Supplementary Information

Kindlin-1 controls cutaneous epithelial stem cell proliferation by modulating Wnt ligand and TGFβ availability

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Supplementary Figure 1–8
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Supplementary Figure 1

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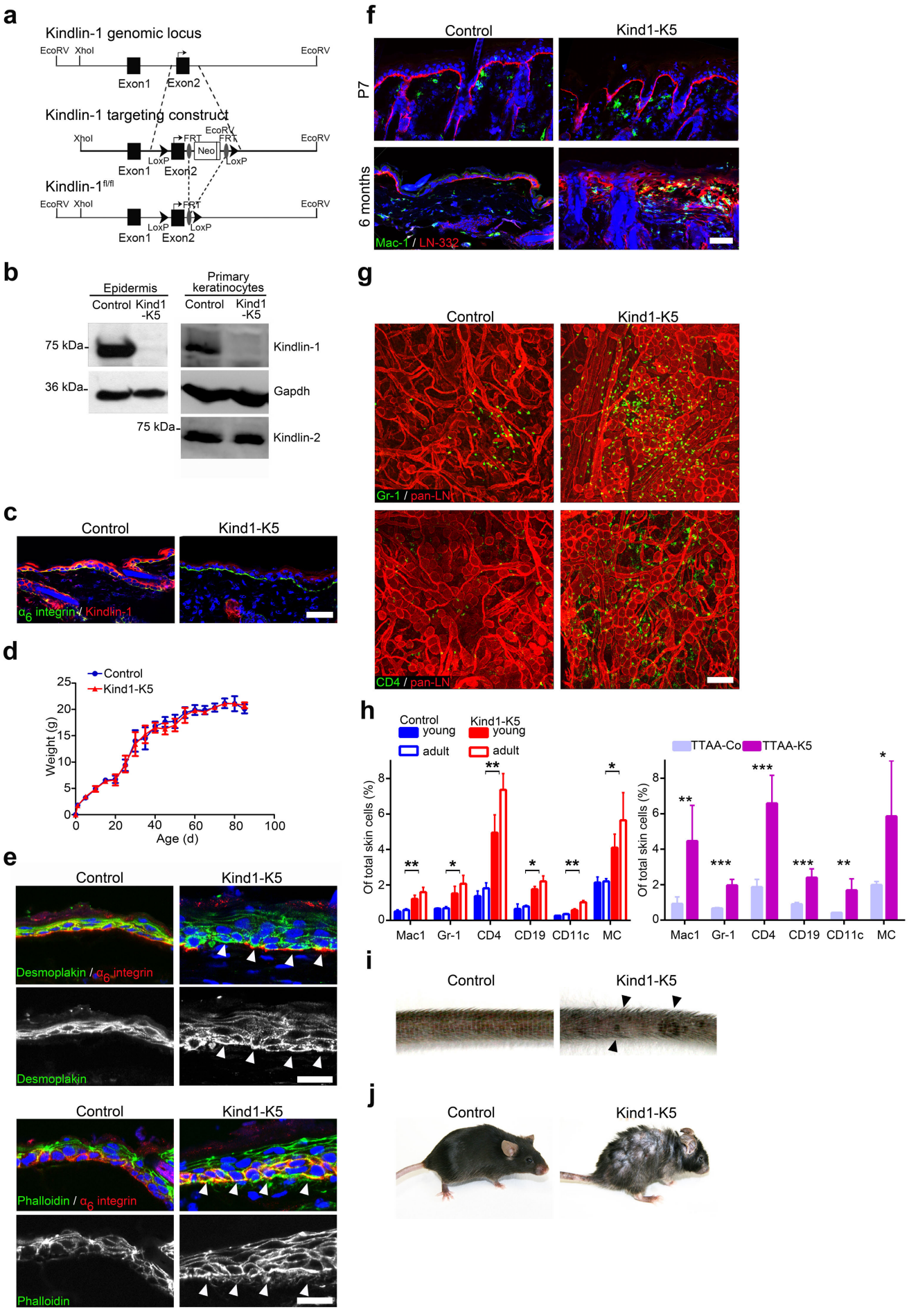
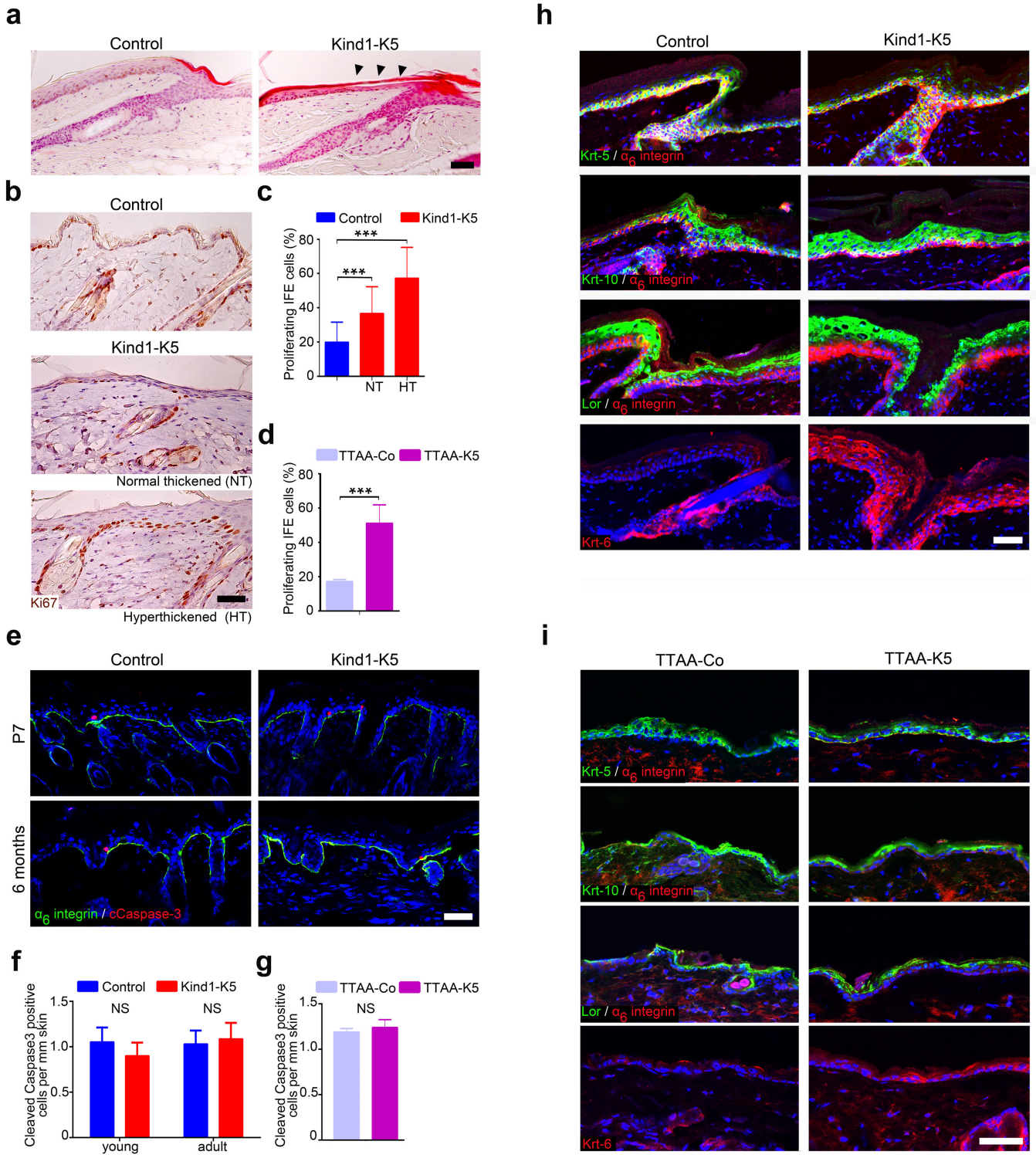


Supplementary Figure 1 Conditional deletion of Kindlin-1 in skin. (**a**) Scheme depicting the conditional Kindlin-1 targeting strategy. (**b**,**c**) Western blotting of epidermal and primary keratinocyte lysates for Kindlin-1 and -2 (**b**) and immunofluorescence for Kindlin-1 (red) and α_6 integrin (green) in back skin sections from P21 old mice (**c**). Nuclei are stained with DAPI (blue). (**d**) Weight curve of control and Kind1-K5 male littermates reported as mean ± SD (*n*=8 Control; 6 Kind1-K5). (**e**) Immunofluorescence staining of P44 back skin for Desmoplakin (green) (upper panel) and Phalloidin (green) (lower panel) co-stained for α_6 integrin (red). Arrowheads indicate basal localization. Nuclei are stained with DAPI (blue). (**f**) Immunofluorescence staining of back skin from P7 and 6 months old mice for LN-332 (red) and Mac-1 (green) detecting macrophage infiltrations. Nuclei are stained with DAPI (blue). (**g**) Immunofluorescence staining of ear whole mounts from 4 months old mice for Gr-1 (green) staining granulocytes (upper panel) and CD4 (green) staining T cells (lower panel). Blood vessels are stained with pan-LN (red). (**h**) Number of innate and adaptive immune cells (macrophages: Mac-1, granulocytes: Gr-1, T cells: CD4, B lymphocytes: CD19, dendritic epidermal cells: CD11c, mast cells: MC) in the dermis of Kind1-K5 (left) and TTAA-K5 mice (right) quantified as percentage of total skin cells and shown as mean ± SD (*n*=4 per genotype). (**i**) Pigment deposits in tails from 6 months old Kind-K5 mice (see arrowheads). (**j**) Appearance of control and Kind1-K5 mice after one year of age. Scale bars indicate 50 µm (**c**,**f**,**g**) and 25 µm (**e**).

Supplementary Figure 2



Supplementary Figure 2 Skin characterization of Kind1-K5 and TTAA-K5 mice. (**a**) H/E staining of 6 months old tail skin. Arrowheads indicate atrophic skin areas in Kind1-K5 mice. (**b**) Ki67 staining of back skin sections from 6 months old mice with upper panel showing normal and lower panel hyperthickened epidermis in Kind1-K5 mice. (**c,d**) Ki67-positive Kind1-K5 (**c**) and TTAA-K5 (**d**) IFE keratinocytes quantified as percentage of total interfollicular cells and shown as mean ± SD (*n*=3 per genotype, ≥20 10x objective fields were counted). (**e-g**) Back skin sections from P7 and 6 months old mice, respectively, immunostained for cleaved Caspase-3 (cCaspase-3; red) and α6 integrin (green) (**e**), and quantification of cleaved Caspase-3 positive cells per mm IFE in young (≤ P21) and adult (≥ 3 months) skin (**f**) or adult skin of TTAA-K5 mice (**g**) reported as mean ± SD (*n*=5 young and 4 adult Control; 7 young and 3 adult Kind1-K5 mice, 5 adult TTAA-Co and TTAA-K5). Nuclei in (**e**) are stained with DAPI (blue). (**h,i**) Differentiation of epidermal keratinocytes in back skin of 10 weeks old Kind1-K5 (**h**) and TTAA-K5 (**i**) mice shown with indicated differentiation markers (keratin 5, Krt-5; keratin 10, Krt-10; loricrin, Lor; all in green) and co-stained with α_6 integrin (red). Keratinocyte activation is shown by Krt-6 (red) expression (lower panel). Nuclei are stained with DAPI (blue). All scale bars indicate 50 μm.

Supplementary Figure 3

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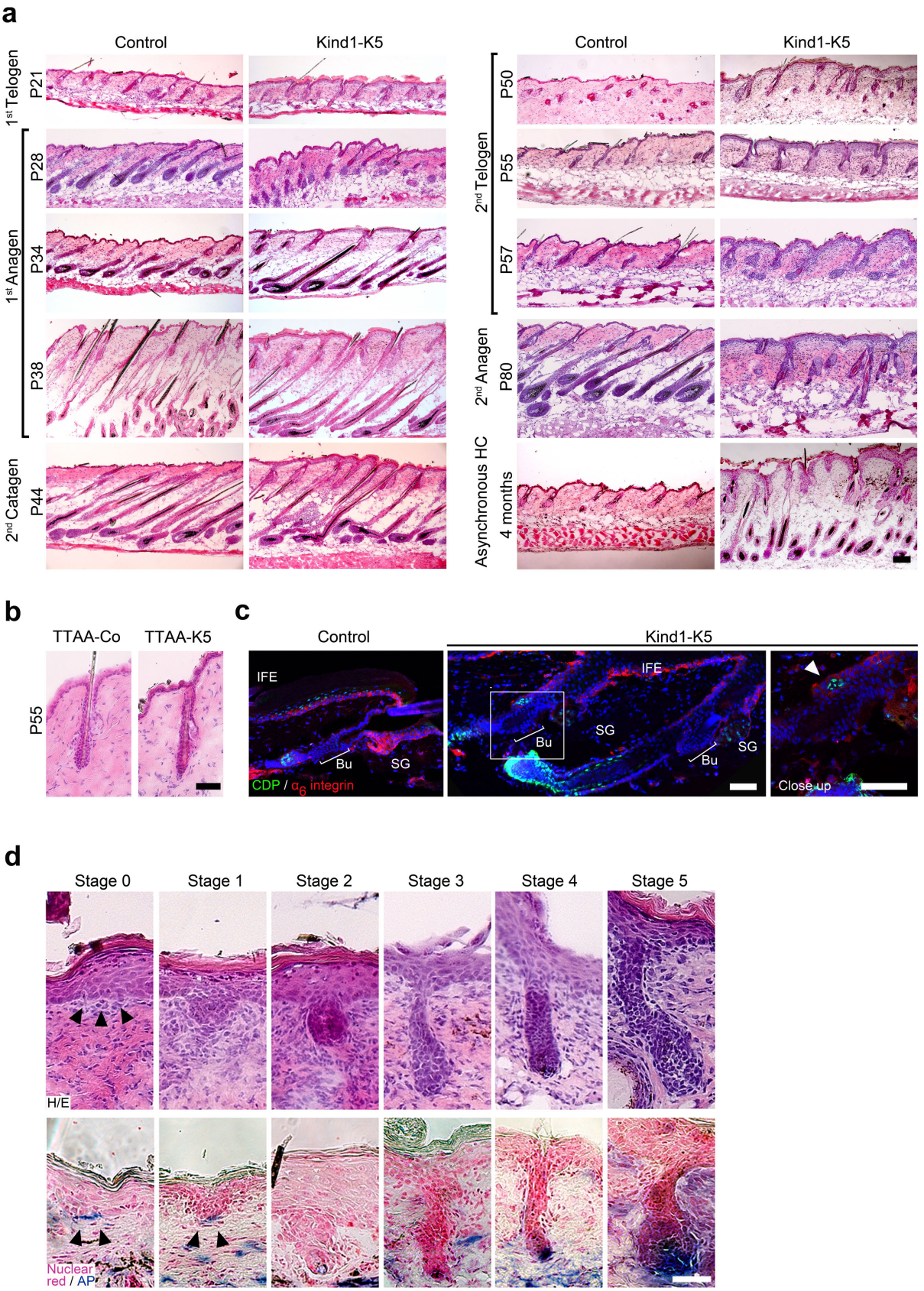

Supplementary Figure 3

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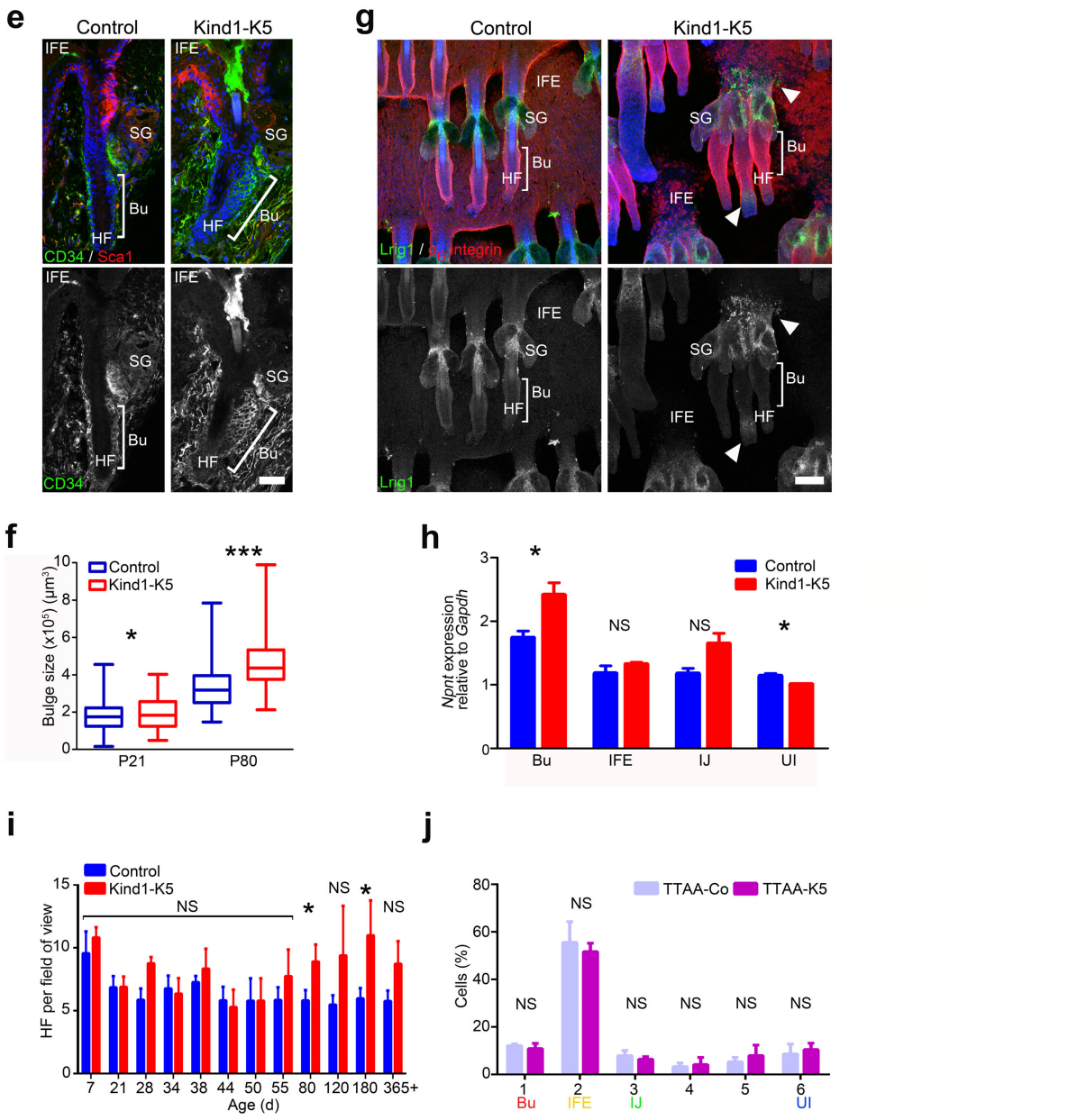

Supplementary Figure 3 HF and SC compartment analysis in skin from Kind1-K5 and TTAA-K5 mice. (**a**) H/E staining of back skin sections at indicated time points for HC analysis. (**b**) H/E staining of P55 back skin from TTAA-Co and TTAA-K5 mice. (**c**) Immunostaining of back skin from P80 mice for CDP (green) and α_6 integrin (red). Arrowhead in right panel indicates ectopic CDP expression. Nuclei are stained with DAPI (blue). (**d**) Ectopic HF development in back skin of Kind1-K5 mice analyzed by H/E (upper panel) and alkaline phosphatase (AP) staining (lower panel). HF stages 1–5 were classified as described before[69]; arrowheads indicate early hair germ formation. (**e**) Immunostaining of P80 back skin HF for CD34 (green) and Sca1 (red). Nuclei are stained with DAPI (blue). (**f**) Bulge size analysis reported as boxplots (P21 *n*=3 Control; 4 Kind1-K5; P80 *n*=5 per genotype; 20-67 HFs per animal from different whole mounts; boxplot whisker ends show min/max distribution and middle line reports the median). (**g**) Immunostaining of tail whole mounts from P80 mice for Lrig1 (green) and α_6 integrin (red). Arrowheads indicate ectopic Lrig1 expression. Nuclei are stained with DAPI (blue). (**h**) FACS-sorted keratinocyte subpopulations from 3 P80 mice per genotype were pooled and transcript levels of *Npnt* analyzed by qPCR. Expression levels are reported as mean ± SEM relative to *Gapdh* (*n*=3 technical replicates). (**i**) HF numbers in back skin at indicated age are reported as mean ± SD (*n*=2–4 per genotype, ≥10 10x objective fields were counted). (**j**) FACS analysis of primary TTAA-K5 keratinocytes (*n*=4 per genotype) described in **Fig. 3e,f**. The color code corresponds to the cell population denoted in **Fig. 3a**. Scale bar indicates 50 μm in (**a**–**d**) and 100 μm in (**e**,**g**). Bu, bulge; SG, sebaceous gland; HF, hair follicle; IFE, interfollicular epidermis; UI, upper isthmus; IJ, interfundibulum junctional zone.

Supplementary Figure 4

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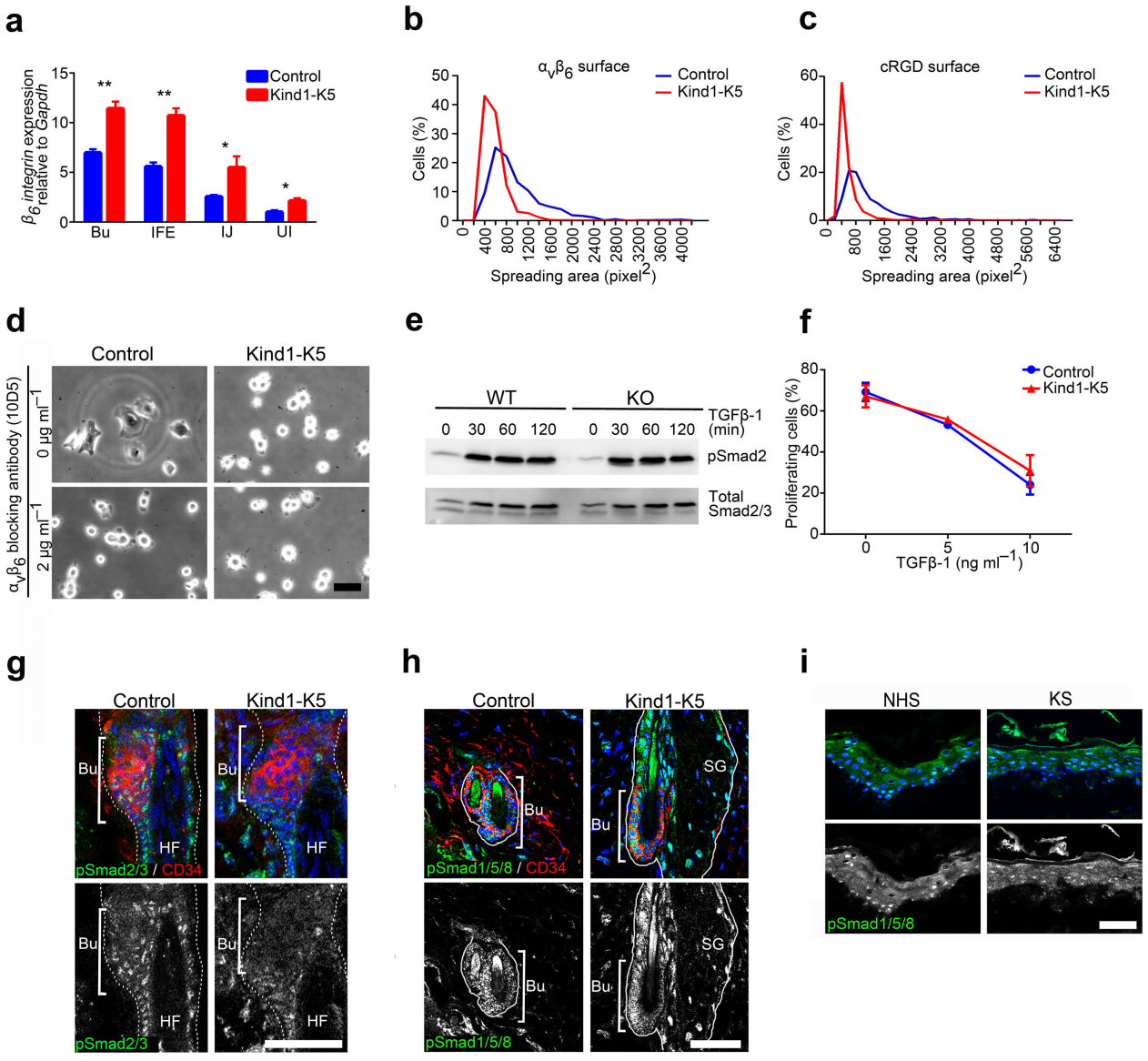

Supplementary Figure 4 Influence of Kindlin-1 on $\alpha_v\beta_6$ integrin, TGFβ and BMP signaling. (**a**) FACS sorted keratinocyte subpopulations from 3 P80 mice per genotype were pooled and transcript levels of *β_6 integrin* were analyzed by qPCR. Expression levels are reported as mean ± SEM relative to *Gapdh* (*n*=3 technical replicates). (**b,c**) Representative experiment showing spreading area on anti-$\alpha_v\beta_6$ integrin antibody (*n*=814 Control and 826 Kind1-K5 cells; **b**) and cRGD-coated (**c**) surfaces (*n*=731 Control and 694 Kind1-K5 cells) (*n*=3 biological replicates). (**d**) Spreading of primary keratinocytes on cRGD-coated surfaces in the absence or presence of anti-$\alpha_v\beta_6$ integrin blocking antibody. (**e**) Treatment of keratinocytes with TGFβ-1 for indicated time points followed by western blot analysis of pSmad2 and total Smad2/3. (**f**) Proliferation analyzed with the EdU incorporation assay after treatment with indicated TGFβ-1 concentrations reported as mean ± SEM (*n*=3 biological replicates). (**g**) Immunofluorescence staining of P44 back skin HF for pSmad2/3 (green) and CD34 (red). Nuclei are stained with DAPI (blue). (**h**) Immunofluorescence staining of a 6 months old back skin HF for pSmad1/5/8 (green) and CD34 (red). Nuclei are stained with DAPI (blue). (**i**) Immunostaining of biopsy skin sections from healthy (NHS) and individuals with KS for pSmad1/5/8 (green). Nuclei are stained with DAPI (blue). All scale bars indicate 50 μm. Bu, bulge; SG, sebaceous gland; IFE, interfollicular epidermis; UI, upper isthmus; IJ, interfundibulum junctional zone.

Supplementary Figure 5

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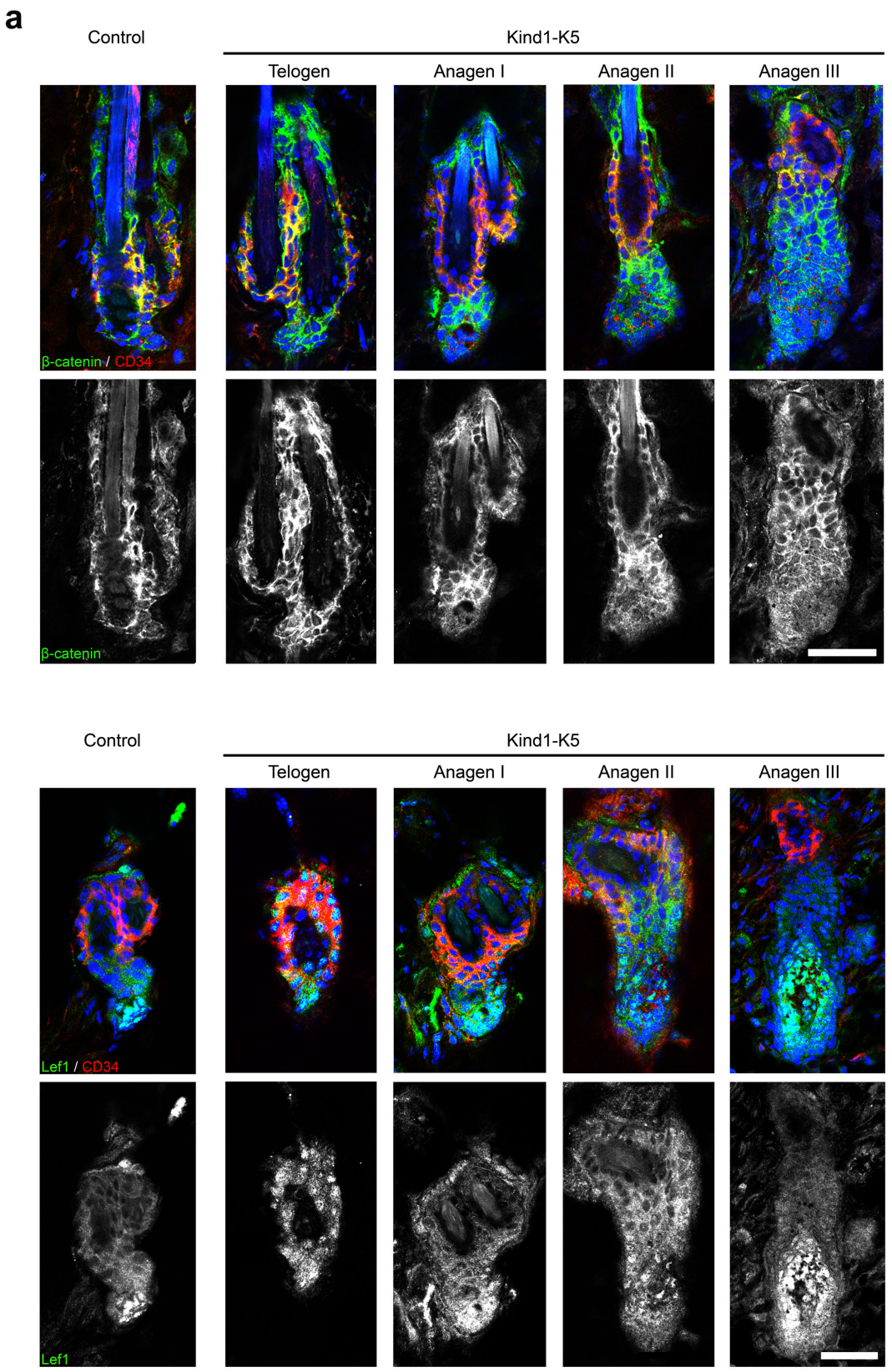
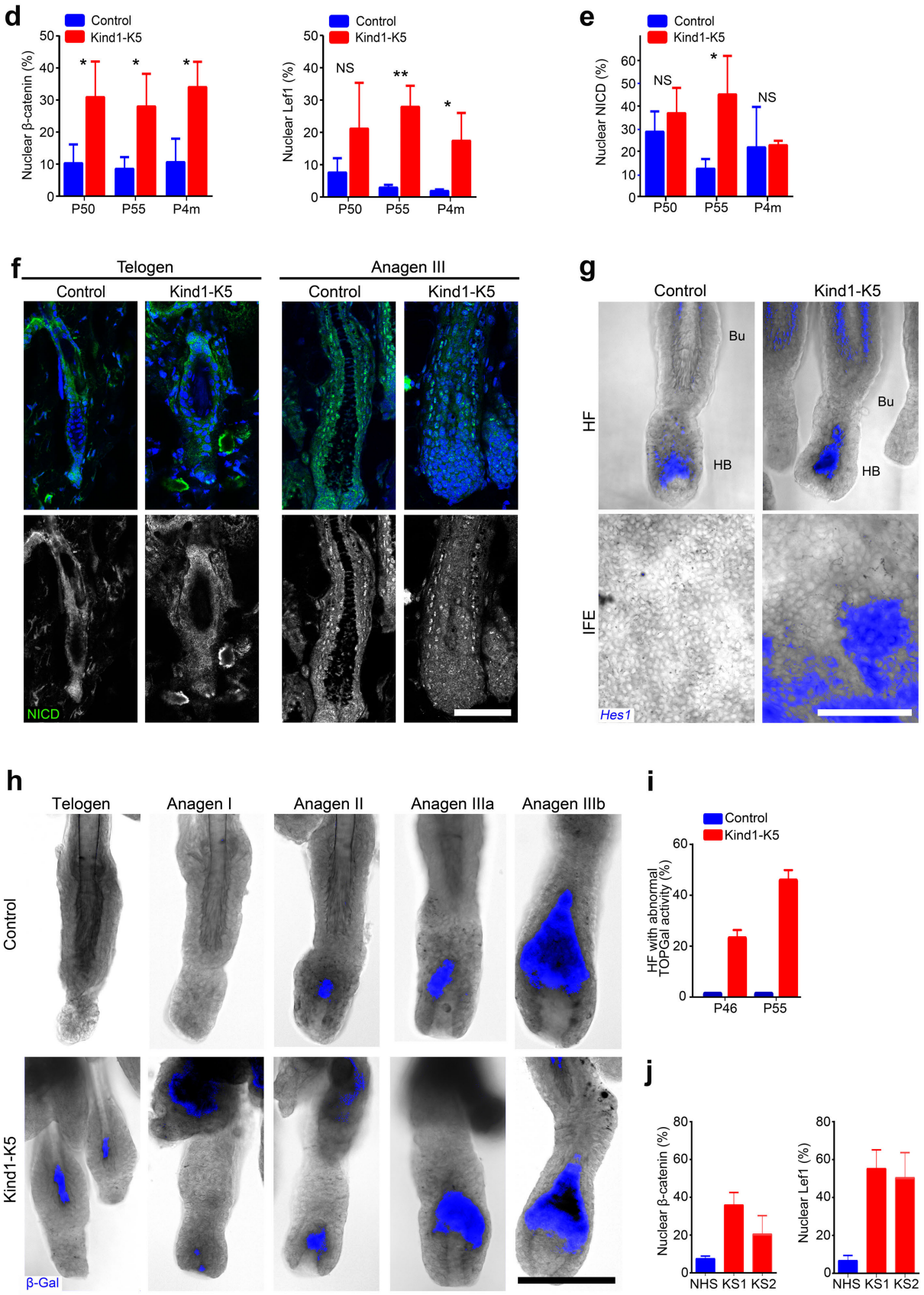


Supplementary Figure 5



Supplementary Figure 5 Kindlin-1 loss leads to elevated Wnt-β-catenin and Notch signaling in skin. (**a**) Immunofluorescence staining of back skin from Kind1-K5 mice during premature anagen induction at indicated HC stage (illustrated in **b**) for β-catenin (upper panel) and Lef1 (green) (lower panel) and co-staining with CD34 (red). Nuclei are stained with DAPI (blue). (**b**) Scheme of HF during the HC transition from telogen to full anagen. (**c**) FACS-sorted keratinocyte subpopulations from 3 P80 mice per genotype were pooled and transcript levels of *Lef1* were analyzed by qPCR and shown as mean ± SEM relative to *Gapdh* (*n*=3 technical replicates). (**d**,**e**) Percentage of IFE keratinocytes at indicated time points with nuclear β-catenin (**d**, left), Lef1 (**d**, right) and NICD (**e**) reported as mean ± SD (*n*=3 mice per genotype with >1000 IFE cells counted per genotype). (**f**) Immunofluorescence staining of telogen (left panel) and anagen (right panel) HF from 4 months old mice for NICD (green) and DAPI (blue). (**g**) *In situ* hybridization of epidermal tail whole mount from 3 months old mice for *Hes1*; the blue *in situ* signal was overlaid with the grayscale images of the HF and IFE. (**h**) Comparison of TOPgal reporter activity at indicated HC during anagen induction in tail skin from control and Kind1-K5 mice; the blue β-Gal activity was overlaid with a grayscale image of the HF. (**i**) Percentage of tail skin HFs with abnormal TOPGal activity quantified at indicated time points (*n*=3 per genotype). (**j**) Percentage of IFE keratinocytes with nuclear β-catenin (left) and Lef1 (right) in healthy humans (NHS) and subject 1 (KS1) and 2 (KS2) with KS disease reported as mean ± SD (*n*=8 40x field of view, >1000 IFE keratinocytes per subject). Scale bars indicate 50 μm (**a**) and 100 μm (**f–h**). Bu, bulge; IFE, interfollicular epidermis; UI, upper isthmus; IJ, interfundibulum junctional zone.

Supplementary Figure 6

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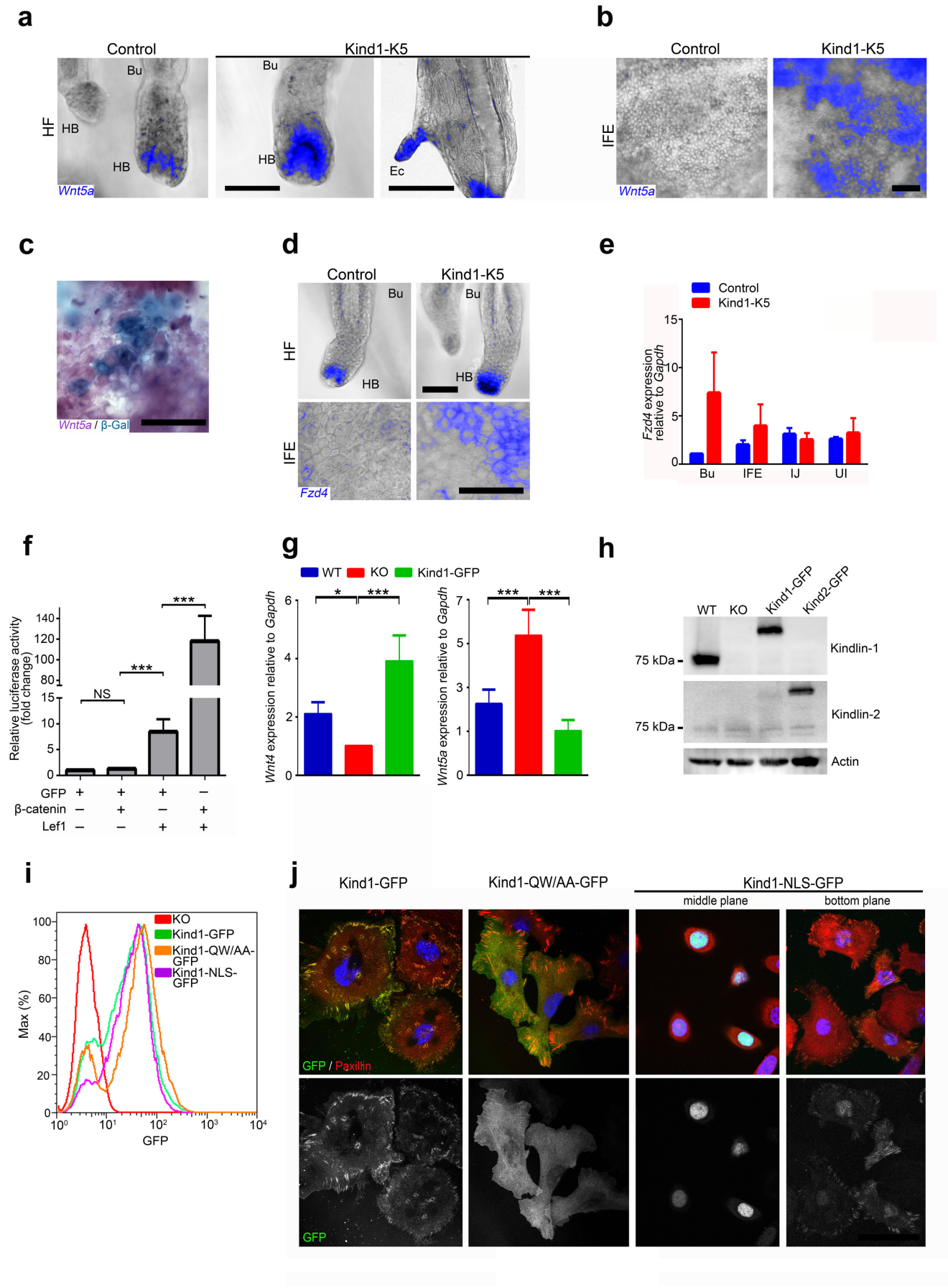

Supplementary Figure 6 Kindlin-1 controls Wnt-β-catenin signaling. (**a**,**b**) *In situ* hybridization of epidermal tail whole mount from P48 (HFs) (**a**) and 4 months (IFE, **b** and Ec, **a**) old mice for *Wnt5a*. (**c**) IFE TOPgal reporter activity overlays with *Wnt5a* expression in 4 months old Kind1-K5 tail epidermal whole mounts. For double labeling β-Gal staining (blue) was developed before epidermal whole mounts were probed for *Wnt5a* (purple). (**d**) *In situ* hybridization of epidermal tail whole mount from 2 months (HFs) and 8 months (IFE) old mice for *Fzd4*. The blue *in situ* signal was overlaid with the grayscale images of the HF and IFE (**a**,**b**,**d**). (**e**) FACS-sorted keratinocyte subpopulations from 3 P80 mice per genotype were pooled and transcript levels of *Fzd4* were analyzed by qPCR and shown as mean ± SEM relative to *Gapdh* (*n*=3 technical replicates). (**f**) TOPFlash activity in ketratinocytes from control mice transiently transfected with Lef1 and β-catenin expression plasmids. Values were corrected for the renilla control, are represented as fold increase relative to lowest value and reported as mean ± SEM (*n*=4 biological replicates). (**g**) qPCR of *Wnt4* and *Wnt5a* transcript levels in floxed (WT), AdenoCre-treated Kindlin-1 deficient (KO) and Kindlin-1-GFP keratinocytes shown as mean ± SEM relative to *Gapdh* (*n*=3 biological replicates). (**h**–**j**) Western blot for Kindlin-1 and 2 (**h**), FACS analysis for GFP (**i**) and immunostaining for paxillin (red) and GFP (green) (**j**) of Kindlin-1 deficient kerationcytes stably transduced with indicated GFP-tagged Kindlin-1 constructs. Nuclei are stained with DAPI (blue). All scale bars indicate 50 µm. Bu, bulge; Ec, ectopic hair follicle; HB, hair bulb; HF, hair follicle; IFE, interfollicular epidermis; UI, upper isthmus; IJ, interfundibulum junctional zone.

Supplementary Figure 7

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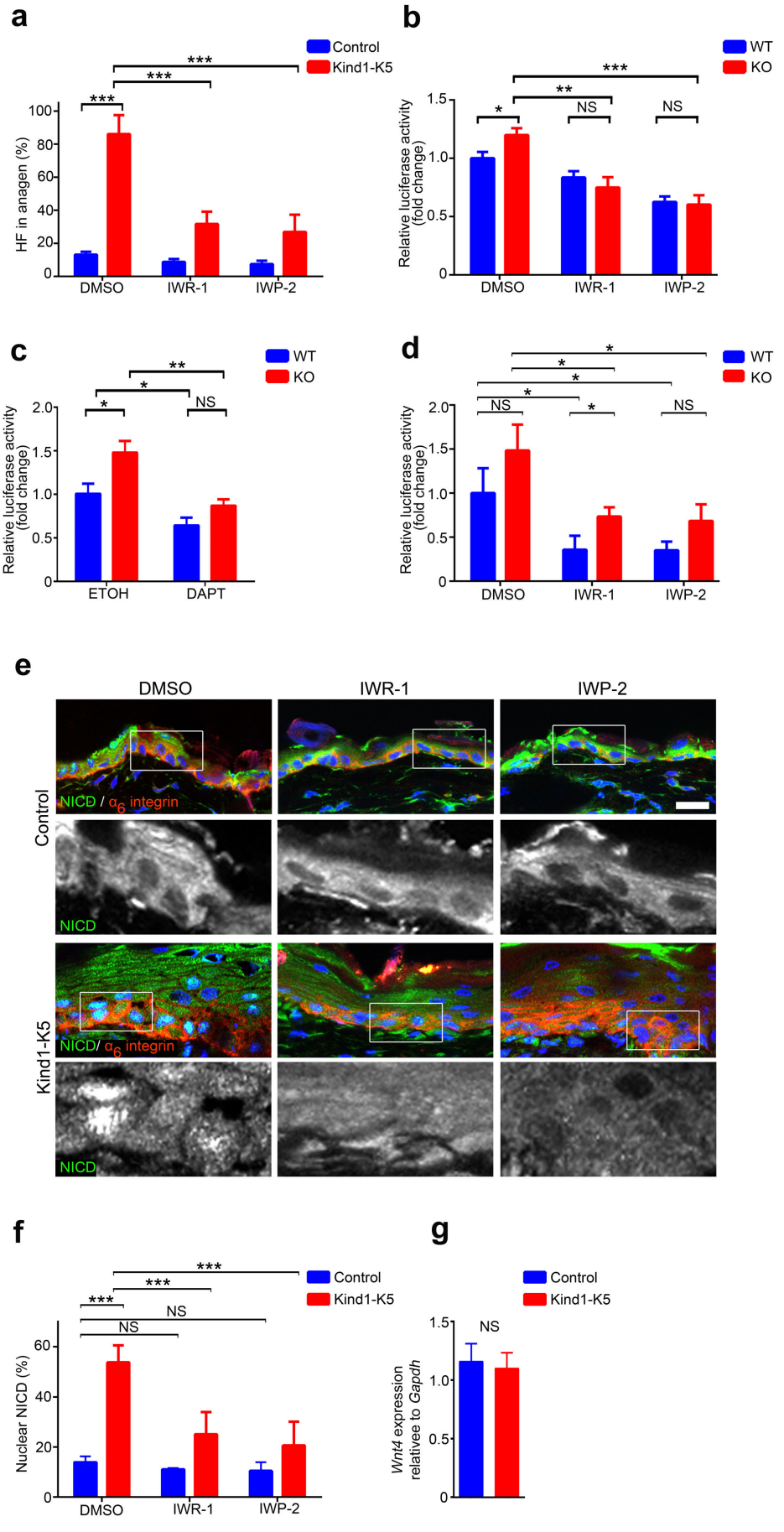

Supplementary Figure 7 Kindlin-1 loss induced Wnt-β-catenin and Notch signaling *in vivo* and *in vitro*. (**a**) Quantification of inhibition of premature anagen induction reported as percentage of anagen HFs after indicated treatments at P56 (reported as mean ± SD, *n*=4 mice per genotype, ≥8 10x objective fields were counted). (**b–d**) TOPFlash and pHes1-Luc reporter assay with floxed (WT) and Adeno-Cre treated Kindlin-1 deficient keratinocyte (KO) lines. Values were corrected for the renilla control, are represented as fold increase relative to WT cells and reported as mean ± SEM. (**b**) TOPFlash reporter activity measured 24 h after treatment with 50 μM of indicated Wnt inhibitor (*n*=5, all biological replicates). (**c**,**d**) pHes1-Luc reporter activity analyzed 24 h after treatment with 2.5 μM DAPT (Notch signaling inhibitor) (*n*=4, all biological replicates) (**c**) or with 50 μM of indicated Wnt inhibitor (*n*=3, all biological replicates) (**d**). (**e**) Immunofluorescence staining of back skin for NICD (green) and α_6 integrin (red) after treatment with Wnt inhibitor. Nuclei are stained with DAPI (blue). Scale bar indicates 25 μm. (**f**) Percentage of IFE cells with nuclear NICD in back skin treated with Wnt inhibitors and shown as mean ± SD (*n*=4 per genotype, ≥1000 IFE keratinocytes counted per mouse). (**g**) qPCR of *Wnt4* transcript levels in floxed (WT) and AdenoCre-treated Kindlin-1 deficient (KO) keratinocytes treated with 2.5 μM DAPT for 24 h shown as mean ± SEM expression relative to *Gapdh* (*n*=4 biological replicates).

Supplementary Figure 8

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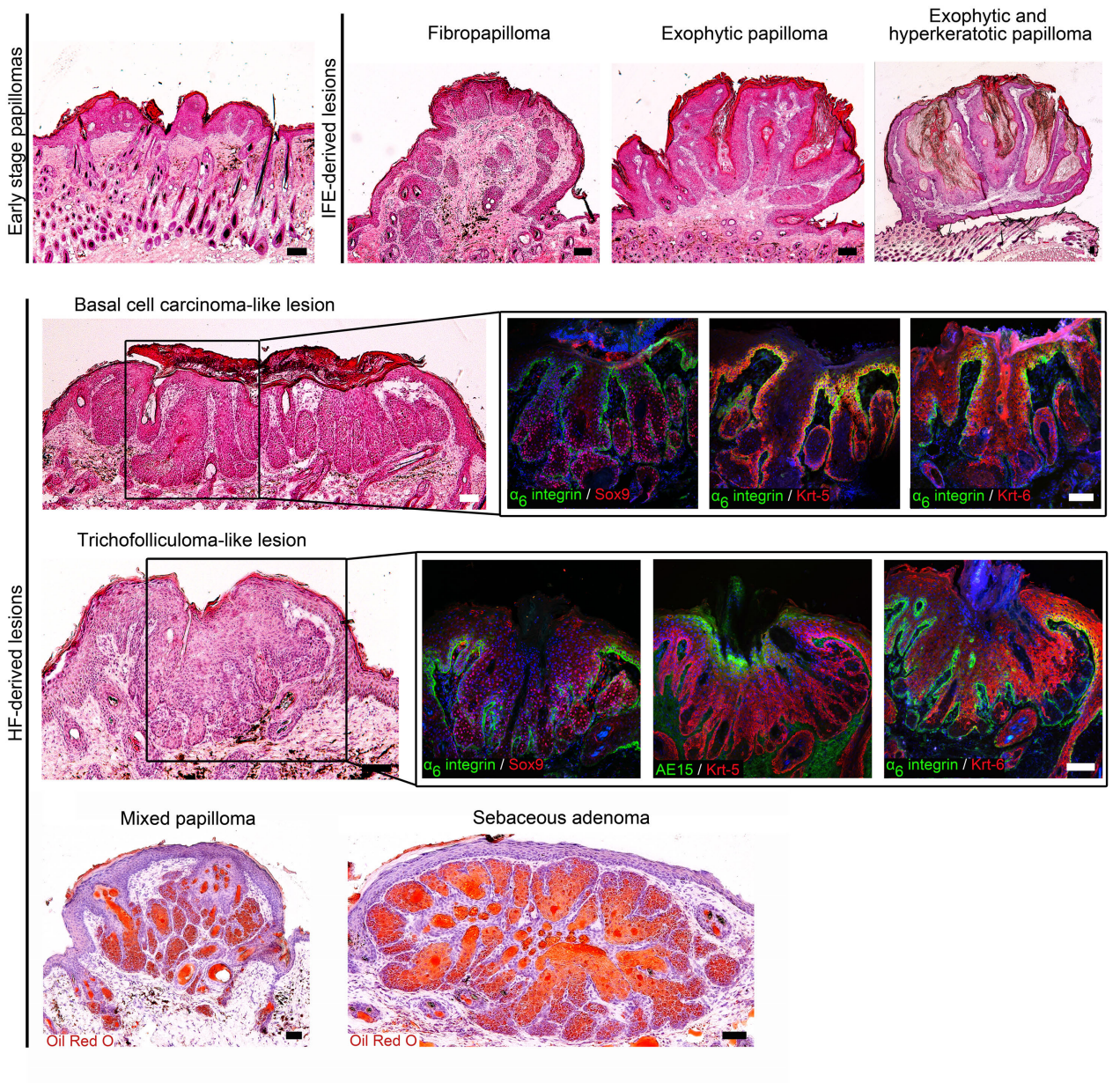
a



Supplementary Figure 8

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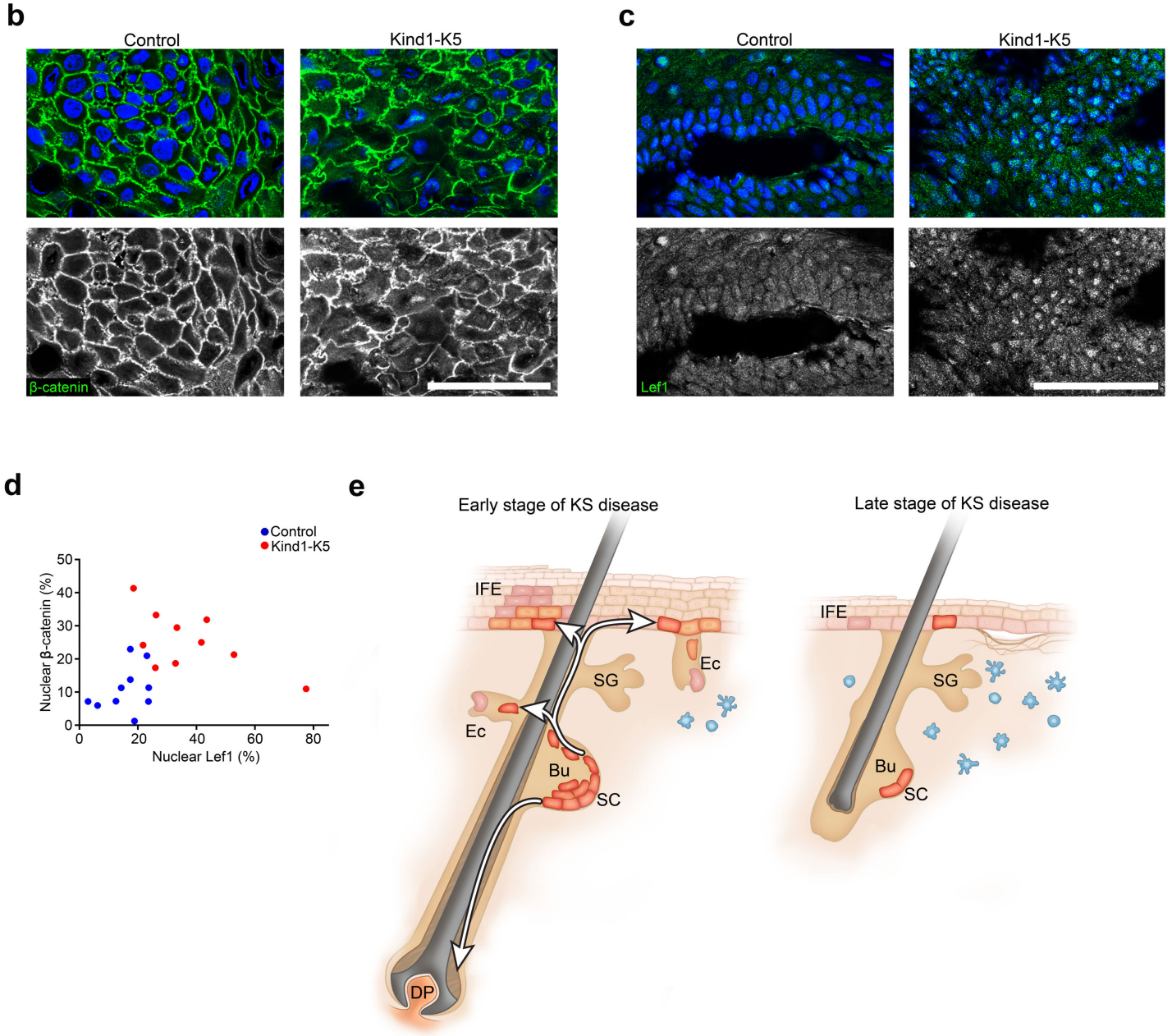

Supplementary Figure 8 Elevated Wnt-β-catenin signaling in skin tumors from Kind1-K5 mice. (**a**) Histological analysis of skin lesions after two stage carcinogenesis. H/E staining of representative skin lesions. BCC- and trichofolliculoma-like lesions display characteristic staining for Sox9 (red), Krt-5 (red), Krt-6 (red), AE15 (green) and α_6 integrin (green). Mixed papilloma and sebaceous adenoma were positive for OilRed O. Nuclei are stained with DAPI (blue). (**b**,**c**) Tumor sections immunostained for β-catenin (green) (**b**) and Lef1 (green) (**c**). Nuclei are stained with DAPI (blue). (**d**) Percentage of cells with nuclear β-catenin (y-axis) and Lef1 (x-axis) in tumors (*n*=10 per genotype; ≥ 1500 cells counted per tumor). (**e**) Model showing early and late cellular consequences of Kindlin-1 deletion in mouse keratinocytes. The early stage shows elevated epithelial SC proliferation and mobilization leading to expanded SC compartments, epidermal hyperthickening, premature HF induction and ectopic HF development from existing HF and IFE (early KS disease stage). The late stage is characterized by an inflammatory response to skin blistering and SC exhaustion leading to a progressive HF loss and skin atrophy. Scale bars indicate 100 µm (**a**) and 50 µm (**b**,**c**). Bu, bulge; SC, stem cell; SG, sebaceous gland; HF, hair follicle, DP, dermal papilla; IFE, interfollicular epidermis; Ec, ectopic HF.

Supplementary Table 1 *P*-values for relative amount of SC subpopulations over time analyzed by FACS (see **Fig. 3g**). *P*-values were calculated with unpaired t-test. Bu, bulge; UI, upper isthmus; IJ, interfundibulum junctional zone.

	N (animal number)		SC compartment		
Age	**Control**	**Kind1-K5**	**Bu**	**IJ**	**UI**
21 d	4	3	*P*= 0.0004	*P*= 0.0146	*P*= 0.9161
24 d	5	3	*P*= 0.0008	*P*= 0.3161	*P*= 0.0082
28 d	5	3	*P*= 0.0047	*P*= 0.0874	*P*= 0.1014
40 d	5	3	*P*= 0.0008	*P*< 0.0001	*P*= 0.0302
44 d	3	3	*P*= 0.0009	*P*= 0.0004	*P*= 0.0182
50 d	5	3	*P*= 0.0004	*P*= 0.0032	*P*= 0.2708
55 d	3	3	*P*= 0.0012	*P*< 0.0001	*P*= 0.0452
80 d	7	4	*P*< 0.0001	*P*< 0.0001	*P*= 0.0003
4 months	4	3	*P*= 0.2501	*P*= 0.0074	*P*= 0.0010
6 months	8	5	*P*= 0.0863	*P*= 0.4542	*P*= 0.0047
>11 months	4	3	*P*= 0.2265	*P*= 0.0232	*P*= 0.3730

Supplementary Table 2 Microarray data with significant gene expression changes of ≥ 2 fold. First sheet shows all genes sorted by the difference score. In the following sheets genes are divided in the indicated categories (Wnt signaling; Inflammation and Wound healing; Proliferation and Cell cycle; Metabolism). N, NHS skin; K, KS skin; AVG, average; DiffScor, difference score.

Supplementary Table 3 Wnt ligand and receptor transcript analysis. qPCR of primary keratinocytes for Wnt ligands and Wnt receptors reported as mean ± SEM expression relative to *Gapdh* (*n*= indicated biological replicates). *P*-values were calculated with unpaired t-test. ND, not determined.

Gene Symbol	Control		Kind1-K5		Log_2 fold change	*P*-value	*n*
	mean	SEM	mean	SEM			
Wnt1	0.496	0.089	1.50	0.089	1.649	0.001	3
Wnt2	0.506	0.053	1.494	0.053	1.578	2×10^{-4}	3
Wnt2b	0.785	0.039	1.215	0.039	0.633	0.001	3
Wnt3	ND		ND		ND		
Wnt3a	0.813	0.054	1.187	0.054	0.550	0.008	3
Wnt4	1.562	0.095	0.438	0.095	–2.088	3×10^{-5}	4
Wnt5a	0.024	0.003	1.976	0.003	6.400	2×10^{-10}	3
Wnt5b	0.99	0.162	1.012	0.162	0.033	0.885	4
Wnt6	0.366	0.032	1.634	0.032	2.167	1×10^{-5}	3
Wnt7a	1.200	0.057	0.800	0.057	–0.589	0.007	3
Wnt7b	1.263	0.158	0.737	0.158	–0.822	0.078	3
Wnt8a	ND		ND		ND		
Wnt8b	ND		ND		ND		
Wnt9a	1.220	0.131	0.780	0.131	–0.667	0.017	3
Wnt9b	1.000	0.256	2.090	0.256	1.067	0.500	3
Wnt10a	1.408	0.122	0.591	0.122	–1.300	0.009	3
Wnt10b	0.877	0.074	1.124	0.074	0.3611	0.077	3
Wnt11	1.107	0.193	0.893	0.193	–0.021	0.326	4
Wnt16	1.209	0.087	0.791	0.087	–0.622	0.027	3
Fzd1	1.064	0.242	0.936	0.242	–0.216	0.721	4
Fzd2	1.065	0.243	0.935	0.243	–0.180	0.726	3
Fzd3	0.364	0.010	1.636	0.010	2.169	6×10^{8}	3
Fzd4	0.378	0.145	1.623	0.145	2.482	0.001	4
Fzd5	0.858	0.123	1.142	0.123	0.433	0.153	4
Fzd6	0.942	0.106	1.058	0.106	0.173	0.468	4
Fzd7	0.642	0.124	1.358	0.124	1.128	0.015	3
Fzd8	0.743	0.134	1.257	0.134	0.813	0.035	4
Fzd9	0.766	0.066	1.234	0.066	0.694	0.007	3
Fzd10	0.983	0.048	1.017	0.048	0.050	0.640	3
Lrp5	1.024	0.079	0.976	0.079	–0.071	0.681	4
Lrp6	0.840	0.048	1.160	0.048	0.470	0.003	4
Ror1	0.690	0.086	1.310	0.086	0.933	0.036	3
Ror2	0.682	0.135	1.318	0.135	1.302	0.067	4

Supplementary Table 4 List of qPCR oligonucleotides.

Gene Symbol	PCR primer
Gapdh	Fw: TCCTGCACCACCAACTGCTTAGC Rev: TGGATGCAGGGATGATGTTCTGG
Npnt	Fw: ATTGATGAATGTGCGACTGG Rev: CTGCTACACTGGTGCTGTCC
Itgb6	Fw: ATGGGGATTGAGCTGGTCTG Rev: GACAGGTGGGTGAAATTCTCC
Lef1	Fw: TCTGGCTACATAATGATGCCCA Rev: GGACATGCCTTGCTTGGAGTT
Wnt1	Fw: CGACTGATCCGACAGAACCC Rev: CCATTTGCACTCTCGCACA
Wnt2	Fw: CCTCCGAAGTAGTCGGGAATC Rev: GCAGGACTTTAATTCTCCTTGGC
Wnt2b	Fw: AACATCCATTACGGTGTTCGC Rev: CCTGTGCGTCGGAAGTCTG
Wnt3a	Fw: AATTTGGAGGAATGGTCTCTCGG Rev: CAGCAGGTCTTCACTTCACAG
Wnt4	Fw: GTCAGGATGCTCGGACAACAT Rev: CACGTCTTTACCTCGCAGGA
Wnt5a	Fw: GGACCACATGCAGTACATTGG Rev: CGTCTCTCGGCTGCCTATTT
Wnt5b	Fw: TCCTGGTGGTCACTAGCTCTG Rev: TGCTCCTGATACAACTGACACA
Wnt6	Fw: GCAAGACTGGGGGTTCGAG Rev: CCTGACAACCACACTGTAGGAG
Wnt7a	Fw: TGAACTTACACAATAACGAGGCG Rev: GTGGTCCAGCACGTCTTAGT
Wnt7b	Fw: CTTCACCTATGCCATCACGG Rev: TGGTTGTAGTAGCCTTGCTTCT
Wnt9a	Fw: GGCCCAAGCACACTACAAG Rev: AGAAGAGATGGCGTAGAGGAAA
Wnt9b	Fw: CAGAGAGGCTTTAAGGAGACGG Rev: CCTGGGGAGTCGTCACAAG
Wnt10a	Fw: CAGATCGCCATCCATGAGTG Rev: ACCGCAAGCCTTCAGTTTACC
Wnt10b	Fw: ATCGCCGTTCACGAGTGTC Rev: GGAAACCGCGCTTGAGGAT
Wnt11	Fw: ATGCGTCTACACAACAGTGAAG Rev: GTAGCGGGTCTTGAGGTCAG

Wnt16	Fw: GCAGGCTGTCGCCAAGTTA Rev: GTCTGCCTCTGGTCTTTTTCTC
Fzd1	Fw: GAGTTCTGGACCAGTAATCCGC Rev: ATGAGCCCGTAAACCTTGGTG
Fzd2	Fw: CTTCTCGCAAGAGGAGACTCG Rev: GTGGTGACCGTGAAGAAAGTG
Fzd3	Fw: TGATGAGCCATATCCCCGACT Rev: GCCTATGAAATAGCGAGCAAATG
Fzd4	Fw: AACCTCGGCTACAACGTGAC Rev: GGCACATAAACCGAACAAAGGAA
Fzd5	Fw: GAGTCACACCCACTCTACAACA Rev: CGGAATCGTTCCATGTCAATGAG
Fzd6	Fw: TAATGGACACTTTTGGCATC Rev: ATCCAATGTCTCTTGGGACT
Fzd7	Fw: GACCAAGCCATTCCTCCGTG Rev: CAGGTAGGGAGCAGTAGGGTA
Fzd8	Fw: CCGCTGGTGGAGATACAGTG Rev: CGGTTGTAGTCCATGCACAG
Fzd9	Fw: CGCACGCACTCTGTATGGAG Rev: GCCGAGACCAGAACACCTC
Fzd10	Fw: CATGCCCAACCTGATGGGTC Rev: GCCACCTGAATTTGAACTGCT
Lrp5	Fw: ACGTCCCGTAAGGTTCTCTTC Rev: GCCAGTAAATGTCGGAGTCTAC
Lrp6	Fw: TGCAAACAGACGGGACTTGAG Rev: CGGGGACAATAATCCAGAAACAA
Ror1	Fw: AACCCTTGATGAGCCGATGAA Rev: CAGCGGATACTGGGAGGTG
Ror2	Fw: AAGTGGAAGATTCGGAGGCAA Rev: CTTCAGCCACCGCACATTG