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

Eleftheria Girousi ¹, Lukas Muerner ², Ludovica Parisi ¹, Silvia Rihs ¹, Stephan von Gunten ², Christos Katsaros ¹ and Martin Degen ¹,*

* Correspondence: Martin Degen
  martin.degen@zmk.unibe.ch
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 (-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 (Forward) | Sequence (Reverse) | AmpliCon (BP) |
|-------------------------------|--------------------|--------------------|---------------|
| Interferon Regulatory Factor 6 (IRF6) | GCTCTCCATATCATGGCCCTC | CTACAGCCCAAGCCCTTTAATAA | 200 |
| Grninyhead-like Regulatory Factor 3 (GIRFL3) | CCCCCATGCAGAAGAGACTA | ACTGCCTGACTGATGTTG | 91 |
| E-Cadherin 1 (CDH1)           | AGAACCGATTCACACATCACT | TCTTGACCGTACCTGATGCAA | 101 |
| Fibronectin (FN)              | CCACTATGGGTACGATGCAA | AGAGAAGAACGGCCCTTTTGGC | 200 |
| Vimentin (VIM)                | TGTCACAAGATGAGATGTTT | TGCTACATTTCCTGCTCTGG | 117 |
| Snail1 (SNAIL)                | AGAGTCGACTCGGAAGCC | CAGAGTTGAGCGTCAGCC | 164 |
| Proliferating cell nuclear antigen (PCNA) | CAGACACTAGAGCTGGCACC | TAGCGCGAAGATTGGCCGTG | 133 |
| Ki67                          | TGGTTCTCCCAGCTGAGACG | TGGTTCTGATTGATGAGGCC | 109 |
| Involucrin (IVL)              | GGCCCTCAAGATGCTTCTCAT | CACCCCTACACAAAAAG | 131 |
| Loricin (LOR)                 | AGACCCAGAGAAGACGCGG | AGGAGACTGATGACTGCGG | 200 |
| Keratin 10 (KRT10)            | TGGTTCAATGAAAAGCAGGGA | GGATTTTCTCACAGGCAGT | 151 |
| Keratin 13 (KRT13)            | CTGAGGAAGGATGCTTACCA | ATACGGCACTGGCTCTTCT | 162 |
| Transglutaminase 1 (TGM1)     | CCCCCGCAGATGAGATCACA | ATCCCTACGGTACAGCACA | 73 |
| Filaggrin (FLG)               | CTGGACACTCAAGTCTCCTCAT | TTTCGTTTTCTGCTGTGC | 103 |
| Galectin-7 (GAL7)             | TGCTGCCCTCCCCATGCGACC | CTCTGGTCTGGAAGACCC | 126 |
| S100A8                        | ATGCGCTCTACAGGGATGACCT | AGAATGAAAGAGCTCTGGGAAGTTA | 142 |
| S100A9                        | GCACCGCCCAACCCCTGACCA | TGCTTCCAGGCTTCCTCATGAT | 124 |
| Desmoglein-1 (DG1)            | CTGCTGCGAGTGCACCTCCTCAT | CATTGCGGACAGGCTCAAGGC | 124 |
| Desmocollin-2 (DSC2)          | CACAGAGACCTGCAGAGGATGAC | GATGGTCCTCGACCTGCTGT | 130 |
| Glyceroldehyde 3-phosphate dehydrogenase (GAPDH) | CTGCTCTCCACACGCACGCC | TCTCTCTGCTCTGCTGCGGC | 199 |

**Supplementary Table 1.** Sequence of the qPCR primers used in this study. BP: base pairs
**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.
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.
| Pathway                          | Proteins                                                                 | Log fold change | P value |
|---------------------------------|--------------------------------------------------------------------------|-----------------|---------|
| Interferon Signaling            | IFIT1, OAS2, IRF6, IRF6, IFITM1, IFITM5, IRF6, IRF6, IFITM1, IFITM5, IFITM1, IFITM5 | 14              | 5.60E-14 |
| Antigen Presentation: Folding, assembly and peptide loading of class I MHC | HLA-C, HLA-B, HLA-B, HLA-B, HLA-B, HLA-B, HLA-B | 3                | 1.83E-04 |

**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.