Activin enhances skin tumorigenesis and malignant progression by inducing a pro-tumorigenic immune cell response

Maria Antsiferova, Marcel Huber[#], Michael Meyer[#], Aleksandra Piwko-Czuchra[#], Tamara Ramadan, Amanda S. MacLeod, Wendy L. Havran, Reinhard Dummer, Daniel Hohl, and Sabine Werner*

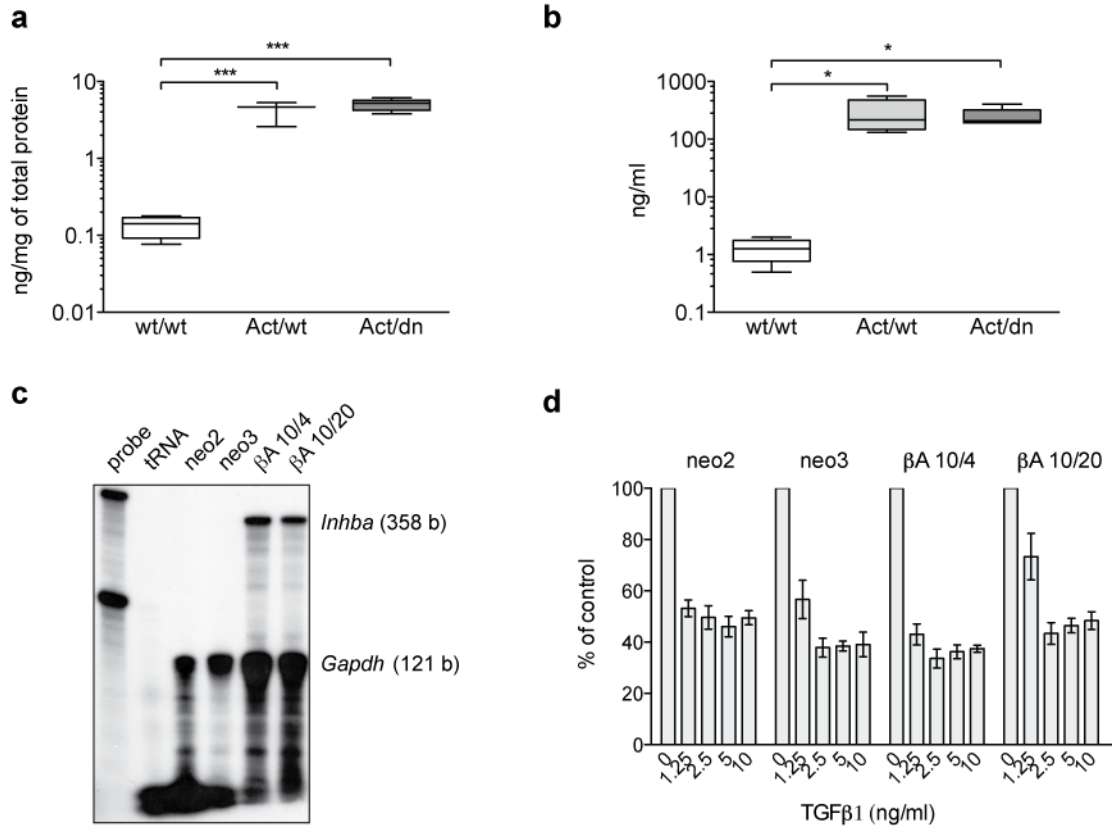
[#]Equal contribution

*Corresponding author

Supplementary Information

Supplementary Figures

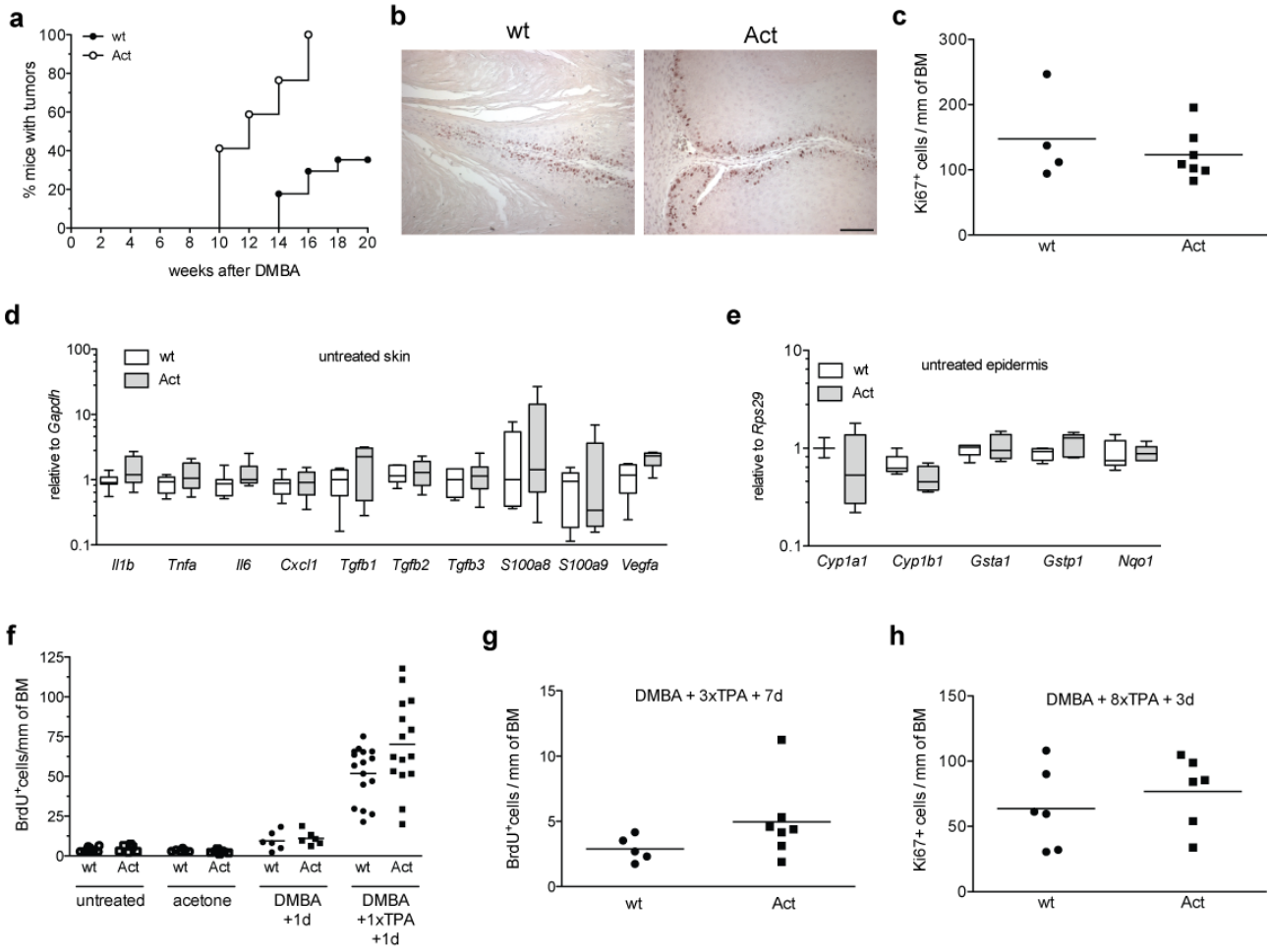
Supplementary Figure S1: Increased activin A levels in skin and serum of activin-overexpressing mice and effect of high activin levels on TGF-β responsiveness of keratinocytes



(a, b) Activin A concentrations in total skin lysates (a) and serum (b) from wt, Act and Act/dnActRIB mice were analyzed by ELISA. N=4-6 per genotype. Mean values ± SD are shown. *p<0.05; ***p<0.001 (one-way ANOVA with Bonferroni post-test). (c) RNA samples (20 µg) from HaCaT keratinocytes stably expressing activin A (βA 10/4, βA 10/20 clones) or transfected with a control vector (neo2, neo3) were analyzed by RNase protection assay to verify the overexpression of activin βA (*Inhba*) in the 10/4 and 10/20 clones. One thousand c.p.m. of the hybridization probes served as size markers (probe). tRNA (10 µg) was used as a negative control. Hybridization of RNA with a *Gapdh* riboprobe served as a loading control. (d) Activin βA transfected HaCaT keratinocytes and vector-transfected control clones were cultured for 48h in the presence of various

concentrations of TGF-β1, and BrdU incorporation was analyzed. The experiment was performed twice with 5 ng/ml TGF-β1 and once with the range of concentrations presented. Data are depicted as percentage of BrdU incorporation in untreated cells, the assay was performed in quadruplicates, mean values ± SD are shown.

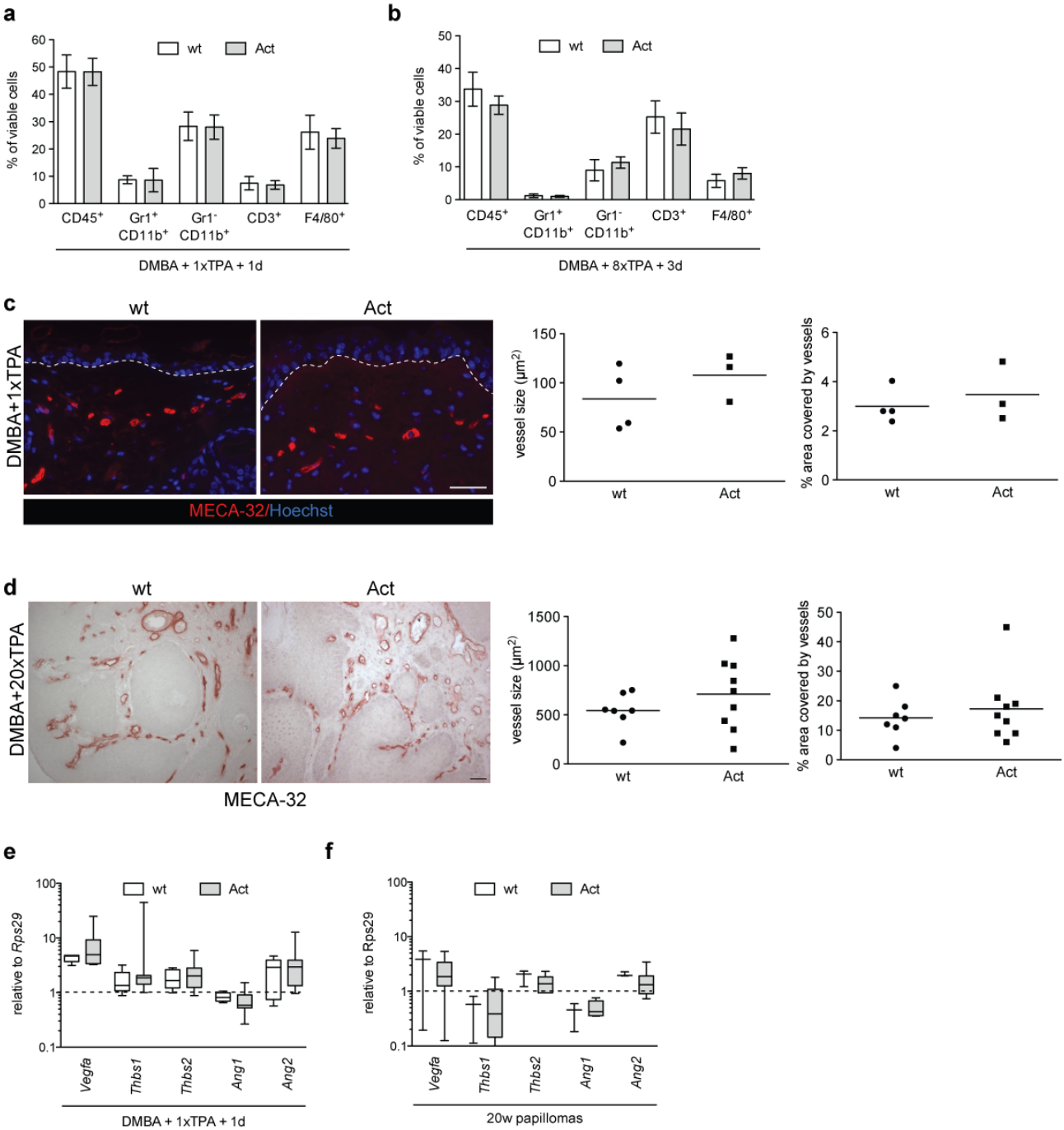
Supplementary Figure S2: Activin overexpression does not significantly affect keratinocyte proliferation in DMBA/TPA-induced tumors or treated skin or expression of cytokines and DMBA-detoxifying enzymes



(a) Kinetics of tumor incidence observed in experiment II. Kaplan-Meier survival curves. N=17 mice per genotype. (b,c) Sections of tumors collected 20 weeks after DMBA initiation were stained with antibodies against Ki67. (b) Representative pictures. (c) $Ki67^+$ cells per mm of basement membrane (BM). N=4 wt mice; N=7 Act mice; n=2-5 microscopic fields of sections per mouse; mean values ± SD are shown. (d, e) RNA samples from untreated total back skin (d) or trypsin-separated epidermis (e) were analyzed for expression of pro-inflammatory cytokines/chemokines and growth factors (d) or enzymes involved in DMBA metabolism (e) by qRT-PCR. *Gapdh* (d) or ribosomal protein S29 (*Rps29*) (e) were used as reference. N=5-10 mice per genotype. For each gene expression in one of the wt mice was arbitrarily set to 1. Box-and-whisker plot is depicted, with boxes showing median and 25^{th} and 75^{th} percentile, whiskers showing minimal and maximal values. (f, g) BrdU incorporation assay was performed at different

time points after acetone, DMBA or DMBA/TPA treatment and the number of epidermal $BrdU^+$ cells per mm of basement membrane (BM) was counted. Mean values ± SD are shown; n=6-41 microscopic fields of skin sections per mouse. N=6-16 mice per group (f) or N=5 wt mice; N=7 Act mice (g). (h) Sections from back skin collected 3 days after the 8^{th} TPA application were stained with antibodies against Ki67, and positive cells per mm of BM were counted. N=5 wt mice; N=7 Act mice; n=3-30 microscopic fields of skin sections per mouse; mean values ± SD are shown.

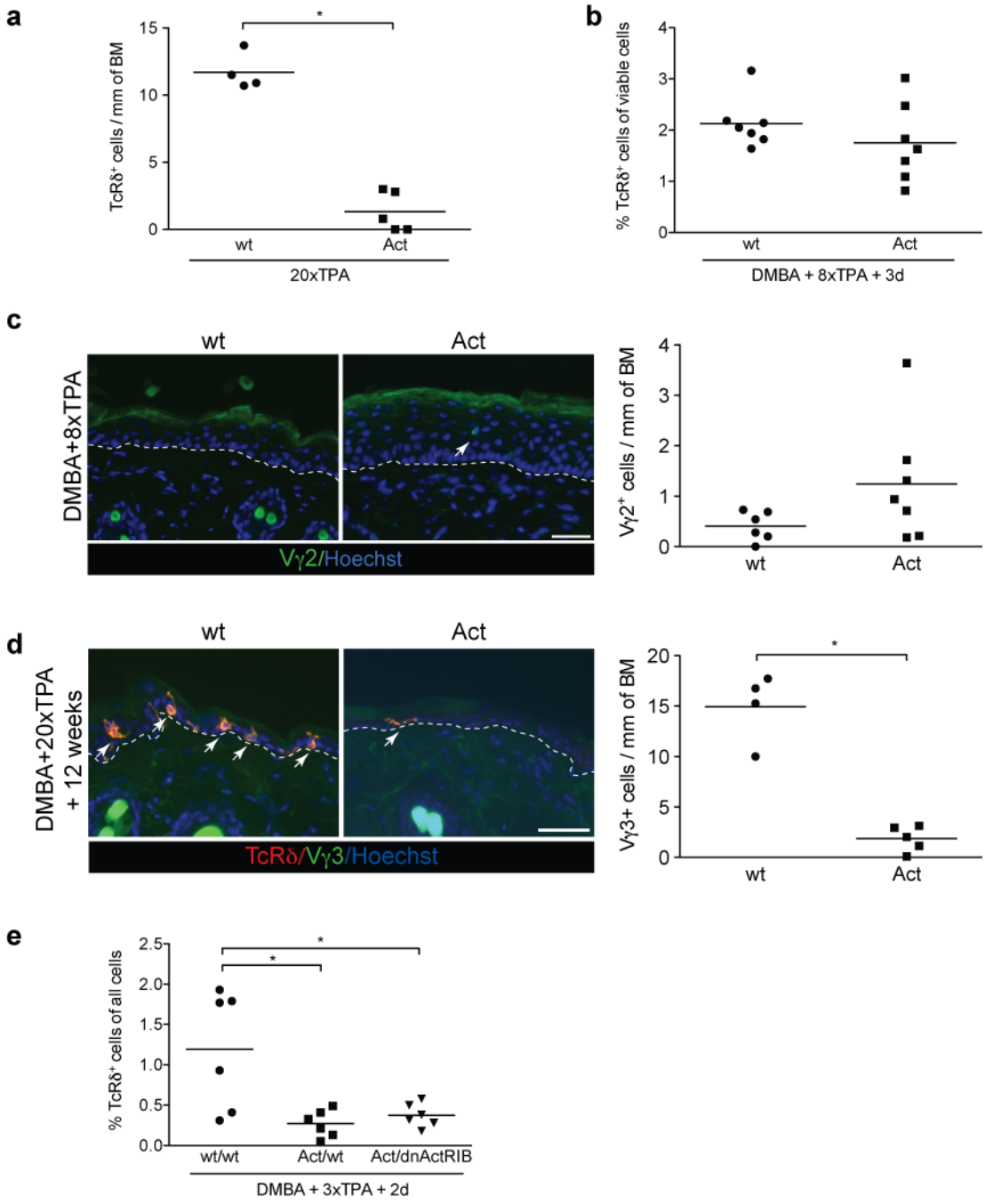
Supplementary Figure S3: Activin overexpression does not affect inflammation or angiogenesis



(a, b) Flow cytometric analysis of dermal single cell suspensions prepared from the skin of wt and Act mice 1 day after the 1st (a) or 3 days after the 8th (b) TPA treatment. The mean percentages of the respective immune cells among viable cells ± SD are shown; N=3-7 mice per genotype. (c, d) Sections of skin collected 1 day after the 1st TPA application (c) or of papillomas collected 20 weeks after initiation (d) were stained with antibodies against MECA-32 to visualize blood vessels and analyzed using

immunofluorescence (c) or immunohistochemistry (d). Size of blood vessels (left) and area of dermis covered by blood vessels (right) were determined. Scatter plot and mean values are shown; N=3-9 mice per genotype. (e, f) RNA samples from TPA-treated back skin (e) or from papillomas collected 20 weeks after initiation (f) were analyzed for expression of angiogenesis-regulating factors by qRT-PCR. *Rps29* was used as reference. N=3-10 mice per genotype. For each gene expression in one of the untreated wt mice was arbitrarily set to 1 (represented by dotted line).

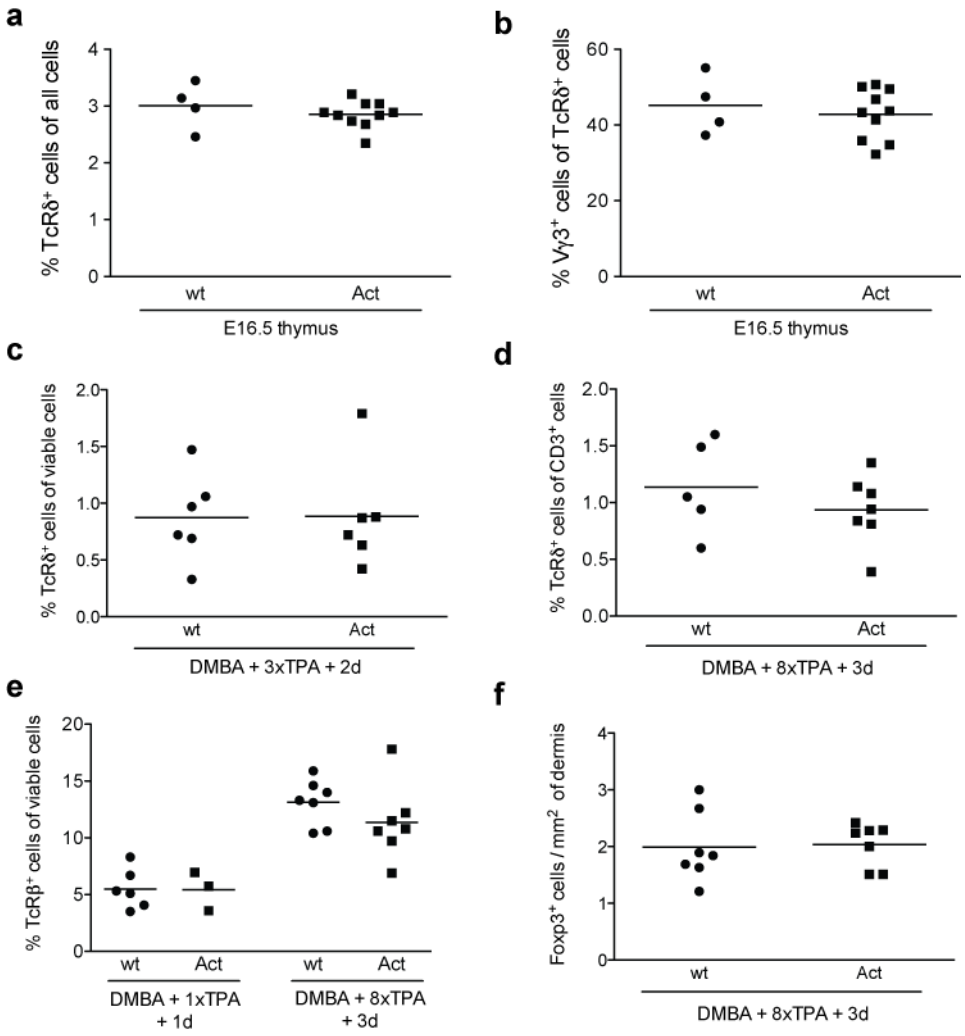
Supplementary Figure S4: Chronic TPA treatment irreversibly depletes Vγ3$^+$ T cells and attracts Vγ2$^+$ T cells to the epidermis of activin overexpressing mice



(a) TcRδ positive cells in the back skin epidermis of mice treated with TPA only for 20 weeks. N=4 wt mice; N=5 Act mice; n=9-22 microscopic fields of skin sections per mouse; *p=0.0159 (Mann-Whitney test). (b) Viable TcRδ$^+$ cells quantified by flow cytometry in dermal single cell suspensions 3 days after the 8th TPA application. N=7 mice per group. (c) Sections from non-tumorigenic skin after the 8th TPA treatment stained for the Vγ2 variant of γδ TcR . Arrows indicate Vγ2$^+$ cells. Vγ2$^+$ cells per mm of BM were

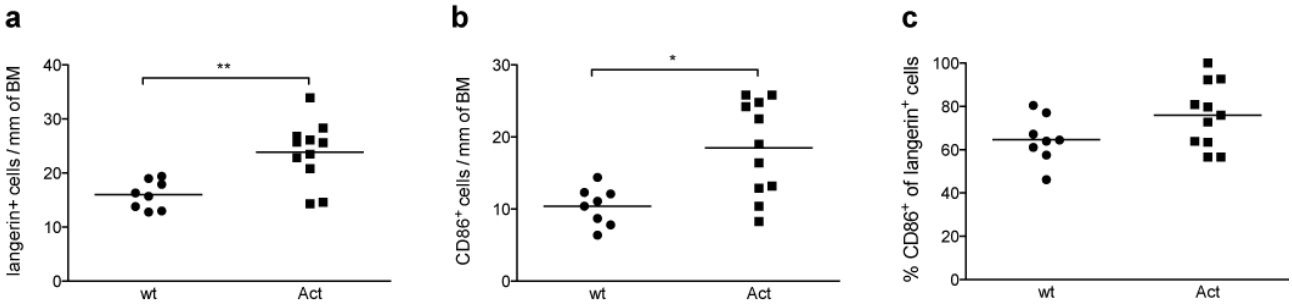
counted. N=6 wt mice; N=7 Act mice; n=3-13 microscopic fields of skin sections per mouse. (d) Sections from non-tumorigenic skin 32 weeks after initiation (12 weeks after 20th TPA application) co-stained for the δ-chain of γδ TcR (TcRδ) and the Vγ3 variant of γδ TcR. Vγ3^{+} cells per mm of BM were counted (all Vγ3^{+} cells co-expressed δTcR). N=4 wt mice; N=5 Act mice; n=6-36 microscopic fields of skin sections per mouse; *p=0.0159 (Mann-Whitney test). In (c) and (d) nuclei were counterstained with Hoechst; dotted line indicates the epidermal-dermal border. (e) Viable TcRδ^{+} cells quantified by flow cytometry in epidermal single cell suspensions prepared from skin 2 days after the 3rd TPA application. N=6 mice per genotype, *p≤0.05 (one-way ANOVA with Bonferroni post-test). Scatter plot and mean values are shown in all panels.

Supplementary Figure S5: Activin overexpression selectively affects epidermal T cells



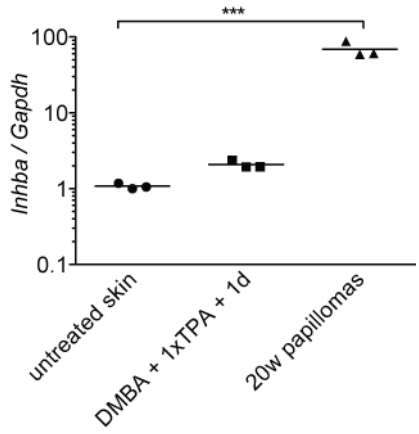
(a, b) Thymocytes were isolated from wt or Act mice at embryonic day 16.5 and analyzed by flow cytometry. The percentage of TcRδ^+ cells among all cells (a) and proportion of Vγ3$^+$ cells among all $\gamma\delta$ T cells (b) are shown. N=4 wt mice; N=10 Act mice. (c, d) Axillary lymph nodes were collected after the 3rd (c) or 8th (d) TPA application, and dissociated cells were analyzed by flow cytometry. The percentage of TcRδ^+ cells among live cells (c) or among CD3$^+$ cells (d) is shown. N=5-6 mice per genotype. (e) Viable TcRβ^+ cells quantified by flow cytometry in dermal single cell suspensions 1 day after the 1st or 3 days after the 8th TPA application. N=3-7 mice per group. (f) Dermal FoxP3$^+$ cells were counted in immunostained sections from back skin 3 days after the 8th TPA application. N=7 mice per genotype; n=6-25 microscopic fields of skin sections per mouse. Scatter plot and mean values are shown in all panels.

Supplementary Figure S6: Activin overexpression does not affect activation of Langerhans cells after long-term TPA treatment



Serial sections of non-tumorigenic back skin after 20 TPA applications were stained with antibodies against langerin or the activation marker CD86. (a) Langerin$^+$ cells / mm of basement membrane (BM) (b) CD86$^+$ cells / mm of BM. (c) Estimated percentage of CD86$^+$ cells out of langerin$^+$ cells. N=8 wt mice, N=11 Act mice, n=9-21 microscopic fields of skin sections per mouse. **p=0.0057; *p=0.0104 (Mann-Whitney test). Scatter plot and mean values are shown.

Supplementary Figure S7: Activin is overexpressed in skin papillomas of wild-type mice



RNA samples from untreated or TPA-treated back skin and from papillomas collected 20 weeks after initiation were analyzed for expression of activin βA (*Inhba*) by qRT-PCR. *Gapdh* was used as a reference. Expression in one of the untreated wt mice was arbitrarily set to 1. N=3. ***p=0.0002 (one-way ANOVA with Bonferroni post-test).

Supplementary Tables

Supplementary Table S1: Antibodies and reagents used for immunostaining

Name	*Catalog number*	*Source*
BrdU-FITC	1 202 693	Roche
BrdU-POD	1 585 860	Roche
Primary antibodies to human antigens		
Activin βA	AF338	R&D Systems, Minneapolis, MN
Pan-cytokeratin (C11)	ab7753	Abcam, Cambridge, UK
Primary antibodies to mouse antigens		
γδTcR-FITC	553177	BD Biosciences, San Diego, CA
γδTcR-biotin	13-5711	eBioscience, San Diego, CA
CD3	A0452	DAKO, Glostrup, Denmark
CD4-FITC	11-0041	eBioscience
CD68	MCA1957B	Serotec, Düsseldorf, Germany
CD86	550542	BD Biosciences
CD8-PE	12-0081	eBioscience
Cleaved caspase-3	9661	Cell Signaling, Beverly, MA
Keratin 10	PRB-159P	Covance, Princeton, New York
Keratin 13	K13.2	Lutz Langbein, DKFZ Heidelberg, Germany
Keratin 14	PRB-155P	Covance
Keratin 6	PRB-169P	Covance
Ki67	M7249	DAKO
Langerin	-	Nikolaus Romani, Innsbruck Austria
Ly6G	551459	BD Biosciences
MECA-32	553849	BD Biosciences
Pan-keratin	GP14	Progen, Heidelberg, Germany
TcRβ	14-5961	eBioscience
Vimentin	GP53	Progen
Vγ2 TcR-FITC	553226	BD Biosciences
Vγ3 TcR-FITC	553229	BD Biosciences

Secondary antibodies and staining reagents		
Anti-armenian hamster-IgG-Cy2	127-225-160	Jackson Immunoresearch, West Grove, PA
Anti-armenian hamster-Cy3	127-165-160	Jackson Immunoresearch
Anti-guinea pig-IgG-biotin	BA-7000	Vector Laboratories
Anti-guinea pig-IgG-Cy2	106-225-003	Jackson Immunoresearch
Anti-guinea pig-IgG-Cy3	706-165-148	Jackson Immunoresearch
Anti-mouse-IgG-Cy3	115-166-062	Jackson Immunoresearch
Anti-rabbit-IgG-biotin	111-065-003	Jackson Immunoresearch
Anti-rabbit-IgG-Cy2	111-225-003	Jackson Immunoresearch
Anti-rat-Ig-biotin	BA-4001	Vector Laboratories
Anti-goat-IgG-HRP	P0449	DAKO
Streptavidin-PE	12-4317	eBioscience

Supplementary Table S2: Primers used for RT-PCR

Gene	Forward primer	Reverse primer
Human		
Fst	cgtgaatgacaacacactcttc	tttttcccaggtccacagtc
Hprt	tgacactggcaaaacaatgca	ggtccttttcaccagcaagct
Inhba	ggagaacgggtatgtggaga	acaggtcactgccttccttg
Mouse		
Acvr1	aagccggcctctggtgctct	tgggggctggtgacgctctt
Acvr1b	ctccaaagacaagacgctcc	agcagcaataaagccaagga
Acvr1c	tatcacactgcaccttccca	accaagagaggcagaccaga
Acvr2a	cgttcgtctttcttatc	gccctcacagcaacaaaagt
Acvr2b	actacaacgccaactgggag	tggctcgtacgtgacttctg
Ang1	cattcttcgctgccattctg	gcacattgcccatgttgaatc
Ang2	ttagcacaaaggattcggacaat	ttttgtgggtagtactgtccattca
Ccl2	ttctgggcctgctgttcac	gagccaacacgtggatgct
Ccl3	ctgcctgctgcttctcctaca	caacgatgaattggcgtgg
Ccl4	tctctcctcttgctcgtggc	tggtgctgagaaccctgga
Csf2	tcatttttggcctggttttt	tattcgagcagggtctacgg
Cxcl1	gcacccaaaccgaagtcata	tggggacacctttagcatc

Cyp1a1	tcgtggagcctcatgtacctggt	aagcgcttgtccagagtgccg
Cyp1b1	cgcttcatcgcatggcc	gcgaggaccacggtttccg
Gapdh	tcgtggatctgacgtgccgcctg	caccaccctgttgctgtagccgtat
Gsta1_2 [55]	cagagtccggaagatttgga	caaggcagtcttggcttctc
Gstp1	cctctgtctacgcagcactgaatcc	ttccagctctggccctggtcag
Il15	gaggtcaggaaagaatccacc	atgcccaggtaagagcttca
Il1b	ggacagaatatcaaccaacaagtg	tgctgatgtaccagttgggg
Il6	ccggagaggagacttcacag	ttctgcaagtgcatcatcgt
Il7	gcagaccatgttccatgtttc	tggttcattattcgggcaat
Krt14	aaccacgaggaggaaatgg	ccggagctcagaaatctcac
Nqo1	ctggcccattcagagaagac	gtctgcagcttccagcttct
Rps29	ggtcaccagcagctctactg	gtccaacttaatgaagcctatgtcc
S100aA8	gccgtctgaactggagaag	gtgagatgccacacccacttt
S100aA9	cgcagcataaccaccatcat	aagatcaactttcggatcagc
Tcrg (Vγ3-Cγ) [56]	gggtcgactcctggatatctcaggatcag	gggtcgacttgtttcagcagaagaaggaag
Tgfa	ctgagtgactcacccgtggc	gcggagctgacagcagtggat
Tgfb1	agcccgaagcggactactat	tccacatgttgctccacact
Tgfb2	ttcgatcttgggcgtatttc	gcaggataattgctgccttc
Tgfb3	ctctgggttcagggtgttgt	aacctggaggagaactgctg
Thbs1	acaaacaggtgtgcaaaccgcg	gcaggcatcgccaatcccgt
Thbs2	tgagggctggtctccgtggg	tccagcggccatcaattgggc
Tnfa	gaccctcacactcagatcatcttct	ccacttggtggtttgctacga
Vegfa	gtacctccaccatgccaagt	ctgcatggtgatgttgctct

Supplementary Table S3: Antibodies and staining reagents used for FACS analysis

Name	*Clone*	*Source*
γδTcR-FITC	GL3	BD Biosciences
γδTcR-biotin	GL3	eBioscience
CD11b-PE	M1/70	eBiosciences
CD3e-APC	145-2C11	BD Biosciences
CD45-APC	30-F11	eBiosciences

F4/80-PE	BM8	Invitrogen
Gr-1-FITC	RB6-8C5	eBiosciences
Streptavidin-PE	12-4317	eBioscience
TCRβ-PE	H57-597	eBiosciences
Thy1.2 (CD90.2)	53-2.1	BD Biosciences
Vγ3 TcR-FITC	536	BD Biosciences

Supplementary Methods

Genotyping of genetically modified mice

Mice expressing the human activin βA subunit in keratinocytes under control of the keratin 14 (K14) promoter were genotyped by PCR using primers 5`-CCTCGGAGATCATCACGTTT-3` and 5`-CCCTTTAAGCCCACTTCCTC-3`, which hybridize to the activin transgene and produce a 238 bp fragment. Mice expressing dnActRIB in keratinocytes were genotyped by PCR using primers 5`-TTCTTCCCCCTTGTTGTCCT-3` and 5`-AGGCAGTAGAAGGGCTTTCC-3`, which hybridize to the dnActRIB transgene and give a product of 227 bp.

Keratinocyte culture and TGF-β growth inhibition assay

HaCaT keratinocytes[57] stably transfected with an activin-βA expression vector (βA 10/4, βA 10/20 clones) and vector-transfected control clones (neo2, neo3)[58] were cultured in DMEM / 5% FCS supplemented with 400µg/ml G418.

For TGF-β growth inhibition assay, cells were seeded in 96-well plates and cultured for 48h with various concentrations of human TGF-β1 (R&D Systems). BrdU (10µM) was added for the last 5 h of incubation and the amount of BrdU incorporated was analyzed using a colorimetric BrdU ELISA kit (Roche).

RNase protection assay

RNase protection assays were carried out as described previously[3]. A 358 bp fragment corresponding to the 3`-end of the human *Inhba* cDNA and a 121 bp fragment corresponding to nucleotides 580-700 of the human *Gapdh* cDNA were used as templates.

Preparation of protein lysates and enzyme-linked immunosorbent assay (ELISA)

Frozen skin samples were homogenized in lysis buffer containing 10mM Tris/HCl pH8, 1mM EDTA, 0.5mM EGTA, 140mM NaCl, 1% Triton-X100, 0.1% sodium deoxycholate, 0.1% SDS, 10μg/ml aprotinin, 50μg/ml leupeptin, 100μg/ml pepstatin, 0.5mM AEBSF, followed by sonication. IL-1α, IL-1β and activin A levels in skin lysates or serum were determined using ELISA kits (R&D Systems).

Supplementary References

55. Black, A.T. *et al.* Distinct effects of ultraviolet B light on antioxidant expression in undifferentiated and differentiated mouse keratinocytes. *Carcinogenesis* **29**, 219-225 (2008).

56. Kuhnlein, P. *et al*. The canonical T cell receptor of dendritic epidermal gamma delta T cells is highly conserved between rats and mice. *Eur J Immunol*. **26**, 3092-3097 (1996).

57. Boukamp, P. *et al*. Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *J Cell Biol*. **106**, 761-771 (1988).

58. Werner, S. *et al*. Targeted expression of a dominant-negative FGF receptor mutant in the epidermis of transgenic mice reveals a role of FGF in keratinocyte organization and differentiation. *EMBO J*. **12**, 2635-2643 (1993).