Supplementary Material

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

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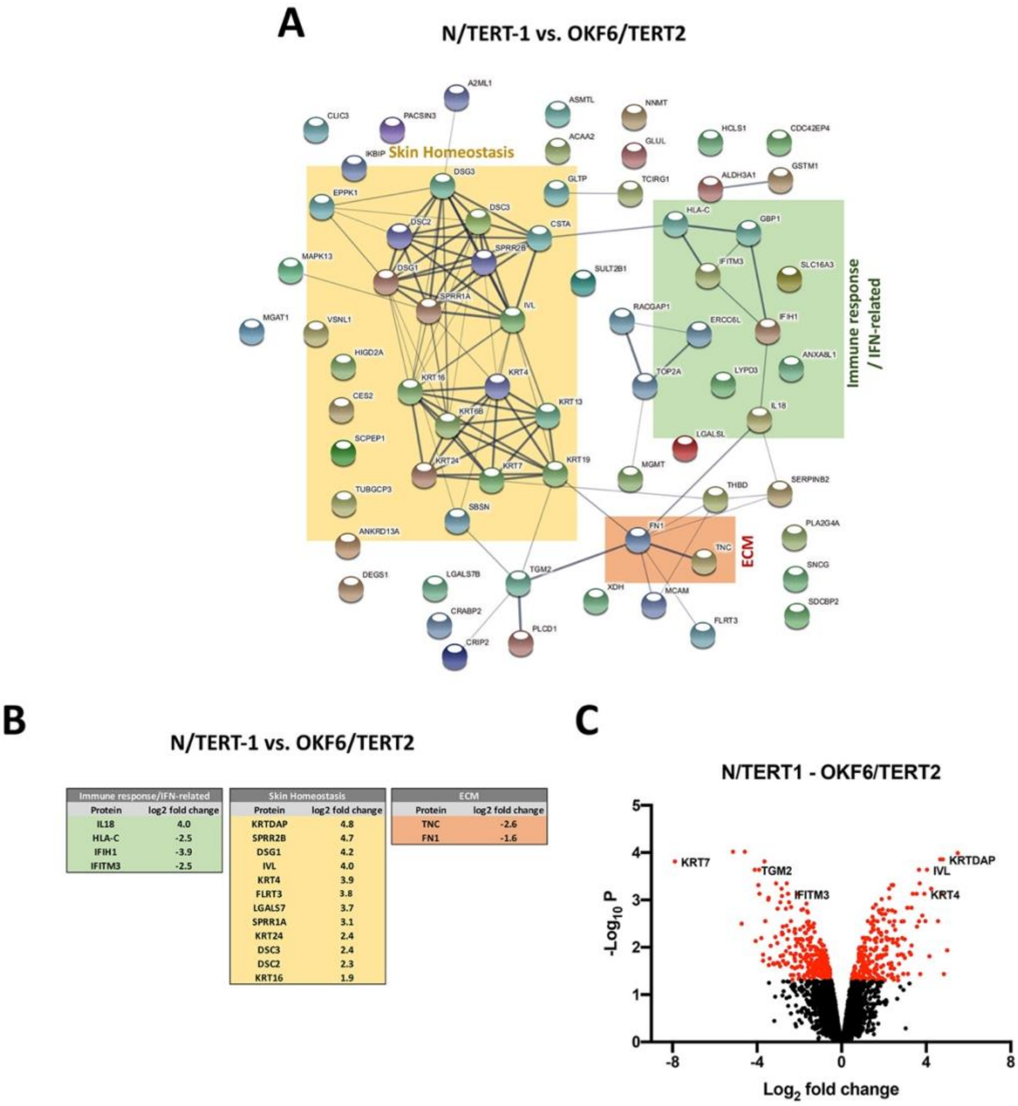
1 Supplementary Figures and Tables

1.1. Supplementary Figures

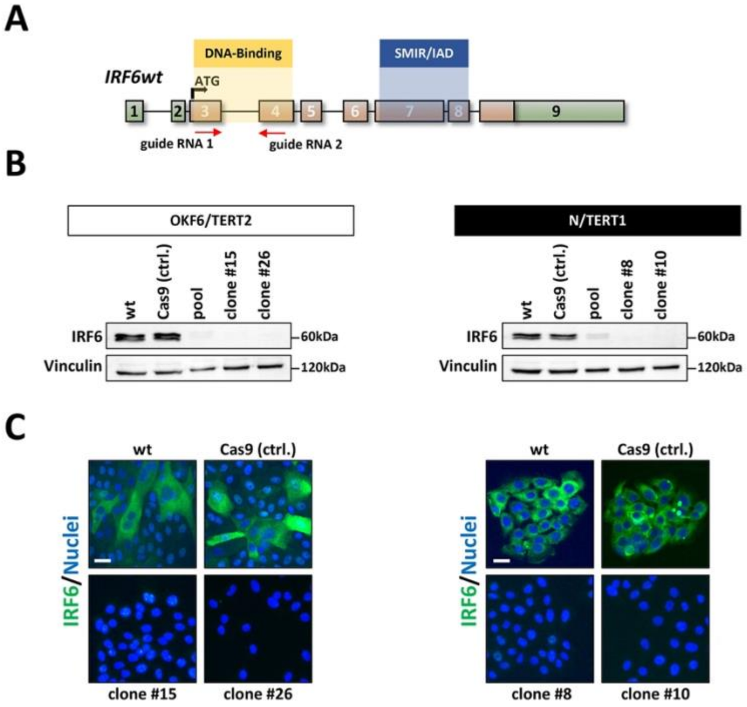


Immune response/IFN-related	
Protein	log2 fold change
IL18	4.0
HLA-C	-2.5
IFIH1	-3.9
IFITM3	-2.5

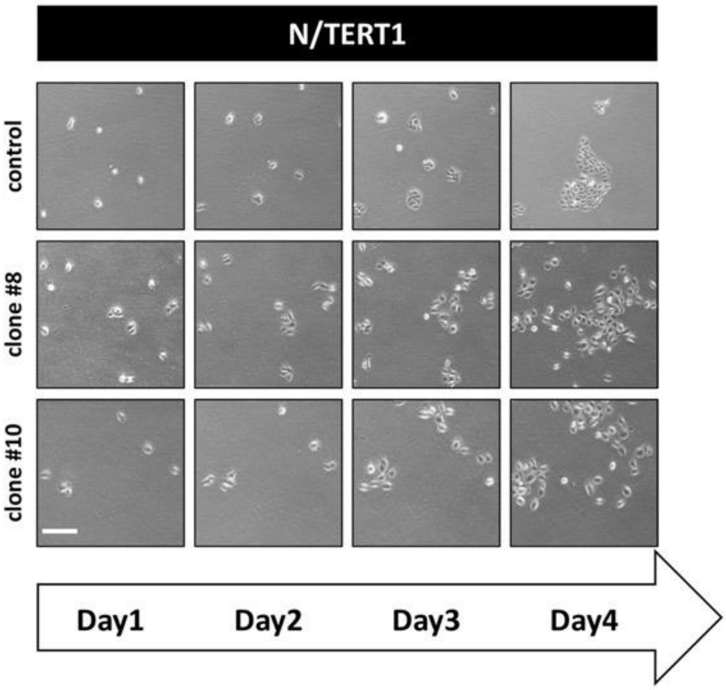
Skin Homeostasis	
Protein	log2 fold change
KRTDAP	4.8
SPRR2B	4.7
DSG1	4.2
IVL	4.0
KRT4	3.9
FLRT3	3.8
LGALS7	3.7
SPRR1A	3.1
KRT24	2.4
DSC3	2.4
DSC2	2.3
KRT16	1.9

ECM	
Protein	log2 fold change
TNC	-2.6
FN1	-1.6

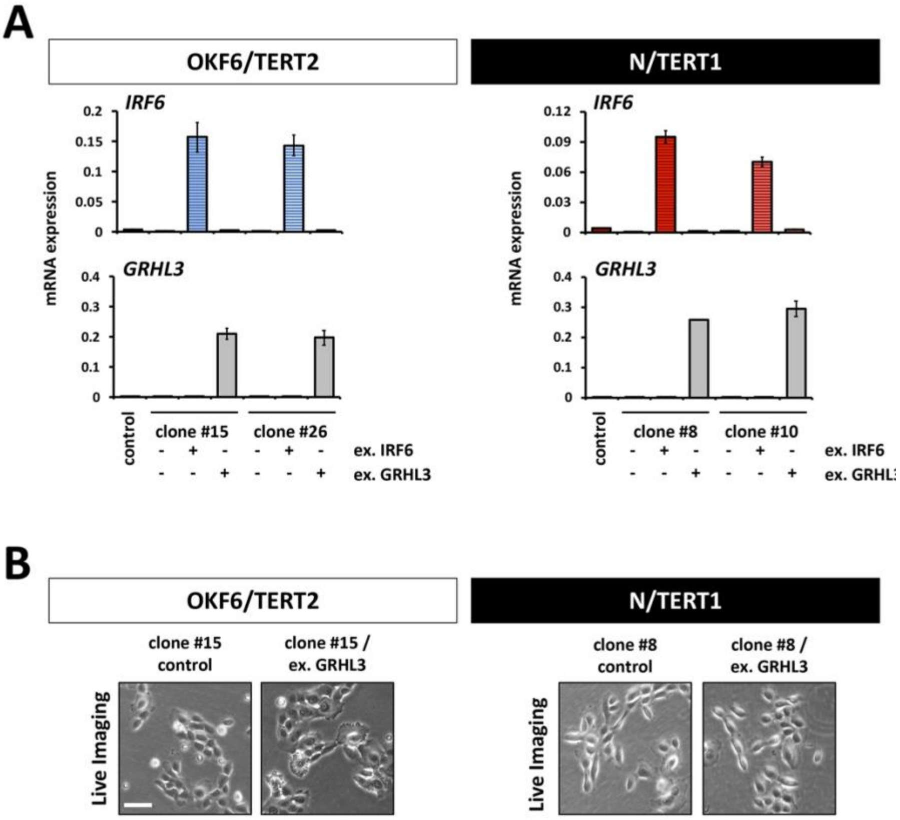
Supplementary Figure 1. (A) Proteomic analysis of OKF6/TERT2 vs. N/TERT1 keratinocytes at high cell-density. Differentially expressed proteins are shown in protein-protein interaction networks using the String database. Yellow: protein cluster "Skin Homeostasis"; Green: protein cluster "immune response / 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).



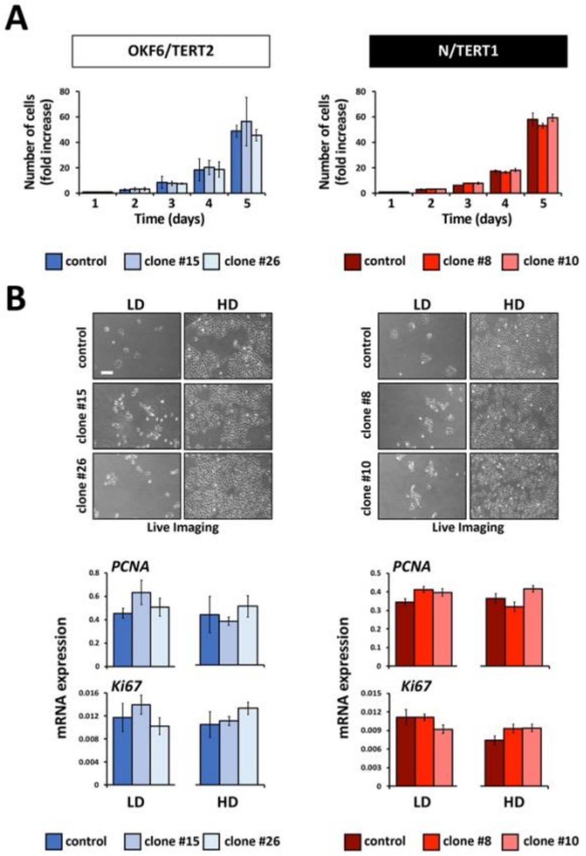
Supplementary Figure 2. (A) Genomic structure of the *IRF6* gene and the position of the IRF6-specific guide RNAs targeting exon 3 and exon 4, which encode for the DNA-binding domain. Green boxes: untranslated exons; Orange boxes: translated exons; SMIR/IAD: SMAD/IRF (SMIR)/interferon association domain (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 µm; ctrl.: control. Full-length immunoblots are shown in **Supplementary Figure 9**.



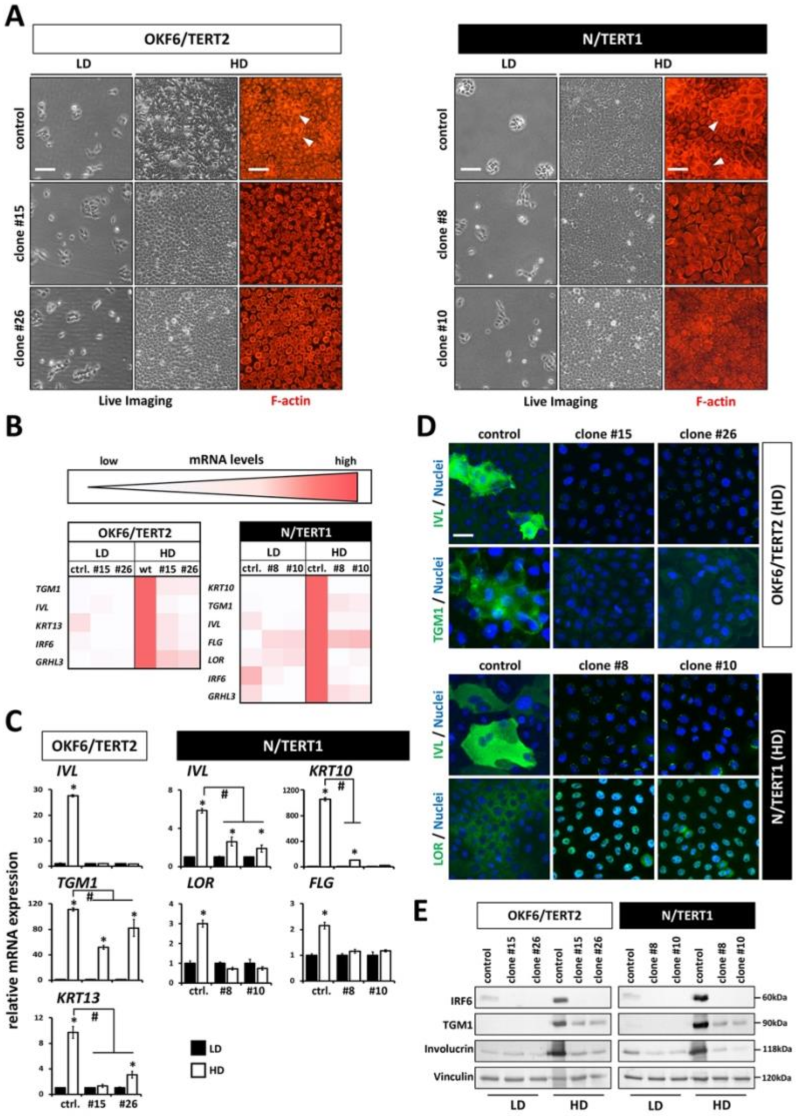
Supplementary Figure 3. N/TERT1 control as well as both cell clones of the IRF6 knockout were seeded at low density and every day a picture of the same spot was taken. Note the formation of cohesive colonies in the control while the cells remain scattered in the absence of IRF6.



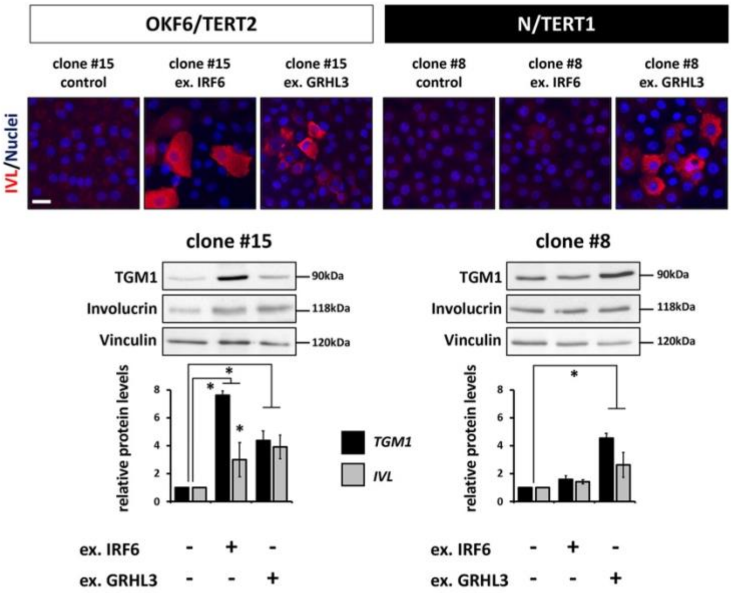
Supplementary Figure 4. **(A)** Confirmation of the IRF6 and GRHL3 over-expression in IRF6 knockout clones by qPCR. Note that in N/TERT (right side) exogenous IRF6 is constantly knocked-out, while in OKF6 (transient transfection of the guide RNA-containing plasmid) IRF6 is strongly overexpressed. ex.: 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 µm.



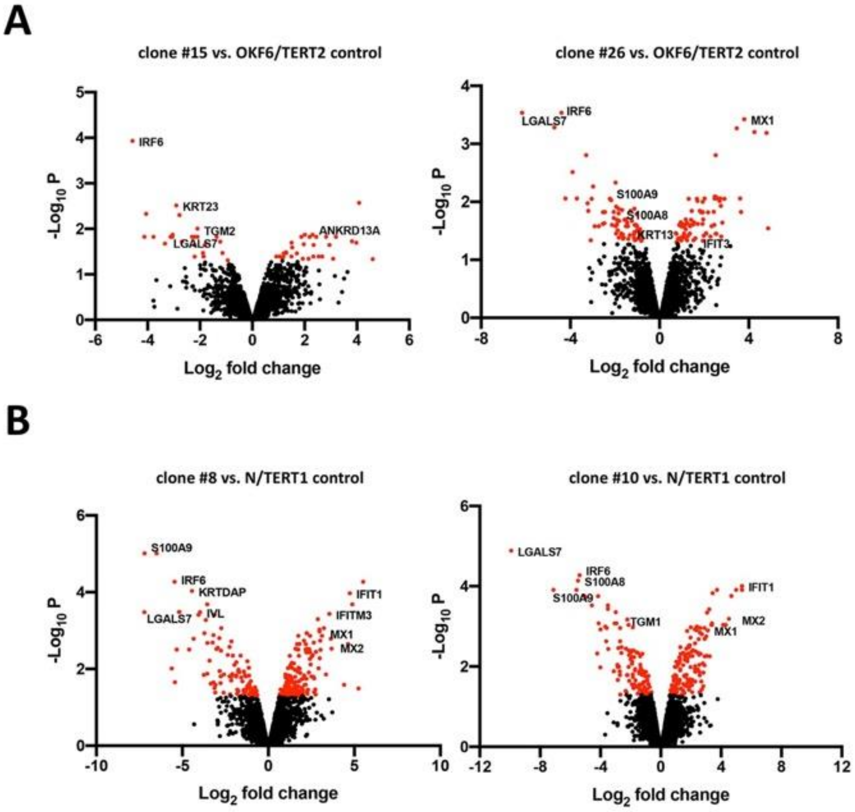
Supplementary Figure 5. (A) Cell growth analysis of control and IRF6 knockout OKF6/TERT2 (left side) and N/TERT1 keratinocytes (right side). Note that *in vitro*, absence of IRF6 does not significantly affect proliferation rate within 5 days. **(B)** Live imaging pictures and qPCR analyses of the cell 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 µm.



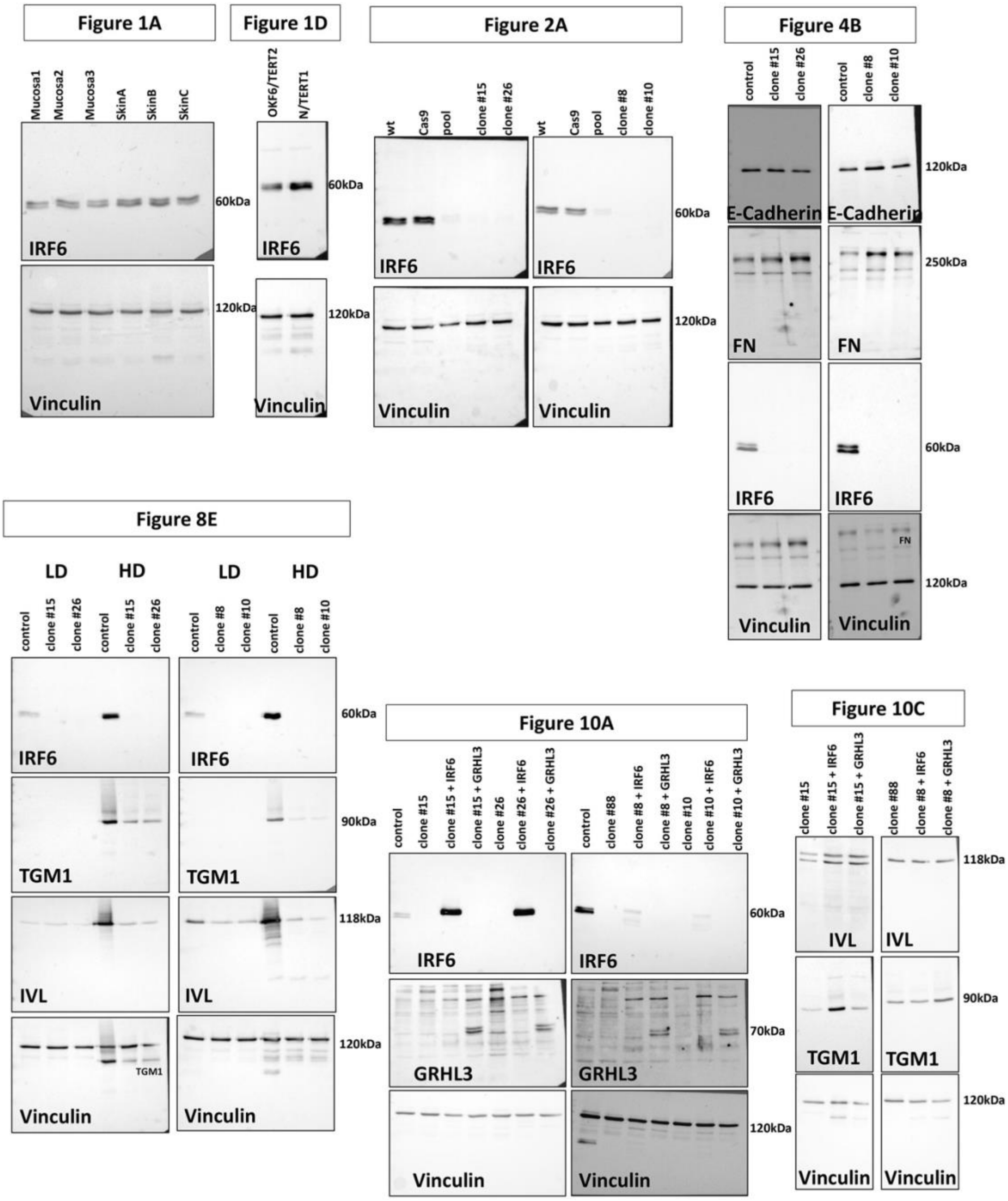
Supplementary Figure 6. (A) Live imaging pictures and F-actin staining (phalloidin, red) analyzing the cell density-driven differentiation, which is clearly visible in controls (arrowheads), but not in the IRF6 knockout clones. Scale bar: 200 µm (Live Imaging); Scale bar: 150 µm (F-actin). **(B)** Heatmap 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 µm. 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 µm. *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. (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. (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

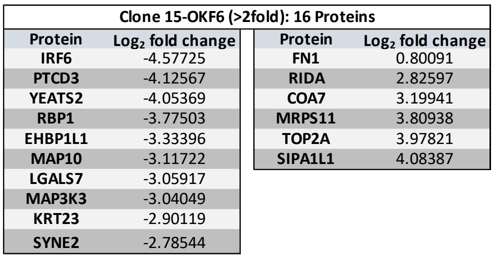
GENE	SEQUENCE FWD	SEQUENCE REV	AMPLICON (BP)
Interferon Regulatory Factor 6 *(IRF6)*	GCTCTTCCATATCATGGCCCTC	CTACAGCCCAGGCCTTAAAAA	200
Grainyhead-like Regulatory Factor 3 *(GRHL3)*	CCCCCATGTCCAGAGGACTA	ACTCGCCTGACTTGATGTGG	91
E-Cadherin 1 *(CDH1)*	AGAACGCATTGCCACATACACT	TCTGATCGGTTACCGTGATCAA	101
Fibronectin *(FN)*	CCATTATTGGGTACCGCATCACA	AGGAGGAACAGCCGTTTGTTGT	200
Vimentin *(VIM)*	TGTCCAAATCGATGTGGATGTTTC	TTGTACCATTCTTCTGCCTCCTG	117
Snail1 *(SNAIL)*	AAGATGCACATCCGAAGCC	CGCAGGTTGGAGCGGTCAGC	164
Proliferating cell nuclear antigen *(PCNA)*	CGACACCTACCGCTGCGACC	TAGCGCCAAGGTATCCGCGT	133
Ki67	TGACTTCCTTCCATTCTGAAGAC	TGGGTCTGTTATTGATGAGCC	109
Involucrin *(IVL)*	GGCCCTCAGATCGTCTCATA	CACCCTCACCCCATTAAAGA	131
Loricrin *(LOR)*	AGACCCAGCAGAAGCAGGCG	AGCAGAACTAGATGCAGCCG	200
Keratin 10 *(KRT10)*	TGGTTCAATGAAAAGAGCAAGGA	GGGATTGTTTCAAGGCCAGTT	151
Keratin 13 *(KRT13)*	CTGAACAAGGAGGTGTCTACCA	ATAGCGGCACTCCGTCTCT	162
Transglutaminase 1 *(TGM1)*	CCCCCGCAATGAGATCTACA	ATCCTCATGGTCCACGTACACA	73
Filaggrin *(FLG)*	CTGGACACTCAGGTTCCCAT	TTTCGTGTTTGTCTGCTTGC	103
Galectin-7 *(GAL7)*	TTGGTTCCTCCCAATGCCAGCA	CTCCTTGCTGTTGAAGACCACC	126
S100A8	ATGCCGTCTACAGGGATGACCT	AGAATGAGGAACTCCTGGAAGTTA	142
S100A9	GCACCCAGACACCCTGAACCA	TGTGTCCAGGTCCTCCATGATG	124
Desmoglein-1 *(DSG1)*	CTGCTGGCATTGGACTCCTCAT	CATTCGGGAACAGGCTCAAAGC	124
Desmocollin-2 *(DSC2)*	CACAGAAGCTCCTGGAGATGAC	GATGGTCTCCTGACCTCCGTTT	130
Glyceraldehyde 3-phosphate dehydrogenase *(GAPDH)*	CTCTGACTTCAACAGCGACACCC	TCCTCTTGTGCTCTTGCTGGGGC	199

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

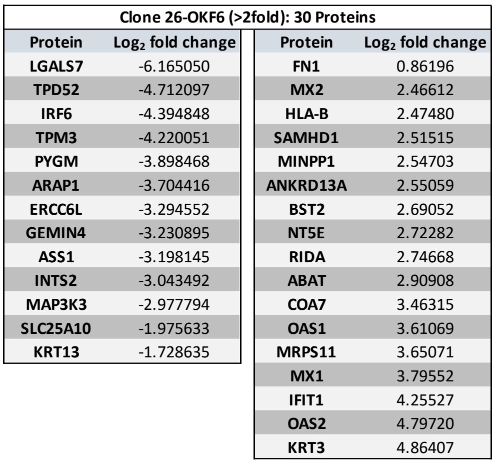
NTERT-OKF6 (>2fold): 92 Proteins			
Protein	Log$_2$ fold change	Protein	Log$_2$ fold change
KRT7	-7.88697	**KRT16**	1.89580
TUBGCP3	-5.14004	**KRT6B**	1.90172
ALDH3A1	-4.72369	**HOOK1**	1.91723
HIGD2A	-4.57925	**DSG3**	1.93726
TGM2	-4.10728	**CSTA**	1.98320
CHD8	-4.06272	**VSNL1**	2.06534
ABI2	-3.93499	**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
PACSIN3	-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	**LGALSL**	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

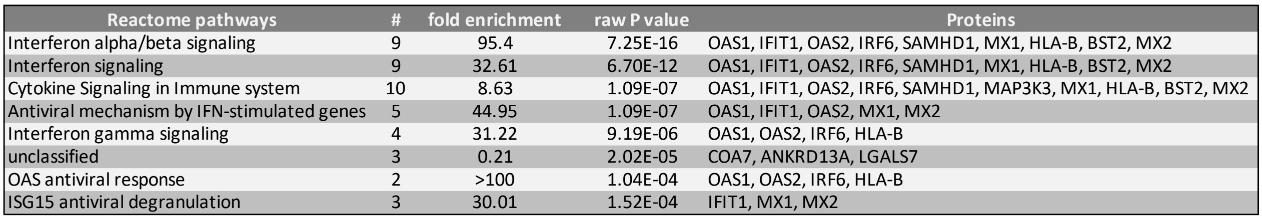
Supplementary Table 2. List of all significantly differentially expressed proteins and their log$_2$ fold change are reported in the proteomic analysis N/TERT1 vs. OKF6/TERT2.



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
EHBP1L1	-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 log$_2$ 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	**TGFBI**	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	**DYNC1LI1**	2.42328
LGALSL	-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	**TGFbI**	1.59164
S100A9	-7.11567	**GLUL**	1.74896
SPRR2B	-5.59424	**PRKD2**	1.96431
S100A8	-5.48779	**TUBGCP3**	1.98718
IRF6	-5.38279	**DYNC1LI1**	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
		TSC22D1	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, DYNC1LI1, 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, DYNC1LI1, 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, TSC22D1, 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 log$_2$ fold change are reported in the proteomic analysis OKF6/TERT2 vs. clones #15 and #26.