frontiers

Supplementary Material

Consistent downregulation of the cleft lip/palate-associated genes *IRF6* and *GRHL3* in carcinomas

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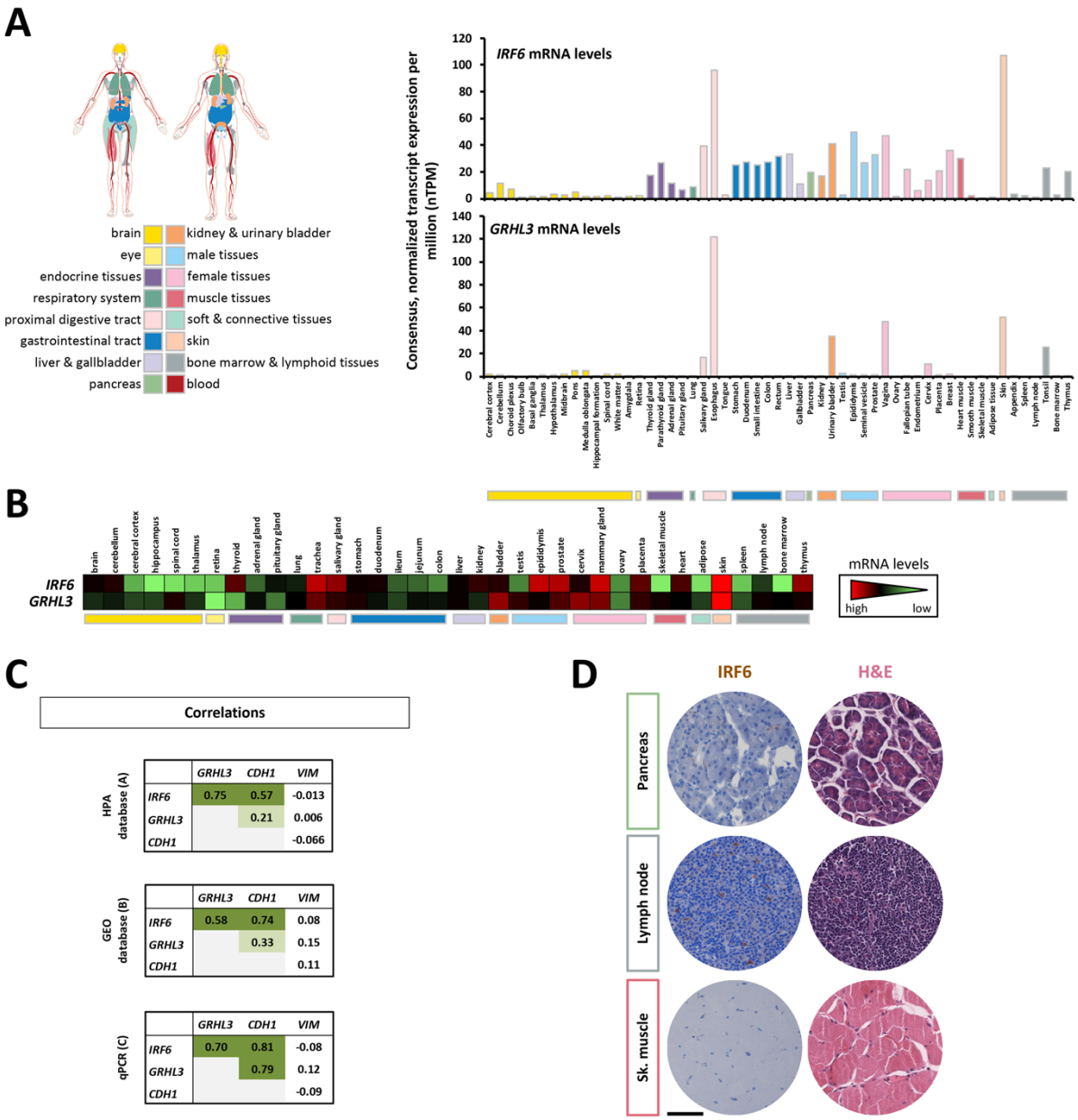
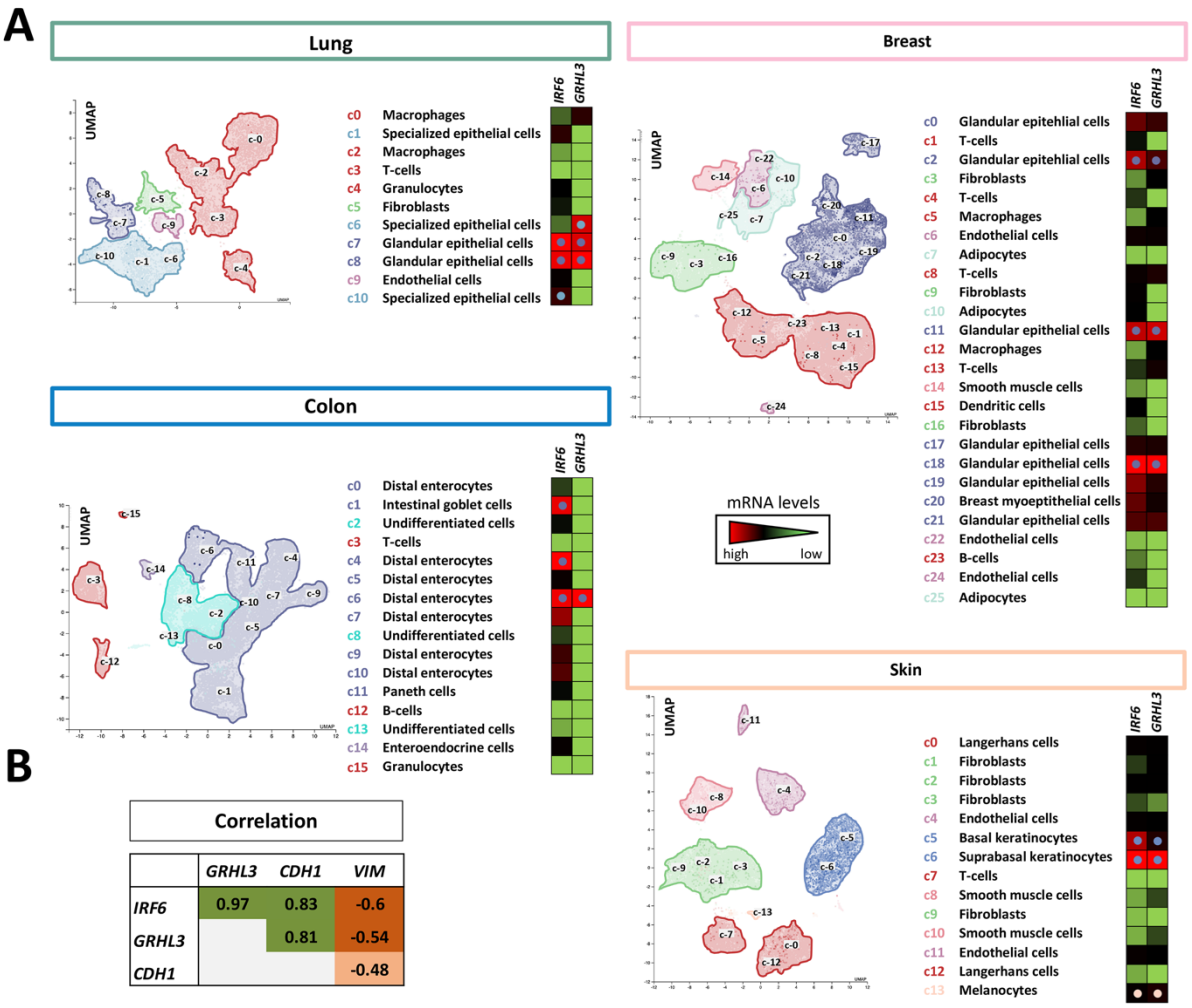


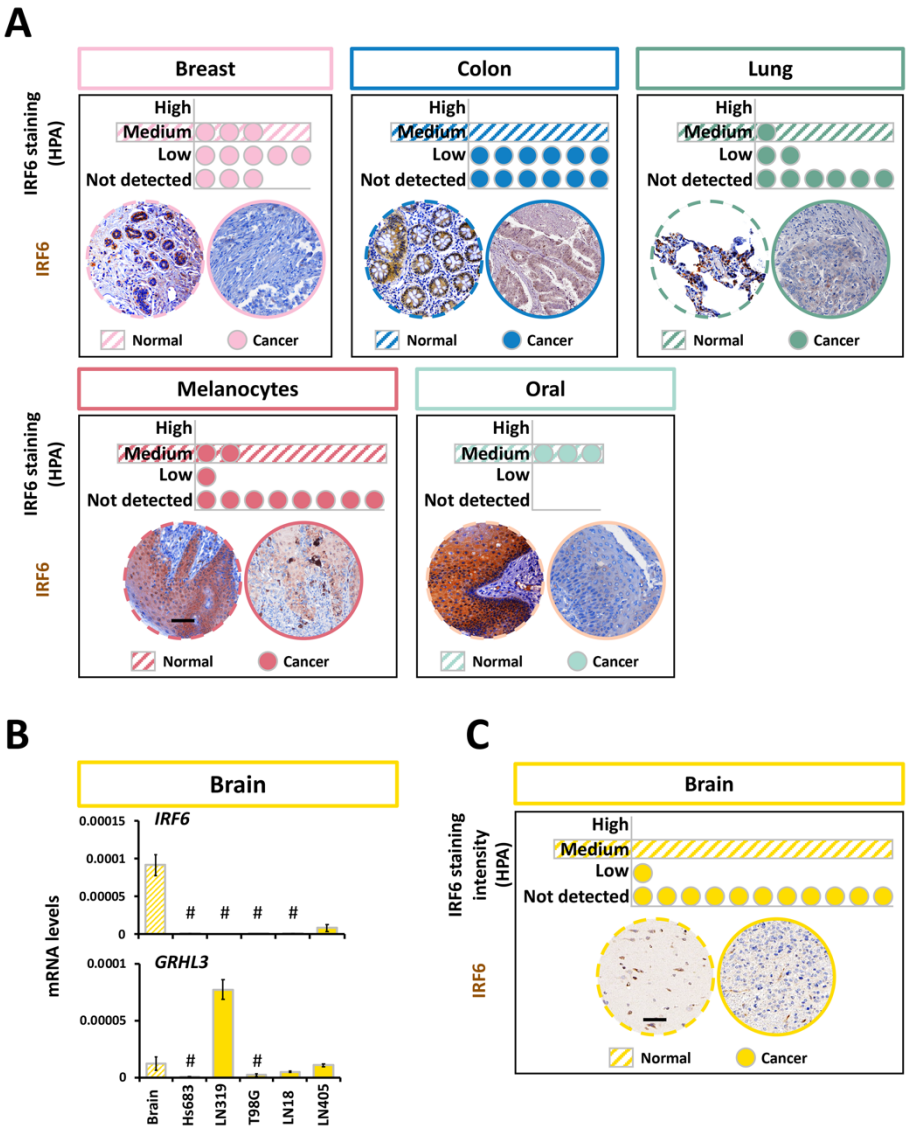
Supplementary Figure 1: IRF6 expression in normal tissues and gene correlations

(A) Consensus *IRF6* and *GRHL3* RNA expression overview from data generated from two different sources: HPA and Genotype-Tissue Expression (GTEx) RNAseq data. Color coding is based on tissue groups and is shown to the left. Data were extracted from the Human Protein Atlas (HPA). **(B)** *IRF6* and *GRHL3* expression in human adult tissues using GSE14938. Scale: light green – low gene expression; red – high gene expression. **(C)** Correlations of *IRF6*, *GRHL3*, *CDH1*, and *VIM* within the various gene expression profiling data sets analyzed in **Figure 1A and Supplementary Figure 1A, B**. Shown is the Pearson's Correlation Coefficient. Dark green: strong positive correlation; Light green: positive correlation. **(D)** Tissue of normal human pancreas, lymph node, and skeletal (sk.) muscle were stained with IRF6 (left). All three tissue do not express detectable levels of IRF6. Corresponding H&E staining of the tissues is shown on the right. Scale Bar: 50 µm.



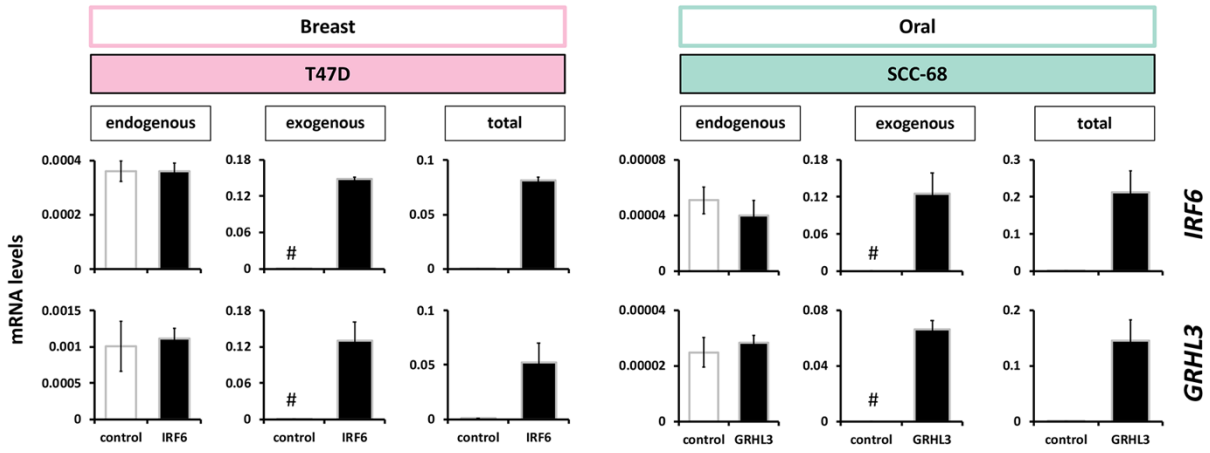
Supplementary Figure 2: Specific expression of *IRF6* and *GRHL3*, and positive correlation of *IRF6*, *GRHL3*, and *CDH1* in normal epithelial cells

(A) Single cell RNA expression levels of *IRF6* and *GRHL3* in breast, colon, lung, and skin tissue. Shown are the UMAP (Uniform Manifold Approximation and Projection) plots with the various cell clusters. The corresponding heatmaps are presented to the right of each plot. Data were extracted from the HPA. Scale: light green – low gene expression; red – high gene expression. Note that the three top expressing cell clusters are indicated by a cell-specific and color-coded dot within the heatmaps for both *IRF6* and *GRHL3*. **(B)** Correlations of *IRF6*, *GRHL3*, *CDH1*, and *VIM* within the normal cell lines analyzed in **Figure 1C**. Shown is the Pearson's Correlation Coefficient. Dark green boxes: strong positive correlation; Dark red boxes: strong negative correlation; Light red box: negative correlation.



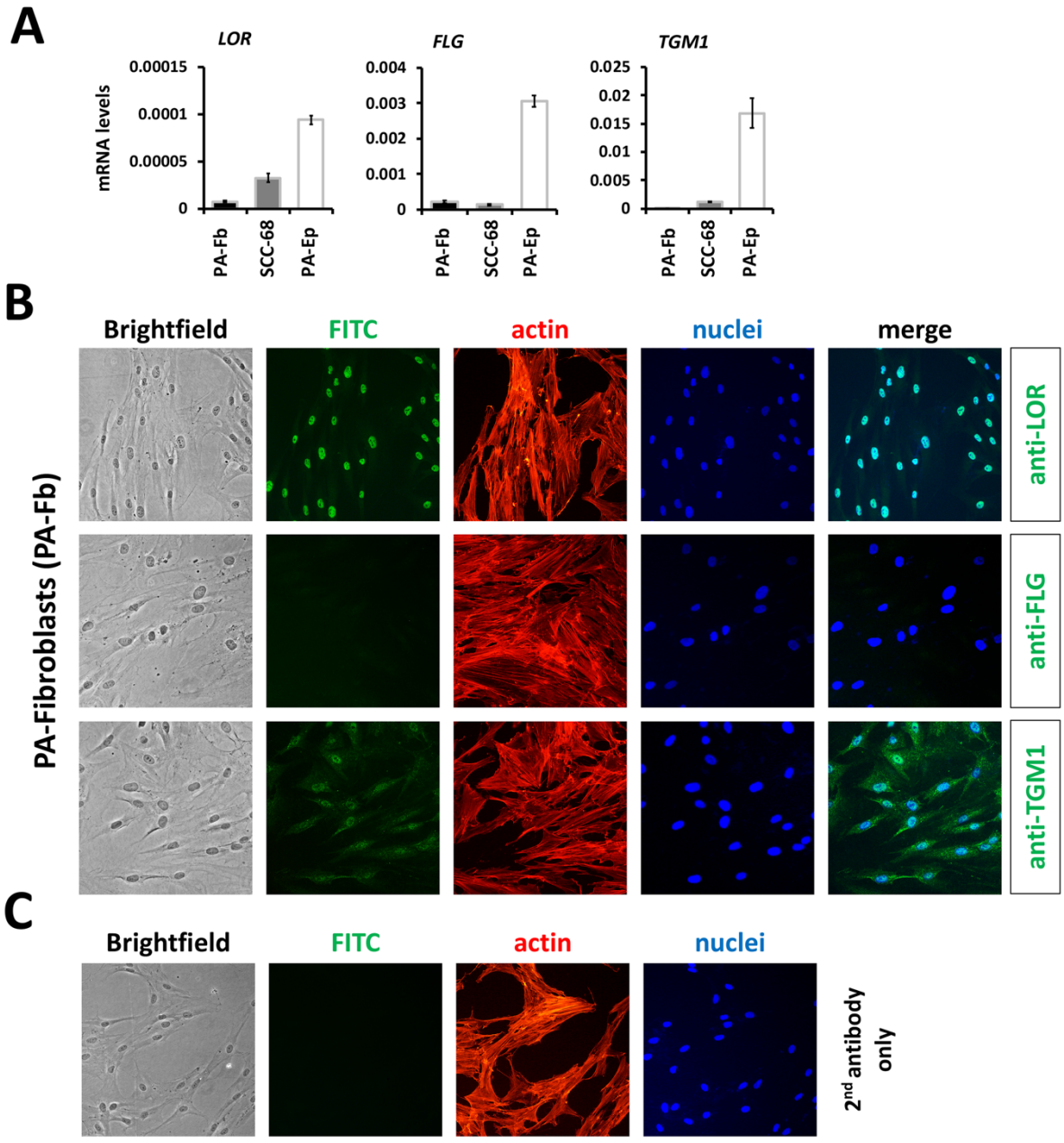
Supplementary Figure 3: Consistent downregulation of IRF6 in carcinomas

(A) The HPA database was used to analyze IRF6 expression in normal and cancerous tissues of the breast, colon, lung, and skin (melanocytes and oral). Expression was quantified as being high, medium, low, and not detected. Each circle represents an individual cancer patient. The striped boxes indicate the IRF6 levels that have been found in the corresponding normal tissues. Note that IRF6 levels in cancer tissue are significantly reduced in breast, colon, lung cancer, and melanoma when compared to their controls. For each tissue type, IHC of normal/cancer tissues is shown for IRF6. Note that IRF6 is readily detectable in the normal tissues, but is mostly absent in the cancer tissues. Scale Bar: 50 µm. **(B)** A panel of brain cancer cell lines was analyzed for the transcript levels of *IRF6* and *GRHL3* compared to brain tissue. # not detectable ($c_T > 32$). **(C)** Only 1/12 brain cancer patients showed low levels of IRF6 while the rest (11/12) was devoid of it. In contrast, IRF6 was found to have a medium staining intensity in normal brain tissue (striped box). Scale Bar: 50 µm. IRF6 staining intensity data collected form the HPA; IHC represents own data.



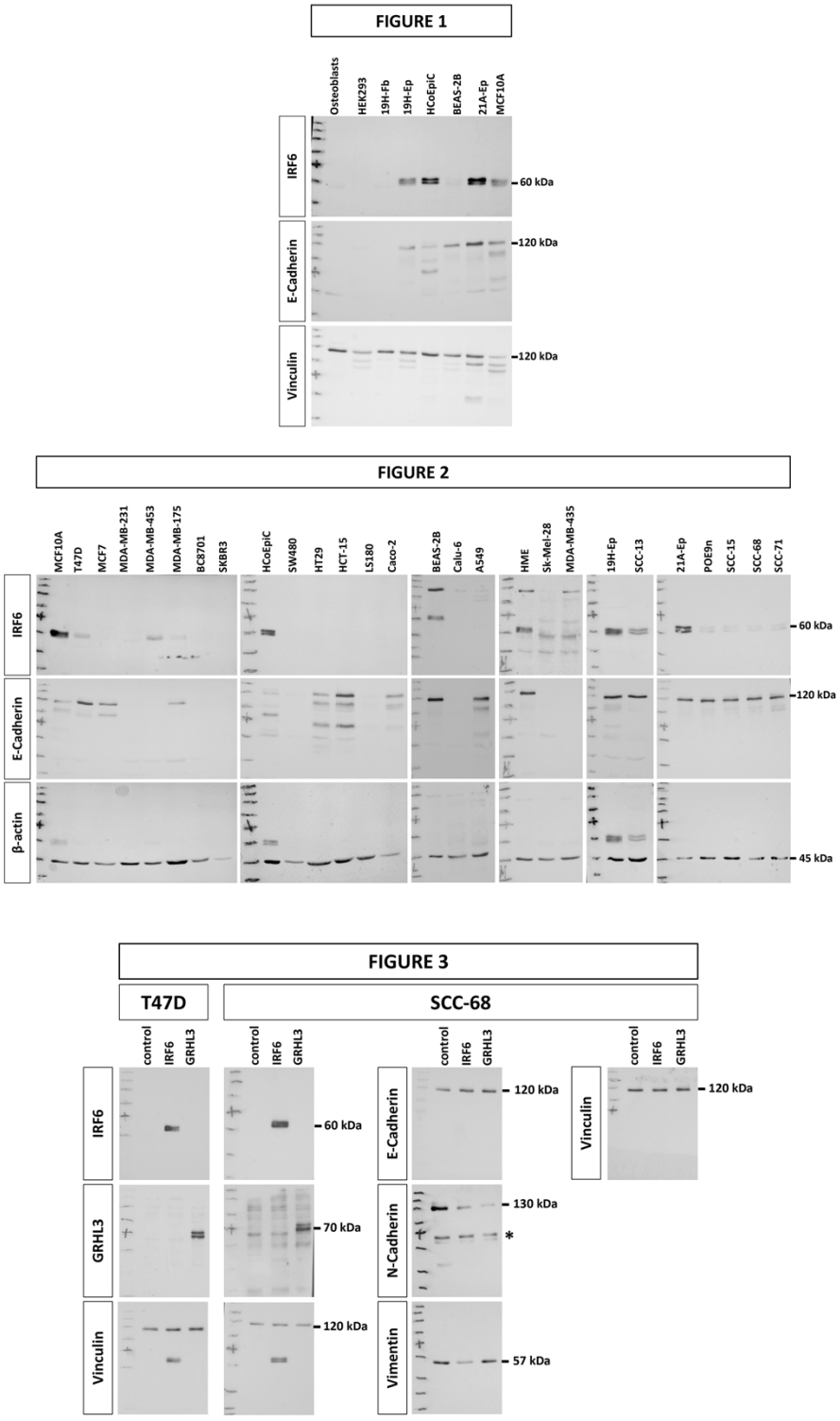
Supplementary Figure 4: Forced expression of IRF6 and GRHL3 in T47D and SCC-68 cell lines

T47D (left) and SCC-68 cell lines were transduced with empty vector control, IRF6, and GRHL3, and *IRF6* and *GRHL3* transcripts analyzed by qPCR. Note that we used specific primer pairs recognizing the endogenous genes only (left panels), the exogenous genes only (middle panels), or both (right panels). # not detectable ($c_T > 32$).



Supplementary Figure 5: Controls for immunofluorescent staining

(A) *LOR*, *FLG*, and *TGM1* mRNA levels were assessed by qPCR in CLP lip fibroblasts (PA-fibroblasts (PA-Fb)), SCC-68, and PA-Ep. Note that the fibroblasts express neglectable levels of all three differentiation markers. **(B)** Staining for LOR, FLG, or TGM1 in PA-Fb revealed nuclear background staining in the FITC channel for LOR and TGM1. **(C)** When only the secondary antibody Alexa Fluor™ 488 goat anti-rabbit IgG (H+L) was used, we did not detect any background fluorescence in the FITC channel.



Supplementary Figure 6: Full-length immunoblots

Full-length blots are shown for all the immunoblot analyses performed in this study. HME: human melanocytes. * background band.

Supplementary Table 1: qPCR primers

GENE	SEQUENCE FWD	SEQUENCE REV	AMPLICON (BP)
Interferon Regulatory Factor 6 *(IRF6)* (endogenous)	GCTCTTCCATATCATGGCCCTC	CTACAGCCCAGGCCTTAAAAA	200
***IRF6* (exogenous)**	CCTTCAAACCCAGGAGAGCTG	CTGCCAGATCCTCTTCTGAGAT	128
***IRF6* (total)**	CAAGTGTGTGACATCCCTCAG	CCACATCATTATCCFFCTCATCC	91
Grainyhead-like transcription factor 3 *(GRHL3)* (endogenous)	ACAGCCTGCACTTCACTGTA	CTCAGATGTGACCTGCCGTC	82
***GRHL3* (exogenous)**	CCCCCACCTGACTGTCTTGA	CTGCCAGATCCTCTTCTGAGAT	199
***GRHL3* (total)**	ACTGTGGAGCACATTGAGGAGG	CTGTGCTCAGACAGTTTACGCC	103
E-Cadherin 1 *(CDH1)*	AGAACGCATTGCCACATACACT	TCTGATCGGTTACCGTGATCAA	101
N-Cadherin *(CDH2)*	GGTGGAGGAGAAGAAGACCAG	GGCATCAGGCTCCACAGT	72
Vimentin *(VIM)*	TGTCCAAATCGATGTGGATGTTTC	TTGTACCATTCTTCTGCCTCCTG	117
Snail1 *(SNAIL1)*	AAGATGCACATCCGAAGCC	CGCAGGTTGGAGCGGTCAGC	164
TWIST2	CTTATGTTTGGGGGGAGGTT	TAGCCAAGCAATCACGGAGA	295
KI67	TGACTTCCTTCCATTCTGAAGAC	TGGGTCTGTTATTGATGAGCC	109
Involucrin *(IVL)*	GGCCCTCAGATCGTCTCATA	CACCCTCACCCCATTAAAGA	131
Filaggrin *(FLG)*	CTGGACACTCAGGTTCCCAT	TTTCGTGTTTGTCTGCTTGC	103
Loricrin *(LOR)*	AGACCCAGCAGAAGCAGGCG	AGCAGAACTAGATGCAGCCG	200
Keratin 10 *(K10)*	TGGTTCAATGAAAAGAGCAAGGA	GGGATTGTTTCAAGGCCAGTT	151
Transglutaminase1 (*TGM1*)	CCCCCGCAATGAGATCTACA	ATCCTCATGGTCCACGTACACA	73
Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*)	CTCTGACTTCAACAGCGACACCC	TCCTCTTGTGCTCTTGCTGGGGC	199

Sequences (5'-3') of the primer pairs used for the qPCR analyses. FWD: forward; REV: reverse; BP: base pairs.

Supplementary Table 2: Cells

NAME	TISSUE	DISEASE	MORPHOLOGY	MEDIUM	SOURCE
BMMSC (Bone marrow-derived mesenchyamal stem cells)	Bone marrow	healthy	fibroblastic	BMMSC	ScienCell
HUVEC (human umbilical vein endothelial cells)	Umbilical Cord	healthy	endothelial	1)	Gift from Elizaveta Fasler-Kan, University of Bern, Switzerland
HEK293 (human embryonic kidney)	kidney	healthy	epithelial	DMEM	ATCC, CRL-1573
PA-Ep	Lip	CLP (wt for *IRF6*)	epithelial	KSFM	Laboratory for Oral Molecular Biology, University of Bern, Switzerland
E6-Ep	Lip	CLP (wt for *IRF6*)	epithelial	KSFM	Laboratory for Oral Molecular Biology, University of Bern, Switzerland
19H-Ep	Forehead	healthy	epithelial	KSFM	Laboratory for Oral Molecular Biology, University of Bern, Switzerland
19H-Fb	Forehead	healthy	fibroblastic	DMEM	Laboratory for Oral Molecular Biology, University of Bern, Switzerland
21A-Ep	Gingiva	healthy	epithelial	KSFM	Laboratory for Oral Molecular Biology, University of Bern, Switzerland
21A-Fb	Gingiva	healthy	fibroblastic	DMEM	Laboratory for Oral Molecular Biology, University of Bern, Switzerland
FRSK3-Ep	Foreskin	healthy	epithelial	KSFM	Laboratory for Oral Molecular Biology, University of Bern, Switzerland
FRSK3-Fb	Foreskin	healthy	fibroblastic	DMEM	Laboratory for Oral Molecular Biology, University of Bern, Switzerland
Wi38	fetal lung	healthy	fibroblastic	DMEM + NEAA	Gift from Prof. Beat Trüb, University of Bern, Switzerland
Hepatocytes	Liver	healthy	epithelial	total RNA	ScienCell (Catalog#5205)
BEAS-2B	Lung	healthy	epithelial	KSFM	ATCC, CRL-9609
HCoEpiC	Colon	healthy	epithelial	total RNA	ScienCell (Catalog#2955)
Osteoblasts	Alveolar Bone	healthy	fibroblastic	DMEM	Laboratory for Oral Molecular Biology, University of Bern, Switzerland
Melanocytes	Skin	healthy	stellar, multipolar	1)	ScienCell (Catalog#2205)
MCF10A	Mammary Gland	healthy	epithelial	DMEM/F12/Horse serum/EGF/Hydrocortisone, Cholera Toxin, Insulin	ATCC, CRL-10317
T47D	Mammary Gland	carcinoma; ductal	epithelial	DMEM	ATCC, HTB-133
MCF7	Mammary Gland	adenocarcinoma	epithelial	DMEM	ATCC, HTB-22
MDA-MB-453	Mammary Gland	carcinoma; metastatic	epithelial	DMEM	ATCC, HTB-131
MDA-MB-175	Mammary Gland	carcinoma; medullary	epithelial	DMEM	ATCC, HTB-25
MDA-MB-231	Mammary Gland	adenocarcinoma	epithelial	DMEM	ATCC, CRM-HTB-26
BC8701	Mammary Gland	carcinoma; ductal	epithelial	DMEM	Gift from Elizaveta Fasler-Kan, University of Bern, Switzerland
SKBR3	Mammary Gland	adenocarcinoma	epithelial	DMEM	ATCC, HTB-30
SW480	Colorectal	adenocarcinoma	epithelial	Leibovitz	ATCC, CCL-228
HT29	Colorectal	adenocarcinoma	epithelial	McCoys 5A	ATCC, HTB-38
HCT-15	Colorectal	adenocarcinoma	epithelial	RPMI-1640	ATCC, CCL-225
LS180	Colorectal	adenocarcinoma	epithelial	Eagles MEM	ATCC, CL-187
Caco-2	Colorectal	adenocarcinoma	epithelial	DMEM	ATCC, HTB-37
SW620	Colorectal	adenocarcinoma	epithelial	Leibovitz	ATCC, CCL-227
DLD-1	Colorectal	adenocarcinoma	epithelial	RPMI-1640	ATCC, CCL-221
Colo 205	Colorectal	adenocarcinoma	epithelial	RPMI-1640	ATCC, CCL-222
HCT-116	Colorectal	carcinoma	epithelial	McCoys 5A	ATCC, CCL-247
Calu-6	Lung	carcinoma; anaplastic	epithelial	DMEM	ATCC, HTB-56
A549	Lung	carcinoma	epithelial	DMEM	CRM-CCL-185
Sk-Mel-28	Skin	malignant melanoma	epithelial	DMEM	ATCC, HTB-72
MDA-MB-435	Skin	melanoma	epithelial	DMEM	ATCC, HTB-129
D10	Skin	melanoma	epithelial	DMEM	Gift from Elizaveta Fasler-Kan, University of Bern, Switzerland
SCC-13	Skin	Squamous Cell Carcinoma	epithelial	KSFM	J. Rheinwald laboratory (Harvard Medical School, Boston, USA)
POE9n	oral cavity	Dysplastic Lesion	epithelial	KSFM	J. Rheinwald laboratory (Harvard Medical School, Boston, USA)
SCC-15	oral cavity	Oral Squamous Cell Carcinoma	epithelial	KSFM	J. Rheinwald laboratory (Harvard Medical School, Boston, USA)
SCC-68	oral cavity	Oral Squamous Cell Carcinoma	epithelial	KSFM	J. Rheinwald laboratory (Harvard Medical School, Boston, USA)
SCC-71	oral cavity	Oral Squamous Cell Carcinoma	epithelial	KSFM	J. Rheinwald laboratory (Harvard Medical School, Boston, USA)
Hs683	Brain	Oligodendroglioma	epithelial	DMEM	ATCC, HTB-138
LN319	Brain	Astrocytoma	epithelial	DMEM	Gift from Jean-Louis Boulay, University of Basel, Switzerland
T98G	Brain	Glioblastoma Multiforme	epithelial	DMEM	ATCC, CRL-1690
LN18	Brain	Glioblastoma	epithelial	DMEM	Gift from Elizaveta Fasler-Kan, University of Bern, Switzerland
LN405	Brain	Glioblastoma	epithelial	DMEM	Gift from Elizaveta Fasler-Kan, University of Bern, Switzerland

Cells used in this study with their names, culture media, and sources. NEAA: non-essential amino acids. 1) only RNA/protein extracts were used. 2) Only RNA was used.

Supplementary Table 3: Literature search strategy

	Keywords	MeSH
IRF6	"IFN-regulatory factor 6" "Interferon regulatory factor 6" IRF-6 IRF6	"IRF6 protein, human" (Supplementary Concept)
GRHL3	"Grainyhead-like 3 protein, human" "Sister-of-Mammalian protein" "Sister-of-Mammalian Grainyhead protein, human" "SOM protein, human" GRHL3 GRHL-3 "Grainyhead like 3" "Grainyhead-like 3"	"GRHL3 protein, human" (Supplementary Concept)
Cancer	Cancer Tumor Tumour* Neoplasm* Carcinoma* Carcinogen*	"Neoplasms" (Mesh)
	Inclusion	**Exclusion**
Criteria	free access to full text Language: English Discussion of connection to cancer in abstract	Reviews, letters, conference abstracts, book chapters

Table shows the keywords, the MeSH terms as well as inclusion/exclusion criteria for the literature search.

Supplementary Table 4: Literature on the expression/function of IRF6 and GRHL3 in cancer

IRF6

Type	Onc	Sup	Outcome(s)	Ref
SKIN/ORAL				
Cutaneous SCC		●	IRF6 downregulation (DNA hypermethylation) correlates with invasiveness and poor differentiation	(1)
		●	IRF6 is a Notch1 target and its downregulation promotes ras-induced tumor formation	(2)
Oral SCC		●	Identification of pathological *IRF6* variants	(3)
	●	●	In response to smoke exposure upregulation of IRF6 that induces NOS2; NOS2 can have dual roles	(4)
		●	Association of an *IRF6* variant with oral SCC	(5)
Vulvar SCC (VSCC)		●	Stepwise downregulation of IRF6 from preneoplastic vulvar lichen sclerosus to VSCC	(6)
Nasorpharyngeal Carcinoma		●	IRF6 downregulation; IRF6 selectively kills cancer stem cells	(7)
Melanoma		●	IRF6 silencing by DNA hypermethylation	(8)
FEMALE TISSUES				
Breast Cancer		●	IRF6 downregulation corelating with Maspin	(9)
		●	Re-expression of IRF6 in breast cancer cell induces cell cycle arrest	(10)
	●		IRF6 as a positive regulator of proliferation/differentiation of MCF10A cells downstream of Notch	(11)
	●		ErbB2-driven IRF6 downregulation blocks anoikis and promotes anchorage-independent growth	(12)
		●	IRF6 downregulation in aggressive cancers	(13)
	●		High IRF6 levels after neoadjuvant therapy associated with relapse in ErbB2-positive cancers	(14)
Cervical Cancer		●	Downregulation of IRF6	(15)
Cervical SCC	●		IRF6 negatively correlates with patient survival time	(16)
GASTROINTESTINAL TRACT				
Rectal Cancer		●	IRF6 levels inversely associated with rectal cancer	(17)
Colorectal Cancer	●		Positive association of IRF6 and colorectal cancer in relation to smoking/NSAID	(17)
	●		Downregulation of IRF6 as an "anti-cancer effect" of Artemisia annua L. polyphenols (pKAL)	(18)
Gastric Cancer		●	Downregulation of *IRF6* due to DNA methylation of the promoter correlating with poor prognosis	(19)
RESPIRATORY SYSTEM				
Lung Cancer	●		Upregulation of IRF6 in asbestos-induced lung cancers	(20)
Large-Cell Lung Cancer; Adenocarcinoma		●	TSCOT, a tumor suppressor is reduced in lung cancer and positively associated with GRHL3 and IRF6	(21)
	●		Upregulation of IRF6 in lung adenocarcinoma and SCC	(22)
BRAIN				
Glioma		●	Downregulation of IRF6	(23)
Subependymal Giant Cell Asctrocytoma	●		High expression of IRF6 makes it a potential target for treatment	(24)
PANCREAS				
Pancreastic Cancer	●		IRF6 elevated in pancreatic cancer correlting with poor overall survival	(25)
KIDNEY&URINARY BLADDER				
Clear Cell Renal Cell Carcinoma		●	Downregulation of IRF6 due to DNA hypermethylation of promoter	(26)
		●	Downregulation of IRF6 that correlates with shorter overall survival of patients	(27)
Urothelial Carcinoma		●	Reexpression of IRF6 inhibits tumor growth; epigenetic regulation of *IRF6*	(28)
BLOOD				
Acute Myeloid Leukemia	●		IRF6 overexpression in an AML cell line	(29)

GRHL3

Type	Onc	Sup	Outcome(s)	Ref
SKIN/ORAL				
Cutaneous SCC		●	Deletion of GRHL3 evokes loss of PTEN resulting in aggressive tumors	(30)
		●	Decreased GRHL3 contributes to tumor progression	(31)
Oral SCC		●	Loss of GRHL3 promotes tumor by downregulating GSK3B and inducing c-myc	(32)
		●	GRHL3, a negative regulator of PI3K/AKT/mTOR signaling, was downregulated in tumors	(33)
		●	GRHL3-FLG axis upregulation reversed cancer cell proliferation	(34)
Basal-Cell Carcinoma		●	Downregulation of GRHL3; reduces thyroid hormone in the tumor microenvironment	(35)
Skin Cancer (non-melanoma)		●	Reduction of GRHL3 can lead to tumors and inflammatory diseases	(36)
		●	Reduction of GRHL3; GRHL3 a target of oncogenic miR-21	(37)
Trichogerminoma (hair follicle)		●	Identification of GRHL3 rearrangements as oncogenic drivers	(38)
FEMALE TISSUES				
Breast Cancer	●		TNFα regulated GRHL3 stimulates endothelial cell migration consistent with an angiogenic fucntion	(39)
	●		ZNF652 represses GRHL3 in normal breast epithelial cells; this response is lost in cancer	(40)
	●	●	Early stage: high expression of GRHL3 (gain of tumor mass); advanced tumors: reduced GRHL3	(41)
		●	Highest GRHL3 levels in stage 1 tumors, lowest in stage 3	(42)
		●	GRHL3 promotes expression of E-Cadherin	(43)
	●		Downregulation of E-Cadherin by GRHL3; induction of migration and invasion by E-Cadherin	(44)
		●	GRHL3 acts downstream of BRD4 to regulate epithelial differentiation; downregulated in tumors	(45)
GASTROINTESTINAL TRACT				
Colorectal Cancer	●		Upregulation of GRHL3 in cancer tissue compared to normal tissue	(46)
	●		GRHL3 mRNA and protein levels were higher in cancer tissue than in normal tissue	(47)
	●		GRHL3 promotes tumor growth via MEK signaling	(48)
RESPIRATORY SYSTEM				
Small-Cell Lung Cancer; Adenocarcinoma	●		Increased GRHL3 in cancer tissue	(49)
Large-Cell Lung Cancer; Adenocarcinoma		●	The tumor suppressor TSCOT is reduced in lung cancer and positively associated with IRF6	(21)
PANCREAS				
Pancreastic Cancer		●	Repression of GRHL3 by SIRT1/CRL4B complex promotes migration and invasion	(51)
KIDNEY&URINARY BLADDER				
Renal Carcinoma			No difference between GRHL3 levels in tumors and controls	(52)
Urothelial Carcinoma		●	Overexpression of GRHL3 reduced migration and invasion potential of cancer cells	(53)
BLOOD				
Diffuse large B-cell Lymphoma	●		Identification of GRHL3 as a predictor of poor surivival in patients	(54)
PROXIMAL DIGESTIVE TRACT				
Esophageal SCC (ESCC)		●	Low GRHL3 levels in P. gingivalis infected ESCC cells stimulates migration/proliferation via PTEN/AKT	(55)

Table indicating the main findings of the studies analyzing expression and/or function of IRF6 (top) and GRHL3 (bottom) in cancer. Onc: oncogene; Sup: Tumor Suppressor.

References Supplementary Table 4

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Supplementary Movie 1: Migration of SCC-68/control cells

Confluent cells were scratched with a pipet tip and cell free gap was monitored using the IncuCyte S3 live imaging device. Pictures were taken at 0, 2, 4, 6, 8, and 10 h after scratching the cell monolayer.

Supplementary Movie 2: Migration of SCC-68/IRF6 cells

Confluent cells were scratched with a pipet tip and cell free gap was monitored using the IncuCyte S3 live imaging device. Pictures were taken at 0, 2, 4, 6, 8, and 10 h after scratching the cell monolayer. Note that the SCC68/IRF6 cells display a significant delay in the closure of the gap when compared to control.

Supplementary Movie 3: Migration of SCC-68/GRHL3 cells

Confluent cells were scratched with a pipet tip and cell free gap was monitored using the IncuCyte S3 live imaging device. Pictures were taken at 0, 2, 4, 6, 8, and 10 h after scratching the cell monolayer. Note that the SCC-68/GRHL3 cells display a significant delay in the closure of the gap when compared to control.