

Supplementary Material

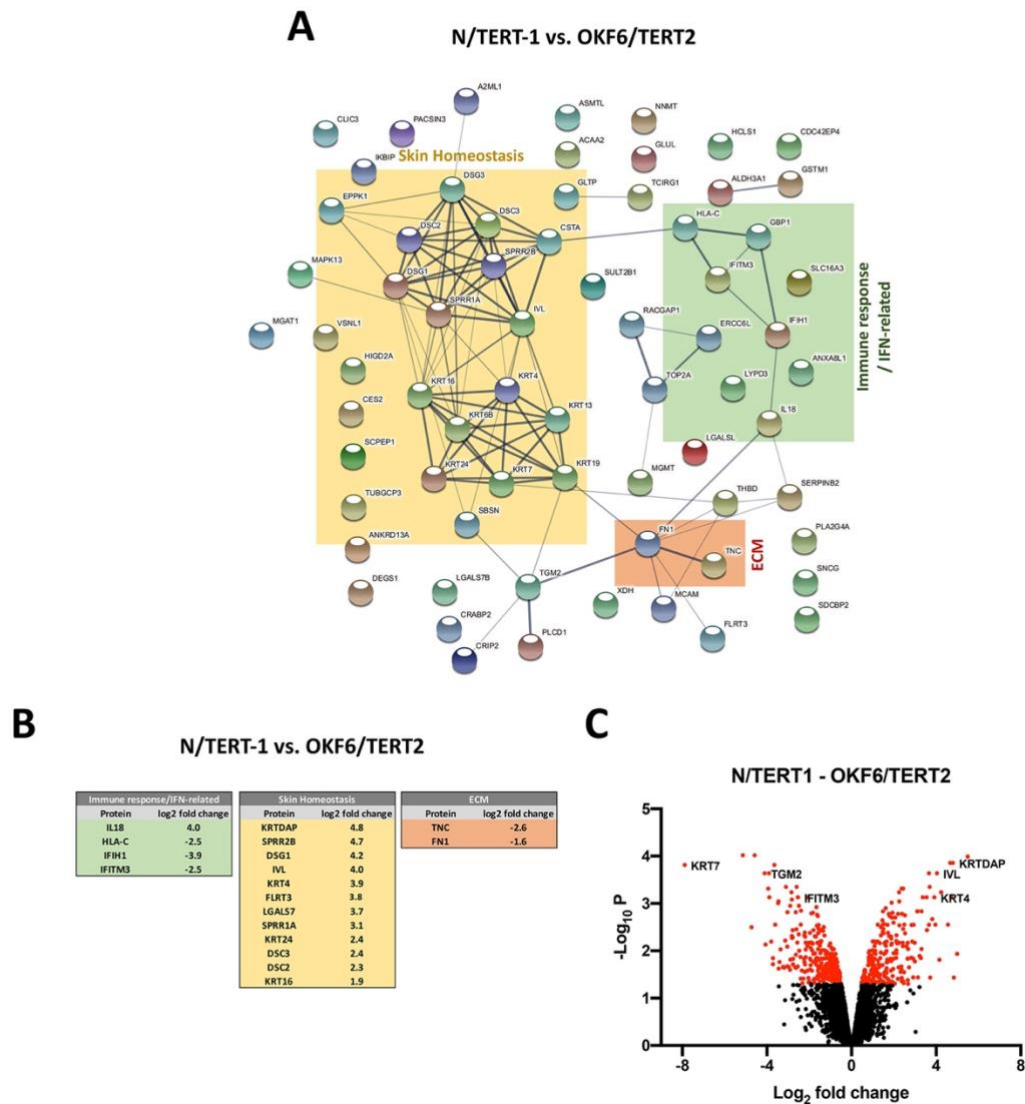
Lack of IRF6 disrupts human epithelial homeostasis by altering colony morphology, migration pattern, and differentiation potential of keratinocytes

Eleftheria Grousi¹, Lukas Muerner², Ludovica Parisi¹, Silvia Rihs¹, Stephan von Gunten², Christos Katsaros¹ and Martin Degen^{1,*}

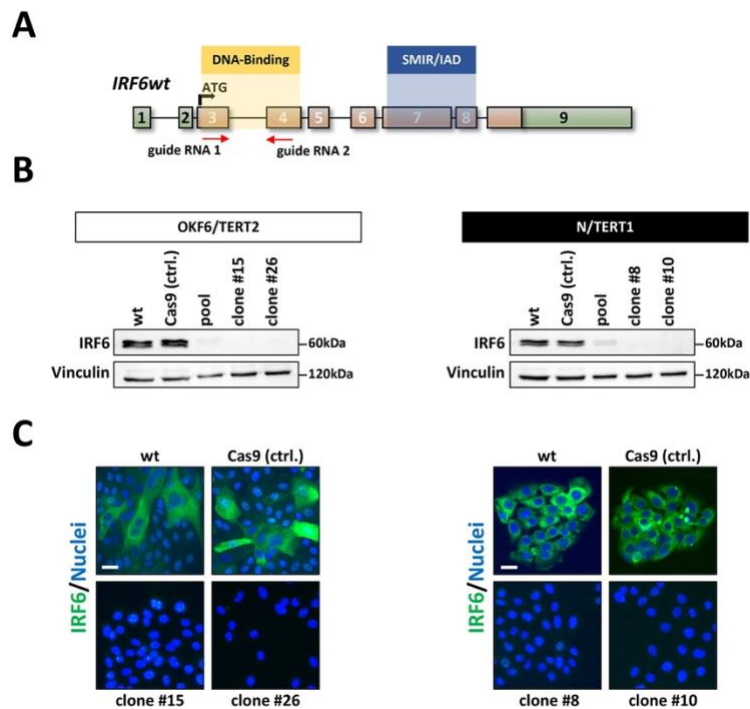
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1 Supplementary Figures and Tables

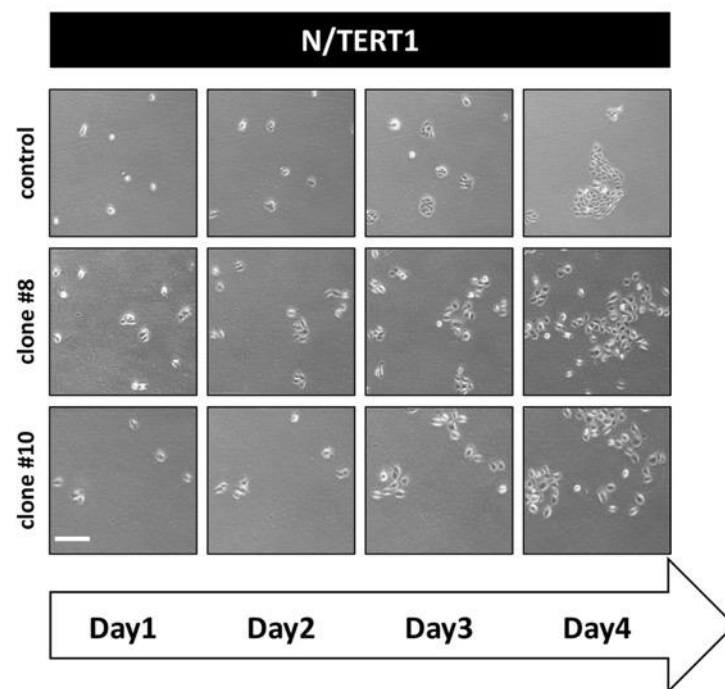
1.1. Supplementary Figures



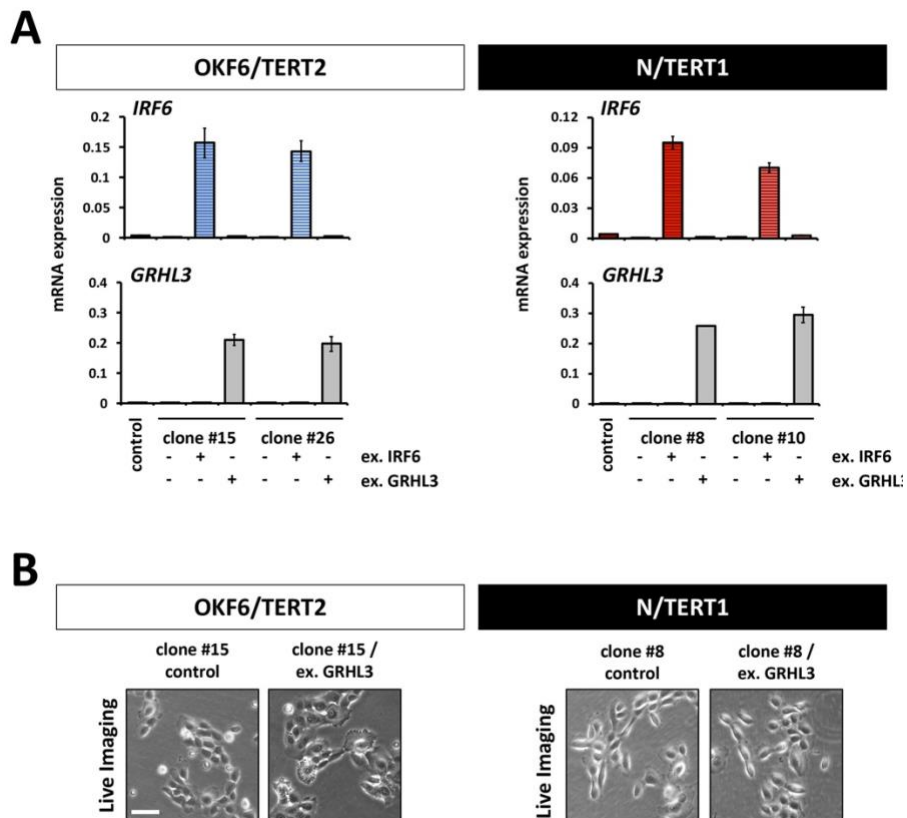
Supplementary Figure 1. (A) Proteomic analysis of OKF6/TERT2 vs. N/TERT1 keratinocytes at high cell-density. Differentially expressed proteins are shown in protein-protein interaction networks using the String database. Yellow: protein cluster “Skin Homeostasis”; Green: protein cluster “immune response / IFN-related”; Red: protein cluster “Extracellular Matrix” (ECM). (B) List of selected proteins including their log₂ fold change belonging to the three main protein clusters. (C) Volcano plot of the proteomic analysis. The red dots represent the significantly differentially expressed proteins ($-\text{Log}_{10} p > 1.3$).



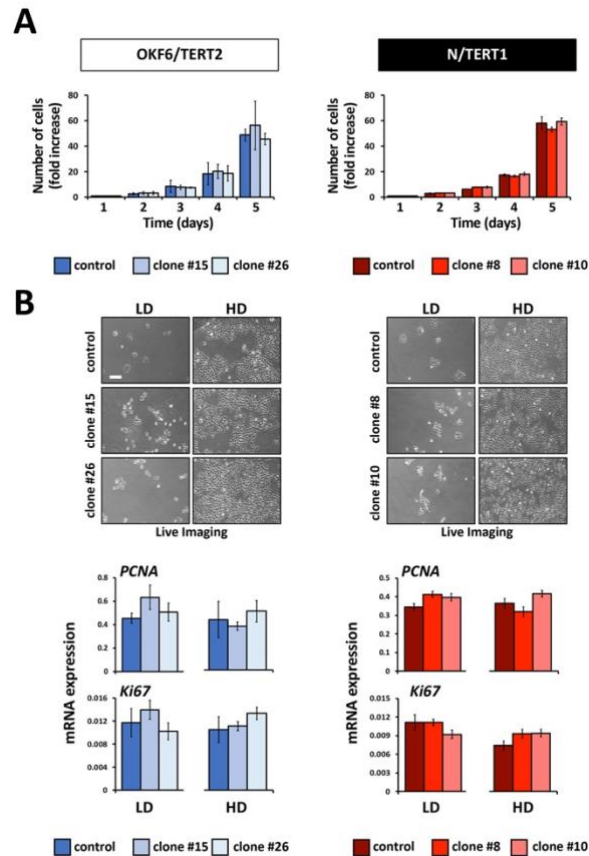
Supplementary Figure 2. (A) Genomic structure of the *IRF6* gene and the position of the IRF6-specific guide RNAs targeting exon 3 and exon 4, which encode for the DNA-binding domain. Green boxes: untranslated exons; Orange boxes: translated exons; SMIR/IAD: SMAD/IRF (SMIR)/interferon association domain (IAD). (B) Confirmation of the IRF6 knockout by immunoblots. The two single cell-derived OKF6/TERT2 clones #15 and #26 (left) and the two single cell-derived N/TERT1 clones #8 and #10 (right) do no longer express detectable amounts of IRF6 compared to IRF6 levels in their respective parental and Cas9-expressing (control) cell lines as well as in the bulk of the cells (pool) after CRISPR/Cas9. Note that the cell pools still show some low levels of IRF6 and that the Cas9-expressing cells are identical to the parental ones. kDa: kilo Dalton; wt: wildtype; ctrl.: control. (C) Immunofluorescent analysis of the IRF6 knockout clones using an anti-IRF6 antibody reveals and confirms absence of IRF6. Note that endogenous IRF6 is mainly expressed in the cytoplasm. Left side: OKF6/TERT2; Right side: N/TERT1. Scale bars: 50 μ m; ctrl.: control. Full-length immunoblots are shown in **Supplementary Figure 9**.



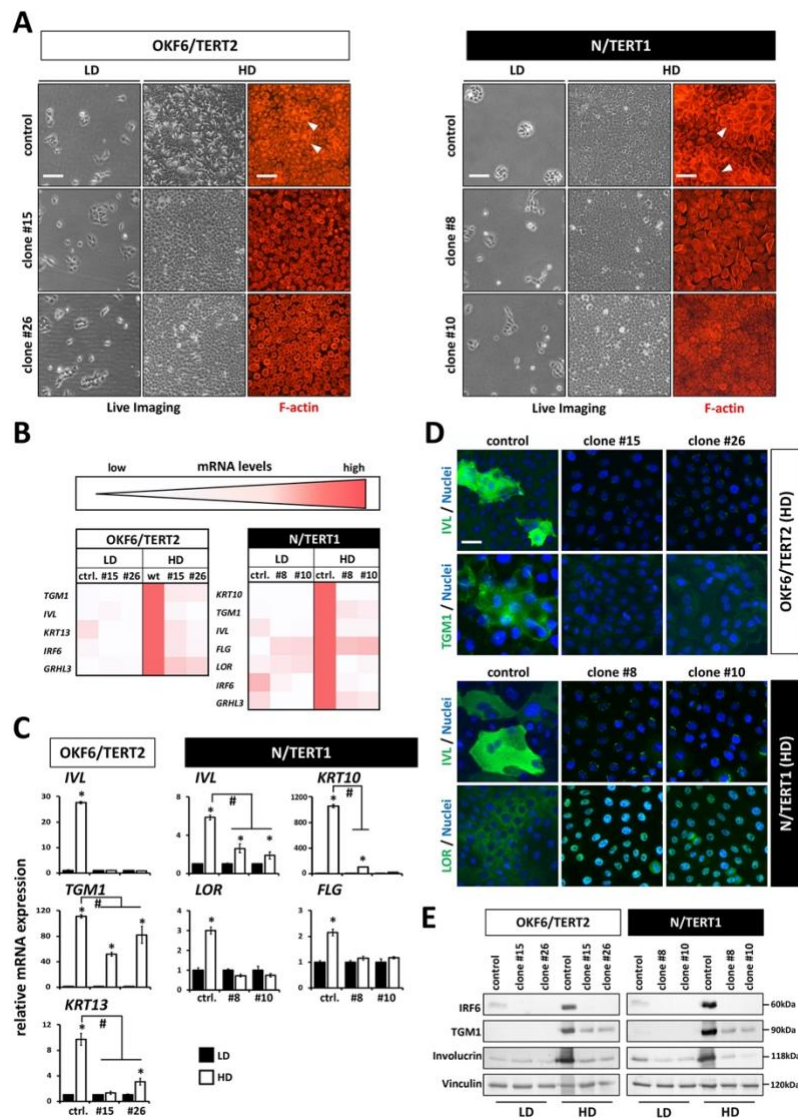
Supplementary Figure 3. N/TERT1 control as well as both cell clones of the IRF6 knockout were seeded at low density and every day a picture of the same spot was taken. Note the formation of cohesive colonies in the control while the cells remain scattered in the absence of IRF6.



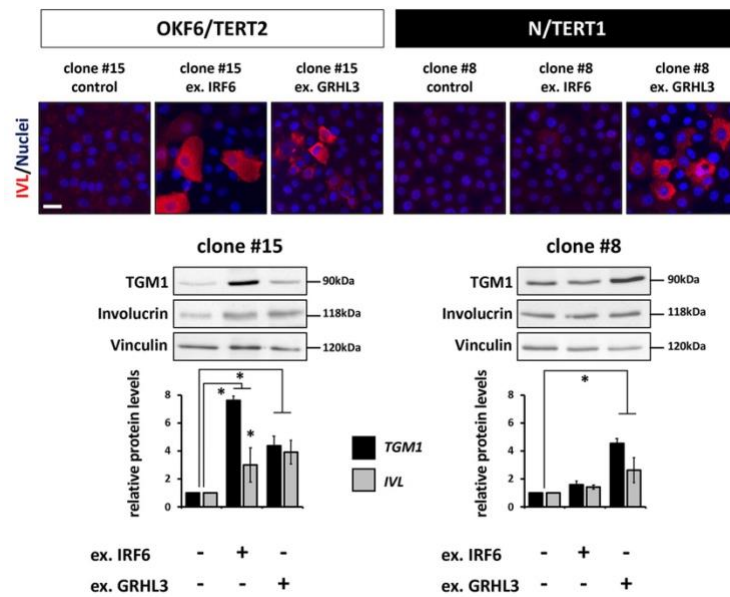
Supplementary Figure 4. (A) Confirmation of the IRF6 and GRHL3 over-expression in IRF6 knockout clones by qPCR. Note that in N/TERT (right side) exogenous IRF6 is constantly knocked-out, while in OKF6 (transient transfection of the guide RNA-containing plasmid) IRF6 is strongly overexpressed. ex.: exogenous; kDa: kilo Dalton. (B) Live imaging pictures of typical cell colonies of the control and GRHL3 over-expressing cells. Note that the morphology defects cannot be rescued by GRHL3. Scale bar: 100 μ m.



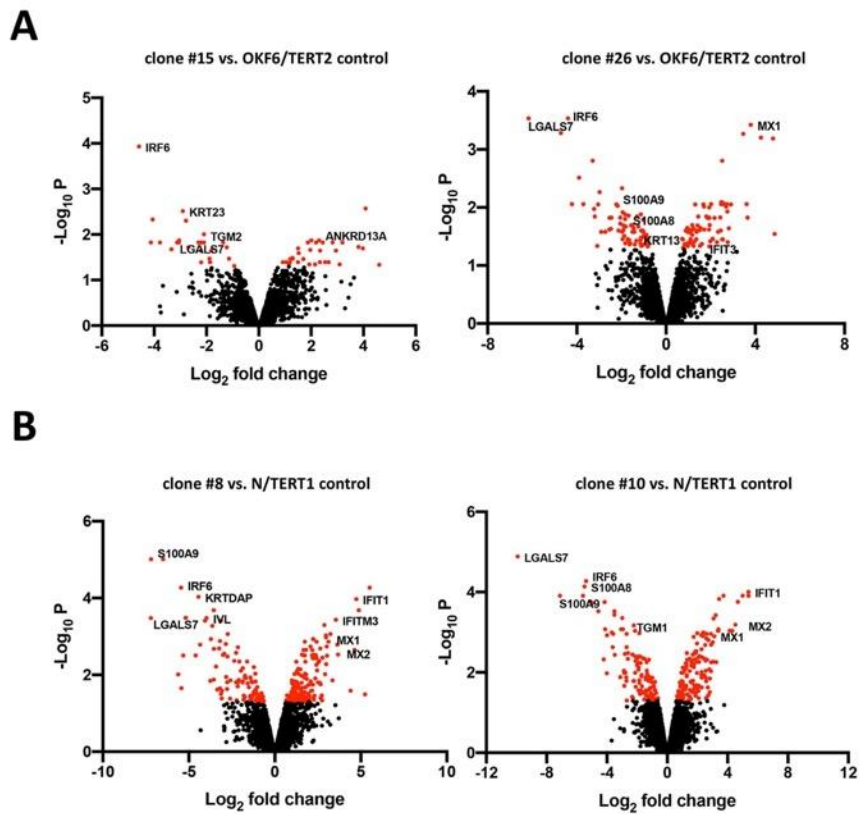
Supplementary Figure 5. (A) Cell growth analysis of control and IRF6 knockout OKF6/TERT2 (left side) and N/TERT1 keratinocytes (right side). Note that *in vitro*, absence of IRF6 does not significantly affect proliferation rate within 5 days. (B) Live imaging pictures and qPCR analyses of the cell cultures grown to low density (LD, left) and high density (HD, right) for the proliferation markers *PCNA* and *Ki67* do not reveal any differences in control when compared to IRF6 knockout keratinocytes. Scale bar: 500 μ m.



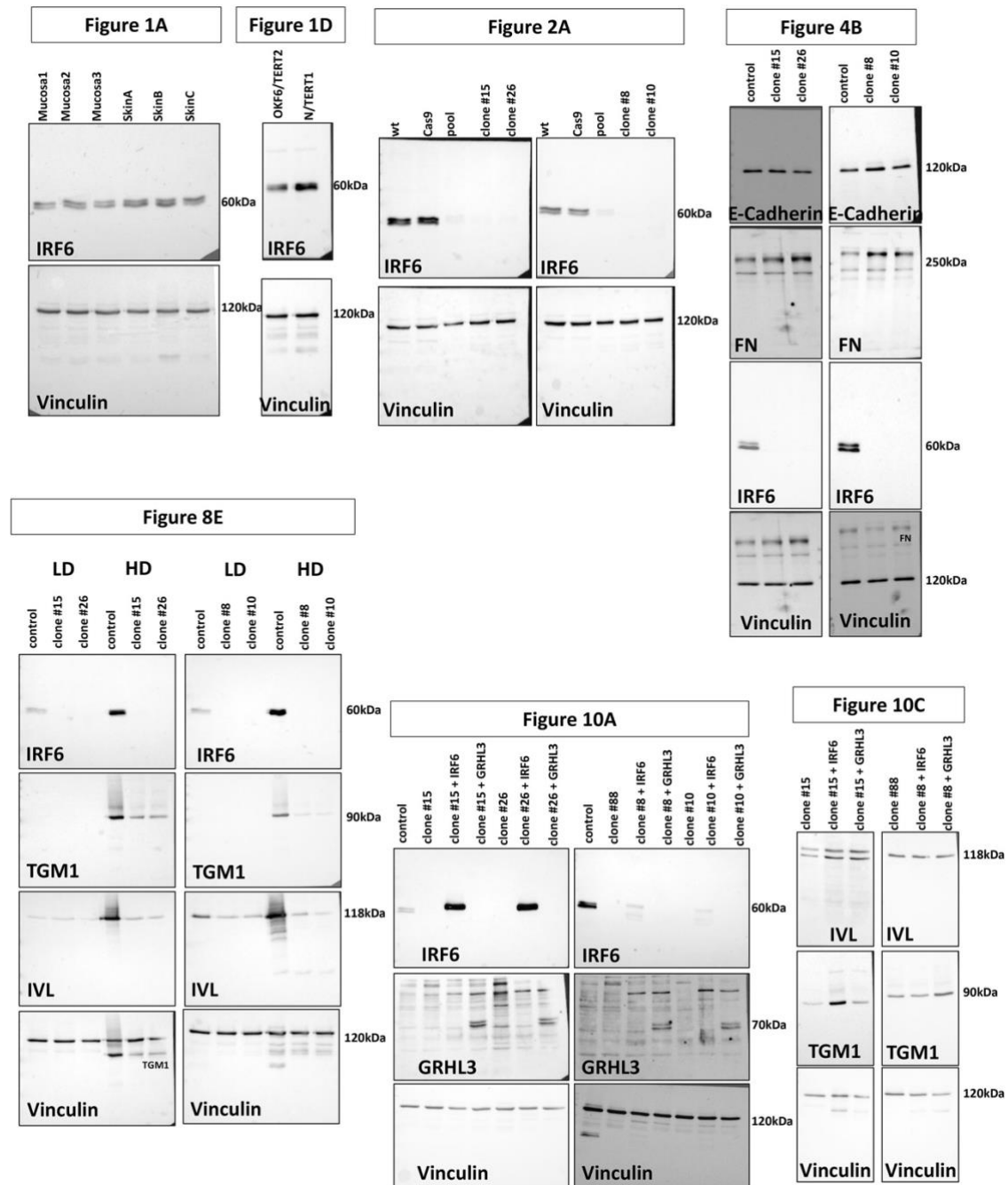
Supplementary Figure 6. (A) Live imaging pictures and F-actin staining (phalloidin, red) analyzing the cell density-driven differentiation, which is clearly visible in controls (arrowheads), but not in the IRF6 knockout clones. Scale bar: 200 μ m (Live Imaging); Scale bar: 150 μ m (F-actin). (B) Heatmap of the qPCR analyses of various differentiation markers in low density (LD) vs. high density (HD) cultures. Note the lack of induction of differentiation markers at HD in the absence of IRF6. ctrl.: control. (C) Graphs of the qPCR analyses of specific differentiation markers showing the lack of induction (*IVL*, *KRT13* in OKF6/TERT2 IRF6 knockout clones; *LOR*, *FLG* in N/TERT1 IRF6 knockout clones) or significantly reduced levels of differentiation markers (*TGM1* and *KRT13* in OKF6/TERT2 clones; *IVL* and *KRT10* in N/TERT1 clones) upon reaching confluence in the IRF6 knockout clones compared to controls. * $p < 0.05$ HD vs. LD; # $p < 0.05$ control vs. clones. ctrl.: control. (D) Immunofluorescent staining for the markers Involucrin (IVL, green) and Transglutaminase (TGM1, green) in OKF6/TERT2 cells and for IVL (green) and Loricrin (LOR, green) in N/TERT1 keratinocytes. Note that all differentiation markers were robustly induced in the control cells in the presence of IRF6 at HD. Scale bar: 50 μ m. DAPI was used to counterstain the cell nuclei (blue). (E) Immunoblots for the proteins IRF6, TGM1, and Involucrin confirms defective differentiation capacity in the absence of IRF6 compared to control. kDa: kilo Dalton. Full-length immunoblots are shown in **Supplementary Figure 9**.



Supplementary Figure 7. Forced expression of IRF6 and GRHL3 is able to rescue the differentiation defects in IRF6-deficient keratinocytes as assessed by staining for the differentiation marker IVL and by immunoblotting for TGM1 and IVL. Quantification of the immunoblots is shown below the blots as relative protein levels. Note that in N/TERT1 keratinocytes, exogenous IRF6 is only minimally expressed and is not able to rescue the differentiation defects. Scale bar: 200 μ m. * p <0.05. Full-length immunoblots are shown in **Supplementary Figure 9**.



Supplementary Figure 8. (A) Volcano plots of the proteomic analysis in OKF6/TERT2 control vs. OKF6/TERT2 clone #15 and clone #26. The red dots represent the significantly differentially expressed proteins. ($\text{Log}_{10} p > 1.3$). (B) Volcano plots of the proteomic analysis in N/TERT1 control vs. N/TERT1 clone #8 and clone #10. The red dots represent the significantly differentially expressed proteins. ($\text{Log}_{10} p > 1.3$).



Supplementary Figure 9. Images of the full-length blots of all immunoblotting experiments. Molecular weights are indicated to the right of the blots. Detected proteins are indicated on the bottom of each blot. Note that sometime membranes were reprobed with different antibodies.

1.2. Supplementary Tables

GENE	SEQUENCE FWD	SEQUENCE REV	AMPLICON (BP)
Interferon Regulatory Factor 6 (<i>IRF6</i>)	GCTCTCCATATCATGGCCCTC	CTACAGCCCAGGCCTTAAAAA	200
Grainyhead-like Regulatory Factor 3 (<i>GRHL3</i>)	CCCCCATGTCCAGAGGACTA	ACTCGCCTGACTTGATGTGG	91
E-Cadherin 1 (<i>CDH1</i>)	AGAACGCATTGCCACATACACT	TCTGATCGGTTACCGTGATCAA	101
Fibronectin (<i>FN</i>)	CCATTATTGGGTACCGCATCACA	AGGAGGAACAGCCGTTTGTGT	200
Vimentin (<i>VIM</i>)	TGTCCAAATCGATGTGGATGTTTC	TTGTACCATTCTCTGCCTCCTG	117
Snail1 (<i>SNAIL</i>)	AAGATGCACATCCGAAGCC	CGCAGGTTGGAGCGGTCAGC	164
Proliferating cell nuclear antigen (<i>PCNA</i>)	CGACACCTACCGCTGCGACC	TAGCGCCAAGGTATCCGCGT	133
<i>Ki67</i>	TGACTTCCTCCATTCTGAAGAC	TGGGTCTGTTATTGATGAGCC	109
Involucrin (<i>IVL</i>)	GGCCCTCAGATCGTCTCATA	CACCCCTACCCCATTAAGA	131
Loricrin (<i>LOR</i>)	AGACCCAGCAGAAGCAGGCG	AGCAGAAGTAGATGCAGCCG	200
Keratin 10 (<i>KRT10</i>)	TGGTTCAATGAAAAGAGCAAGGA	GGGATTGTTCAAGGCCAGTT	151
Keratin 13 (<i>KRT13</i>)	CTGAACAAGGAGGTGTCTACCA	ATAGCGCACTCCGTCTCT	162
Transglutaminase 1 (<i>TGM1</i>)	CCCCCGCAATGAGATCTACA	ATCCTCATGGTCCACGTACACA	73
Filaggrin (<i>FLG</i>)	CTGGACACTCAGGTTCCCAT	TTTCGTGTTTGTCTGCTTGC	103
Galectin-7 (<i>GAL7</i>)	TTGGTTCTCCCAATGCCAGCA	CTCCTTGCTGTTGAAGACCACC	126
<i>S100A8</i>	ATGCCGTCTACAGGGATGACCT	AGAATGAGGAACTCCTGGAAGTTA	142
<i>S100A9</i>	GCACCCAGACACCTGAACCA	TGTGTCCAGGTCCTCCATGATG	124
Desmoglein-1 (<i>DSG1</i>)	CTGCTGGCATTGGACTCCTCAT	CATTCGGGAACAGGCTCAAAGC	124
Desmocollin-2 (<i>DSC2</i>)	CACAGAAGCTCCTGGAGATGAC	GATGGTCTCTGACCTCCGTTT	130
Glyceraldehyde 3-phosphate dehydrogenase (<i>GAPDH</i>)	CTCTGACTTCAACAGCGACACCC	TCCTCTTGCTCTTGCTGGGGC	199

Supplementary Table 1. Sequence of the qPCR primers used in this study. BP: base pairs

NTERT-OKF6 (>2fold): 92 Proteins			
Protein	Log ₂ fold change	Protein	Log ₂ fold change
KRT7	-7.88697	KRT16	1.89580
TUBGCP3	-5.14004	KRT6B	1.90172
ALDH3A1	-4.72369	HOOK1	1.91723
HIGD2A	-4.57925	DSG3	1.93726
TGM2	-4.10728	CSTA	1.98320
CHD8	-4.06272	VSNL1	2.06534
ABI2	-3.93499	TFPI2	2.19941
ACAA2	-3.90295	DSC2	2.25012
IFIH1	-3.88169	PRMT3	2.25523
SYNPO	-3.79531	DEGS1	2.26218
CRIP2	-3.71403	LYPD3	2.36809
KRT19	-3.64902	SERPINB2	2.38662
SCPEP1	-3.60900	ASMTL	2.41977
SNCG	-3.46860	KRT24	2.44271
NNMT	-3.44793	DSC3	2.44751
ERCC6L	-3.35669	TFAP2A	2.49504
SDCBP2	-3.13375	ANKRD13A	2.52221
GLUL	-3.10382	KIF13A	2.53449
IKBIP	-3.03723	PLA2G4A	2.59702
XDH	-3.02992	BCAT1	2.65475
PAC3IN3	-2.89989	EPPK1	2.83843
TCIRG1	-2.83860	MGMT	2.86854
TSC22D1	-2.82578	PRXL2A	2.93042
BST2	-2.82041	CLIC3	2.98070
EFEMP1	-2.81649	SPRR1A	3.10688
RACGAP1	-2.78019	TOP2A	3.11843
CTSL	-2.77956	MAPK13	3.22242
TNC	-2.63100	PLCD1	3.28008
CDC42EP4	-2.60036	NDUFV3	3.30896
GSTM1	-2.59672	CRABP2	3.31282
HCLS1	-2.56463	POF1B	3.37148
RAD50	-2.53215	THBD	3.55118
IFITM3	-2.52856	GLTP	3.56682
HLA-C	-2.49365	SBSN	3.65983
SLC16A3	-2.48634	CYBRD1	3.66300
KIF2A	-2.39657	LGALS7	3.69548
MCAM	-2.38714	FLRT3	3.81578
GBP1	-2.22621	KRT4	3.91641
RIPOR1	-2.20872	IL18	3.96070
PRODH	-2.08056	IVL	4.03843
TNFRSF10B	-1.90141	LGALS1	4.15027
MGAT1	-1.82522	CES2	4.23586
FN1	-1.62474	DSG1	4.24148
		ANXA8L1	4.56315
		SULT2B1	4.66493
		SPRR2B	4.71912
		KRTDAP	4.79373
		A2ML1	4.99856
		MTAP	5.49187

Reactome pathways	#	fold enrichment	raw P value	Proteins
Formation of the cornified envelope	14	24.03	2.77E-15	DSG3, SPRR2B, DSC3, KRT4, KRT19, IVL, KRT6B, KRT16, CSTA, DSC2, DSG1, KRT7, KRT24, SPRR1A
Keratinization	14	14.29	2.14E-12	DSG3, SPRR2B, DSC3, KRT4, KRT19, IVL, KRT6B, KRT16, CSTA, DSC2, DSG1, KRT7, KRT24, SPRR1A
Developmental Biology	16	3.36	2.11E-05	DSG3, SPRR2B, DSC3, KRT4, KRT19, IVL, KRT6B, KRT16, FLRT3, CSTA, DSC2, DSG1, KRT7, MAPK13, KRT24, SPRR1A

Supplementary Table 2. List of all significantly differentially expressed proteins and their log₂ fold change are reported in the proteomic analysis N/TERT1 vs. OKF6/TERT2.

Clone 15-OKF6 (>2fold): 16 Proteins			
Protein	Log ₂ fold change	Protein	Log ₂ fold change
IRF6	-4.57725	FN1	0.80091
PTCD3	-4.12567	RIDA	2.82597
YEATS2	-4.05369	COA7	3.19941
RBP1	-3.77503	MRPS11	3.80938
EHBPI1L1	-3.33396	TOP2A	3.97821
MAP10	-3.11722	SIPA1L1	4.08387
LGALS7	-3.05917		
MAP3K3	-3.04049		
KRT23	-2.90119		
SYNE2	-2.78544		

Clone 26-OKF6 (>2fold): 30 Proteins			
Protein	Log ₂ fold change	Protein	Log ₂ fold change
LGALS7	-6.165050	FN1	0.86196
TPD52	-4.712097	MX2	2.46612
IRF6	-4.394848	HLA-B	2.47480
TPM3	-4.220051	SAMHD1	2.51515
PYGM	-3.898468	MINPP1	2.54703
ARAP1	-3.704416	ANKRD13A	2.55059
ERCC6L	-3.294552	BST2	2.69052
GEMIN4	-3.230895	NT5E	2.72282
ASS1	-3.198145	RIDA	2.74668
INTS2	-3.043492	ABAT	2.90908
MAP3K3	-2.977794	COA7	3.46315
SLC25A10	-1.975633	OAS1	3.61069
KRT13	-1.728635	MRPS11	3.65071
		MX1	3.79552
		IFIT1	4.25527
		OAS2	4.79720
		KRT3	4.86407

Reactome pathways	#	fold enrichment	raw P value	Proteins
Interferon alpha/beta signaling	9	95.4	7.25E-16	OAS1, IFIT1, OAS2, IRF6, SAMHD1, MX1, HLA-B, BST2, MX2
Interferon signaling	9	32.61	6.70E-12	OAS1, IFIT1, OAS2, IRF6, SAMHD1, MX1, HLA-B, BST2, MX2
Cytokine Signaling in Immune system	10	8.63	1.09E-07	OAS1, IFIT1, OAS2, IRF6, SAMHD1, MAP3K3, MX1, HLA-B, BST2, MX2
Antiviral mechanism by IFN-stimulated genes	5	44.95	1.09E-07	OAS1, IFIT1, OAS2, MX1, MX2
Interferon gamma signaling	4	31.22	9.19E-06	OAS1, OAS2, IRF6, HLA-B
unclassified	3	0.21	2.02E-05	COA7, ANKRD13A, LGALS7
OAS antiviral response	2	>100	1.04E-04	OAS1, OAS2, IRF6, HLA-B
ISG15 antiviral degranulation	3	30.01	1.52E-04	IFIT1, MX1, MX2

Supplementary Table 3. List of all significantly differentially expressed proteins and their log₂ fold change are reported in the proteomic analysis OKF6/TERT2 vs. clones #15 and #26.

Clone 8-NTERT (>2fold): 69 Proteins			
Protein	Log ₂ fold change	Protein	Log ₂ fold change
LGALS7	-7.20350	SAMD9	1.75048
S100A9	-7.20046	TGFB1	2.07501
ASS1	-6.49613	B2M	2.17009
CRABP2	-5.61240	STRN4	2.17845
IRF6	-5.45500	SP100	2.20775
PRXL2A	-5.43304	PARP9	2.23000
S100A8	-5.32429	OAS2	2.24215
NAGA	-5.19244	SHFL	2.25079
SPRR2B	-5.16952	NNMT	2.32910
LARS2	-4.60264	TYMP	2.37067
KRTDAP	-4.43477	P3H2	2.37124
SULT2B1	-4.35110	PLCG1	2.41219
DSG1	-4.05767	SIRPA	2.42115
IVL	-3.97754	DYNC1L1	2.42328
LGALS5	-3.74913	HLA-C	2.46036
KRT4	-3.71414	SAMHD1	2.52797
GLTP	-3.63268	ERF	2.57677
SERPINB1	-3.55599	LGALS3BP	2.63867
NT5C	-3.55359	FAM136A	2.76411
GBP6	-3.54427	CPA4	2.87590
SBSN	-3.41310	ISOC2	2.92397
KRT13	-3.23976	PARP14	2.97346
TPD52	-3.19247	POMP	2.97572
TSTD1	-3.12675	BST2	2.98357
TXNL1	-3.01717	HLA-B	3.01237
CLIC3	-2.93995	PIN4	3.03749
HOOK1	-2.85144	MX1	3.11516
ALOX15B	-2.82851	ISG15	3.22845
LDLR	-2.75993	CD82	3.35660
KRT24	-2.73798	IFITM3	3.55231
PLCD1	-2.35364	MX2	3.63423
PCK2	-2.25037	CST3	3.67524
PSPH	-2.14402	IFIH1	4.63579
		IFIT1	4.73719
		HCLS1	4.88137
		POGZ	5.51111

Clone 10-NTERT (>2fold): 80 Proteins			
Protein	Log ₂ fold change	Protein	Log ₂ fold change
LGALS7	-9.92161	TGFB1	1.59164
S100A9	-7.11567	GLUL	1.74896
SPRR2B	-5.59424	PRKD2	1.96431
S100A8	-5.48779	TUBGCP3	1.98718
IRF6	-5.38279	DYNC1L1	2.05977
PRXL2A	-5.07028	AQR	2.13884
SULT2B1	-4.98552	PLCG1	2.25777
KRTDAP	-4.57084	IFIT5	2.26604
MSLN	-4.20912	SP100	2.31100
DSG1	-4.15158	B2M	2.35166
RAB38	-4.12052	EXOC2	2.37689
ASS1	-4.00846	OAS2	2.40769
PLCD1	-3.99994	SIRPA	2.46705
IVL	-3.67956	TYMP	2.51555
SBSN	-3.62328	HLA-C	2.53333
GBP6	-3.58750	POLG	2.55452
TPD52	-3.50781	PARP9	2.58850
KRT4	-3.50587	LGALS3BP	2.61934
GLTP	-3.48481	POMP	2.62617
TPBG	-3.11176	PWP2	2.71520
TXNL1	-3.05724	TCEAL4	2.78543
KRT13	-2.98997	ISOC2	2.88933
TGM1	-2.93689	HIGD2A	2.89495
KRT10	-2.86610	FN1	2.90165
RAB25	-2.85530	PIN4	2.90266
LDLR	-2.81039	SAMHD1	3.00510
ALOX15B	-2.77537	ERF	3.04227
ABCC1	-2.69916	UBE2C	3.06634
HOOK1	-2.69231	NNMT	3.10963
MBOAT2	-2.62034	PARP14	3.20170
CLIC3	-2.60277	RAD50	3.24378
A2ML1	-2.31931	HLA-B	3.35241
BCAT1	-2.30366	ISG15	3.38746
SPRR1A	-2.22283	MX1	3.39074
DSC2	-2.13966	NMI	3.43768
ATP6V0A1	-2.12443	IFITM3	3.72944
PSPH	-1.85559	CST3	4.13453
		MX2	4.30594
		LCP1	4.51717
		TSC2D1	4.68141
		HCLS1	5.00505
		POGZ	5.38042
		IFIT1	5.38061

Reactome pathways	#	fold enrichment	raw P value	Proteins
Interferon Signaling	14	18.62	5.60E-14	IFIT1, OAS2, IRF6, PLCG1, SAMHD1, ISG15, HLA-C, GBP6, MX1, SP100, IFITM3, HLA-B, B2M, MX2
Interferon alpha/beta signaling	10	38.91	3.29E-13	IFIT1, OAS2, IRF6, SAMHD1, ISG15, HLA-C, MX1, IFITM3, HLA-B, MX2
Formation of the cornified envelope	9	18.19	2.80E-09	KRT10, SPRR2B, KRT4, KRT13, TGM1, IVL, DSC2, DSG1, SPRR1A
Cytokine Signaling in Immune system	16	5.07	9.22E-08	IFIT1, OAS2, IRF6, PLCG1, SAMHD1, ISG15, LCP1, HLA-C, GBP6, MX1, SP100, IFITM3, HLA-B, FN, B2M, MX2
Interferon gamma signaling	7	20.05	9.49E-08	OAS2, IRF6, HLA-C, GBP6, SP100, HLA-B, B2M
Keratinization	9	10.81	2.01E-07	KRT10, SPRR2B, KRT4, KRT13, TGM1, IVL, DSC2, DSG1, SPRR1A
Antiviral mechanism by IFN-stimulated genes	6	19.8	8.83E-07	IFIT1, OAS2, PLCG1, ISG15, MX1, MX2
Immune System	24	2.9	1.13E-06	IFIT1, OAS2, IRF6, PLCG1, SAMHD1, ISG15, UBE2C, LCP1, HLA-C, GBP6, MX1, CST3, SP100, S100A9, IFITM3, HLA-B, S100A8, DYNC1L1, DSG1, FN, B2M, MX2, SIRPA, ATP6V0A1
ISG15 antiviral degranulation	5	18.36	1.07E-05	IFIT1, PLCG1, ISG15, MX1, MX2
Neutrophil degranulation	10	5.45	1.64E-05	HLA-C, CST3, S100A9, HLA-B, S100A8, DYNC1L1, DSG1, B2M, SIRPA, ATP6V0A1
Endosomal/Vacuolar pathway	3	65.17	2.36E-05	HLA-C, HLA-B, B2M
Nicotinamide salvaging	3	41.16	7.85E-05	NNMT, PARP14, PARP9
unclassified	21	0.55	1.04E-04	PRXL2A, ISOC2, IFIT5, KRTDAP, A2ML1, SBSN, POLG, HOOK1, TSC2D1, TCEAL4, LGALS7, PIN4, NMI, POMP, HCLS1, TPBG, CLIC3, POGZ, SULT2B1, HIGD2A, TXNL1
Antigen Presentation: Folding, assembly and peptide loading of class I MHC	3	30.08	1.83E-04	HLA-C, HLA-B, B2M

Supplementary Table 4. List of all significantly differentially expressed proteins and their log₂ fold change are reported in the proteomic analysis OKF6/TERT2 vs. clones #15 and #26.