 1:100, Abcam, Cambridge, United Kingdom). To stain for AP, wound dermis was fixed in acetone at 4℃ overnight, and then incubated with NBT/BCIP (Roche, Mannheim, Germany) for 30 min at 37℃, and then rinsed with PBS followed by distilled water to stop the reaction.

**Dual luciferase activity assay**

*Shh* (Accession number: NM_009170.3) 3'UTR fragment containing binding site (5'-GUUUAGT-3') was obtained from mouse skin cDNA and cloned into the psiCHECK-2 vector (Promega, Madison, WI), downstream of the Renilla luciferase gene. For a mutant 3'UTRs reporter vector, the binding site was mutated to (5'-GGGTTCG-3') by a QuikChange Site-Directed Mutagenesis kit according to the manufacturer's protocol (Stratagene, St Clara, CA). Ten nanograms of each reporter construct were co-transfected with human MSI2 overexpressing eukaryote expression vector (hMSI2) or a negative control (his) at a final concentration of 50 nM into 293T cells seeded in 96-well plates. After 24 hours, the activity of Firefly and Renilla luciferase were measured with the Dual-Glo luciferase assay system according to the manufacturer's instructions (Promega, Madison, WI). The primer used was: forward: 5'- AAAGCGCACGGAAGGAG-3'; reverse: 5'- CGCAGGACAAGGGACAT-3'.

**Transcriptome profiling**

Three *K14-rtTA* mice and three DTG (*TRE-MSI2*:: *K14-rtTA*) mice were treated with 2 g/L Dox in drinking water at P21 for 3 days. Total RNA for dorsal skin samples was isolated as described above. RNA sequencing (RNA-seq) was performed on Illumina HiSeq™ 2000 by the Beijing Genomics Institution (BGI, Beijing, China). To analyze RNA-Seq data, tophat2 was used for reads mapping to the mm10 reference and cufflinks to estimate the FPKM. To identify differentially expressed genes, we employed cuffdiff and drew heatmaps using R gplots package. Differentially expressed transcripts were defined by FDR cuffdiff of ≤ 5% and fold changes ≥ 2. We performed KEGG and gene ontology (GO) enrichment analysis using R GO stats package and visualized for pathways with an R pathview package. The obtained transcriptome metadata was deposited as the accession number: SRA320651 in Sequence Read Archive (SRA) database of National Center for Biotechnology Information (NCBI).

**Rescue assay analysis**

Littermate control and DTG male mice were treated with Dox from P18, and depilated at P21. Hh pathway activation was measured as previously described with some modification (Paladini *et al.*, 2005). Briefly, littermate control mice were treated with a single 25 μL topical application of vehicle (95% acetone/5% DMSO) across depilated dorsal skin. DTG mice were treated with a single 25 μL topical application of SAG (0.06 μg per μL in vehicle, 120 mM). Second dose was applied 24 hours later. Hh pathway activation was assessed by studying Gli1 on Western blotting.

## *In situ* hybridization

*In situ* hybridizations for *Shh* were performed as the previously described (Hsu *et al.*, 2014; Yuan *et al.*, 2015). Construct used to generate *Shh In situ* probe was a gift from Maksim V. Plikus (UC Irvine). *Shh* probe and anti-sense probe were generated by DIG RNA Labeling Kit (SP6/T7) according to the manufacturer's instructions (Roche, Mannheim, Germany) and purified by illustra ProbeQuant G-50 Micro Columns kit (GE Healthcare, Pittsburgh, PA). Double DIG-labeled *Shh* probe and anti-sense probe were hybridized at 65°C. To detect the signal, slides were stained with anti-Digoxigenin-AP antibody (Roche, Mannheim, Germany) and color was developed using BM purple substrate (Roche, Mannheim, Germany). Cell nuclei were stained with 0.1% nuclear fast red.

## CLIP-qPCR

Candidate target genes of Msi2 were studied with CLIP-qPCR (Cross-lingking and Immunoprecipitation-qPCR) assay performed as previously described for Ago2-HITS-CLIP with minor modifications (Wang *et al.*, 2015). To obtain Msi2 enriched intact HFs, three back skins from P26 WT mice were treated with dispase at 4℃ for 8 hours to separate epidermis and upper HF portions. Dermis was additionally digested with 0.8% collagenase II (Sigma-Aldrich, St. Louis, MO) at 37℃ for 40 min. Intact HFs and dermal cells were spun at 300 g; follicles were obtained at 20 g. Following 2% trypsinization and 37℃ for 5 min, cell suspensions were strained in a 12-well plate and placed on ice in HL-2000 HybriLinker and irradiateed twice at 400 mJ/cm$^2$. Cells were collected in 1.5 mL microtubes and pelleted by centrifugation and then lysed using PXL buffer (PBS, 0.1% SDS, 0.5% deoxycholate, 0.5%

NP-40, plus protease inhibitor and RNAsin). The lysates were sequentially treated with RQ1 DNase, and spun at 40,000g for 20 min. The supernatant was added to protein A Dynabeads (Dynal, 100.02, Thermo Fisher, Fremont, CA), conjugated with anti-Msi2 antibody (Abcam, Cambridge, United Kingdom) or goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) and incubated for 4 hours at 4℃ with continuous rotation. Beads were washed twice with PXL buffer (1 x PBS, 0.1% SDS, 0.5% deoxycholate, 0.5% NP-40), high salt buffer (5 x PBS, 0.1% SDS, 0.5% deoxycholate, 0.5% NP-40), and PNK buffer (50mM Tris-Cl pH7.4, 10mM $MgCl_2$, 0.5% NP-40). Beads were suspended in 200 μL of PK buffer (100mM Tris-Cl pH7.5, 50mM NaCl, 10mM EDTA) containing 2 mg/mL of Proteinase K (PCR grade, Roche, Mannheim, Germany) and incubated for 30 min at 37℃. RNA was extracted with Trizol LS Reagent (Invitrogen, San Diego, CA). cDNA was synthesized using SuperScript® IV First-Strand Synthesis System (Invitrogen, San Diego, CA) with random hexamers in the kit. Real-time PCR was performed as described above. *GAPDH* was used as an internal control and *Tnbs1* as positive-target control.

**RNA stability measurements**

To measure RNA stability of Msi2 candidate targets, Lovo cells were cultured and transfected with 1 μg human MSI2 overexpressing eukaryote expression vector (hMSI2) or a negative control (his) in Opti-MEM media. Four hours later, the Opti-MEM media was replaced with DMEM (10% FBS, 1% SP) and supplemented with 5 μg/mL Actinomycin D (Thermo Fisher, Fremont, CA) after 90 min of culture. The time point assay was performed as previously described (Bennett *et al.*, 2016). The zero time point was marked starting 5 min

after Actinomycin D addition. RNA was harvested using Trizol at 0, 2, 4, 6 and 8 h and used in qPCR. Relative expression was computed and normalized to the zero hour time point for targets from three technical replicates calculated based on $2^{-\Delta\Delta Cp}$ method normalized to *GAPDH* values. RNA half-lives were calculated from linear regression of log transformed expression values for targets. ANCOVA analysis was performed on the resulting regression lines to assess statistical significance.

**Statistical analysis**

All morphometric analyses were performed in triplicates or greater and the means obtained were used for student's t-tests. Asterisks denote statistical significance (* $p < 0.05$; ** $p < 0.01$; *** $p<0.001$). All data are reported as mean ± SD. Means and standard errors from at least three independent experiments are shown on all graphs.

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