Exploring Grainyhead-like 2 target genes in breast cancer

Wang, Z.

Chapter 5

Identification of direct GRHL2 target genes in luminal-like breast cancer through integration of ChIP-seq and Bru-seq

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Abstract

The transcription factor GRHL2 has been reported to induce and repress gene expression, which is cell context-dependent. While several studies have addressed expression and function of GRHL2 in breast cancer with different conclusions, the profile of GRHL2 target genes in breast cancer has not been characterized. In the present study, ChIP-seq analysis of GRHL2-binding genes in MCF7 cells was integrated with Bru-seq analysis of genes showing transcriptional responses to conditional CRIPR-Cas9 knockout of GRHL2 in MCF7 cells. This identified 48 direct target genes of GRHL2 in MCF7 cells. Signaling pathways and networks associated with these direct GRHL2 target genes were explored using IPA. Notably, in line with our previous report that the *CDH1* promotor lacks GRHL2-binding sites, RNA synthesis of *CDH1*, encoding the epithelial adhesion receptor E-cadherin, was not altered following GRHL2 deletion, demonstrating *CDH1* is not a direct target gene of GRHL2. Instead, the epithelial-specific transcription factor EHF/ESE3, a transcription factor that, like GRHL2, suppresses EMT, was identified as a direct target gene of GRHL2. *EHF* was downregulated at all time points after GRHL2 deletion and, like *GRHL2*, *EHF* was specifically absent in basal B-like breast cancer in a pan-subtype human breast cancer cell line panel. EHF has been implicated in tumor initiating properties. However, its overexpression failed to rescue proliferation in GRHL2-depleted breast cancer cells. Collectively, this study identifies direct target genes of GRHL2 and their related signaling pathways and sheds light on the epithelial factors GRHL2 and EHF in luminal-like breast cancer MCF7 cells.

Introduction

The transcription factor GRHL2 has been reported to activate and repress gene expression [1,2]. Identification of GRHL2 direct target genes is significant for

understanding GRHL2 biological functions and signaling pathways regulated by GRHL2. A negative feedback loop exists between ZEB1 and GRHL2, in which GRHL2 acts as an inhibitory regulator of EMT, inhibiting ZEB1 expression directly [2]. Additionally, GRHL2 is involved in differentiation of epithelial cells, morphogenesis of epithelial tubes and maintenance of epithelial phenotype through activating expression of epithelial-specific genes such as *CDH1* and *CLDN4* [3]. EHF derives from the ETS transcription factor family characterized by epithelial-specific expression [4]. Multiple lines of evidence demonstrate a significant role of EHF in the regulation of cell proliferation and differentiation [4,5].

Integrating RNA-seq and ChIP-seq data is a typical method to identify target genes. However, target genes identified by RNA-seq include direct and indirect target genes. By contrast, Bru-seq analyzes changes in nascent RNA, is not affected by post-transcriptional regulation, and hence monitors direct changes in transcription [6,7]. Here, we integrated ChIP-seq analysis of GRHL2-binding genes in MCF7 cells with Bru-seq analysis of genes showing transcriptional responses to conditional CRISPR-Cas9 knockout of GRHL2 in MCF7 cells. Direct target genes of GRHL2 and their related signaling pathways were identified and the interaction between *GRHL2, EHF*, and *CDH1* in luminal-like breast cancer (MCF7 cells) was explored.

Materials and methods

Cell culture

MCF7 human breast cancer cells were obtained from the American Type Culture Collection. Cells were cultured in RPMI1640 medium with 10% fetal bovine serum, 25 U/mL penicillin and 25 μg/mL streptomycin at 37°C and 5%

CO_2. Generation of MCF7 conditional GRHL2 knockout (KO) cells was previously described (see Chapter 4).

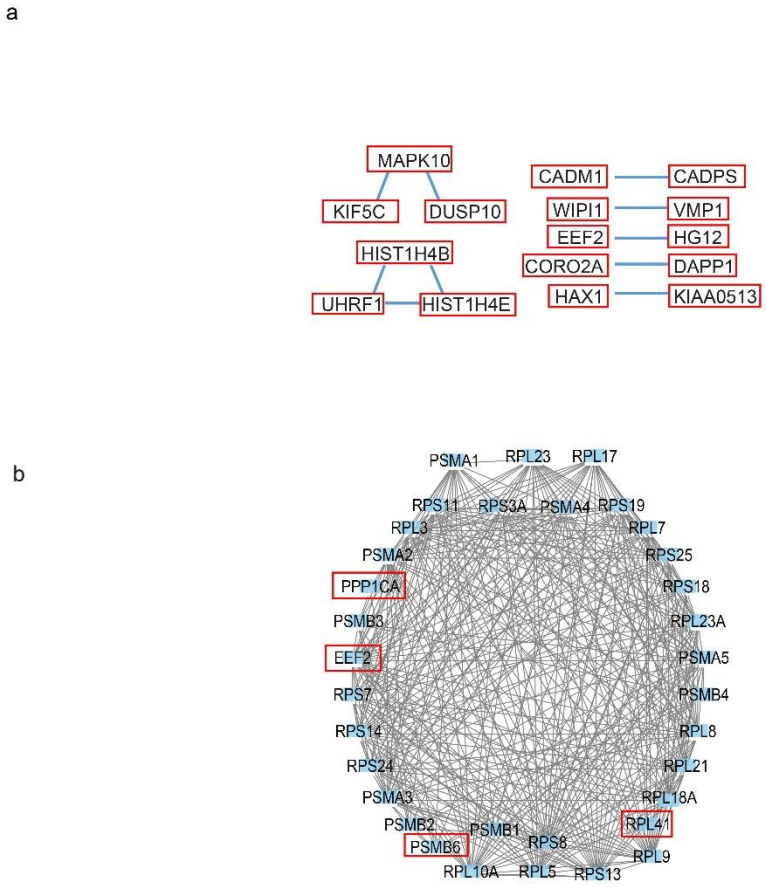


Fig. 1 Protein-protein interaction networks among GRHL2 direct target genes. Based on identification of GRHL2 direct target genes through integration of ChIP-seq and Bru-seq data, **(a)** direct and **(b)** indirect protein-protein interaction networks among GRHL2 direct target genes are predicted. Red rectangles indicate direct target genes of GRHL2.

ChIP-PCR

Chromatin preparation for chromatin immunoprecipitation-sequencing (ChIP-seq) has been previously described [8]. For ChIP-PCR, chromatin fragments were immunoprecipitated with control IgG or anti-GRHL2 antibodies (Sigma; HPA004820). Precipitates were eluted by NP buffer, low salt (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl (pH 8.1), 150mM NaCl), high salt (0.1%

SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl (pH 8.1), 500mM NaCl) and LiCl buffer (0.25M LiCl, 1%NP40, 1% deoxycholate, 1mM EDTA, 10mM Tris-HCl (pH 8.1)). Chromatin was de-crosslinked by 1% SDS at 65°C. DNA was purified by Phenol:Chloroform:Isoamyl Alcohol (PCI) and then diluted in TE buffer. The following primers were used for ChIP-PCR: *EHF* forward: ctgaaaagaacagtcaccacca, *EHF* reverse: tggccaactcacacgttagt, control (an intergenic region upstream of the *GAPDH* locus) forward: atgggtgccactggggatct, control reverse: tgccaaagcctaggggaaga. ChIP-PCR data were analyzed using the $2^{-\Delta\Delta Ct}$ method [9].

Bru-seq

Bru-seq and associated bioinformatics analysis was described previously (see Chapter 4). In short, at different timepoints after doxycycline-induced deletion of GRHL2, MCF7 conditional KO cells were incubated with Bru, cells were lysed, and Bru-labelled nascent RNA was isolated using an anti-BrdU antibodies. Subsequently, cDNA libraries were generated, sequenced, and reads were mapped to Genome Reference Consortium human genome (build 37). To identify GRHL2-regulated genes, reads per kilobase per million mapped reads for each gene in the doxycycline-treated samples were compared to the untreated samples, genes with $p<0.05$ and $FC>2$ or $FC<0.5$ were filtered, and genes responding to Cas9 induction in the context of sgGRHL2 but not in the context of sgCTR were selected.

Bioinformatics analysis

Canonical pathways and networks analysis were performed with the Ingenuity Pathways Analysis (IPA) software (Ingenuity Systems, USA). The STRING database [10] was used to predict direct protein-protein interactions (PPI), which were visualized by Cytoscape v3.7.2 [11].

Table 1. Top 20 canonical pathways identified by GRHL2 direct target genes using IPA.

Canonical Pathways	-log(p-value)	Ratio
Germ Cell-Sertoli Cell Junction Signaling	2.26	0.0181
Diphthamide Biosynthesis	2.19	0.333
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	2.13	0.0162
Activation of IRF by Cytosolic Pattern Recognition Receptors	2.11	0.0328
SAPK/JNK Signaling	1.7	0.0198
Sumoylation Pathway	1.7	0.0198
CDK5 Signaling	1.65	0.0187
Pancreatic Adenocarcinoma Signaling	1.64	0.0185
Cholecystokinin/Gastrin-mediated Signaling	1.58	0.0171
D-myo-inositol (1,4,5,6)-Tetrakisphosphate Biosynthesis	1.46	0.0148
D-myo-inositol (3,4,5,6)-tetrakisphosphate Biosynthesis	1.46	0.0148
Granzyme A Signaling	1.44	0.0588
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	1.41	0.0139
3-phosphoinositide Degradation	1.39	0.0134
D-myo-inositol-5-phosphate Metabolism	1.38	0.0133
HMGB1 Signaling	1.34	0.0127
3-phosphoinositide Biosynthesis	1.33	0.0126
Tec Kinase Signaling	1.33	0.0125

Sulforhodamine B (SRB) assay

Cell proliferation rate was measured by SRB assay. Cells were seeded into 96-well plates. At the indicated time points, cells were fixed with 50% Trichloroacetic acid (TCA, Sigma-Aldrich) for 1 hour at 4 °C and plates were washed with demineralized water 4 times and air-dried at room temperature (RT). Subsequently, plates were incubated with 0.4% SRB (60 µl/well) for at least 2 hours at RT. The plates were washed five times with 1% acetic acid and air-dried. Plates were incubated with 10 mM Tris (150 µl/well) for 30 minutes at RT with gentle shaking. The absorbance value was measured by a Fluostar OPTIMA plate-reader.

Realtime quantitative PCR (RT-qPCR)

Total RNA was isolated using RNEasy Plus Mini Kit (Qiagen). 500 ng RNA was reverse-transcribed into cDNA using the RevertAid H Minus First Strand cDNA

Synthesis Kit (Thermo Fisher Scientific). The cDNA was mixed with SYBR green master mix (Fisher Scientific) for qPCR. RT-qPCR data were collected and analyzed using $2^{-\Delta\Delta Ct}$ method. The following primers were used: *GAPDH* forward: ccatggggaaggtgaaggtc, *GAPDH* reverse: agttaaaagcagccctggtga. *EHF* forward: ctgccctgagtggagattgg, *EHF* reverse: tgcccttgccttcacagaaa.

Colony formation assays

Cell survival was measured by colony formation assay. 450 cells were seeded into a well of 6-well plate after 4 days of doxycycline treatment. After 7 days, cells were fixed with 4% formaldehyde and stained with Giemsa. Images were analyzed by Image J (ColonyArea package).

Table 2. Networks based on direct GRHL2 genes predicted by IPA.

Top Diseases and Functions	Score	Focus Molecules
Cancer, Organismal Injury and Abnormalities, Reproductive System Disease	24	13
Cell Cycle, Cellular Development, Cellular Growth and Proliferation	22	12
Cell Death and Survival, Cellular Development, Cellular Movement	9	6
Cellular Assembly and Organization, Embryonic Development, Organismal Development	2	1
Cell Cycle, Cellular Development, Embryonic Development	2	1
Drug Metabolism, Molecular Transport, Organismal Functions	2	1
Cell Morphology, Cellular Movement, Immune Cell Trafficking	2	1
Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry	2	1

Results

Candidate direct GRHL2 target genes whose transcription is altered in response to GRHL2 depletion

In order to identify direct target genes of GRHL2 in luminal breast cancer (MCF7 cells), integration of ChIP-seq and Bru-seq data was performed. 48 differentially expressed genes identified by Bru-seq were confirmed as direct GRHL2 target genes by ChIP-seq **(Table S1)**, including *EHF/ESE3, E2F2, CDCA7L* and *FOXP2*. Protein-protein interaction (PPI) networks among the direct GRHL2 target genes were constructed using STRING database [10] **(Fig. 1).**

Predicted signaling networks regulated by GRHL2

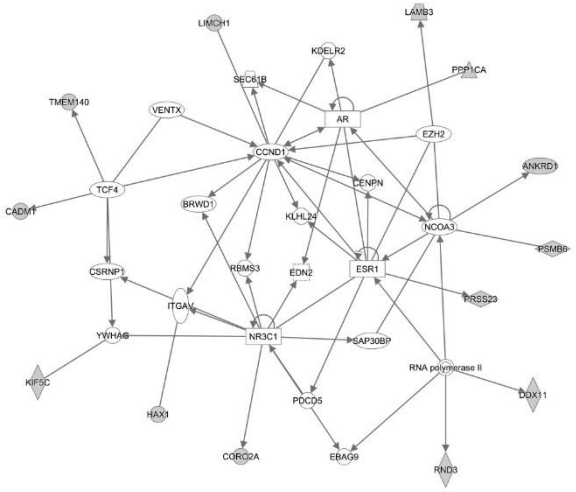
IPA analysis was performed to predict signaling pathways associated with the identified GRHL2 direct target genes. The signaling pathways were ranked according to *p* value. **Table 1** demonstrates the top 20 signaling pathways, including SAPK/JNK signaling and CDK5 signaling that are involved in cell proliferation [12,13]. Germ cell-Sertoli cell junction signaling was identified as the top signaling pathway predicted by genes regulated by GRHL2 loss. Notably, this same pathway was also predicted by the Bru-seq data alone at 2-8 days post deletion of GRHL2 **(see Chapter 4 Table 1)**.

Subsequently, networks related to multiple biological functions and diseases associated with the identified GRHL2 direct target genes were predicted by IPA and then ranked based on the score. The top networks were enriched in cancer, organismal injury and abnormalities, reproductive system disease **(Table 2)**, of which top 3 networks are shown in **Fig. 2.** The main functions of GRHL2 direct target genes were associated with cell cycle, growth, and proliferation **(Table 2)**.

RNA synthesis of CDH1 is not altered after GRHL2 loss

CDH1 encodes E-cadherin, a cell-cell adhesion receptor involved in maintenance of the epithelial phenotype [14]. *CDH1* has been proposed to represent a direct target gene of GRHL2 [3,15,16]. Other studies [3,17,18], and our unpublished results (Wang et al, manuscript under revision) showed that GRHL2 loss gives rise to reduced expression of E-cadherin protein in MCF7 cells [3]. However, our ChIP-seq data revealed that GRHL2 binding sites were not observed in the *CDH1* promoter region, consistent with other findings [3,19,20]. Moreover, we did not observe any downregulation of *CDH1* nascent RNA synthesis in the first 16 days after GRHL2 loss **(Fig. 3a and c)**, Together, these findings indicate that the *CDH1* gene is not a direct target for transcriptional

a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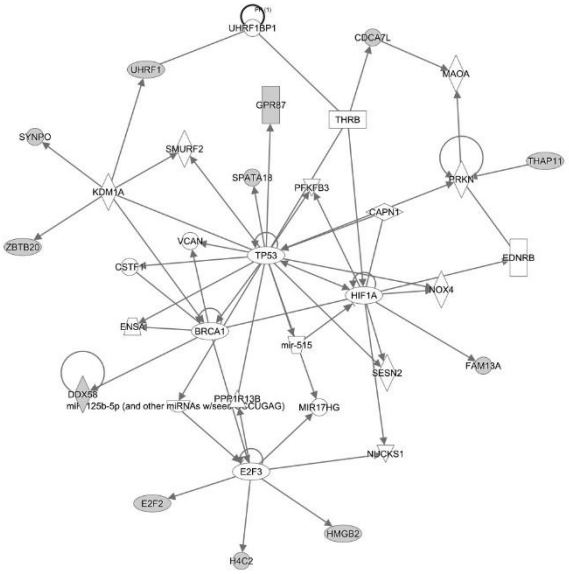
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CAPN1
ZBTB20
VCAN
TP53
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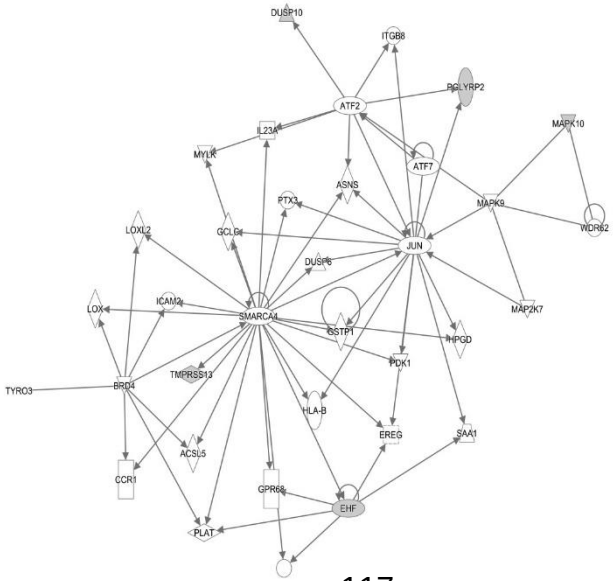
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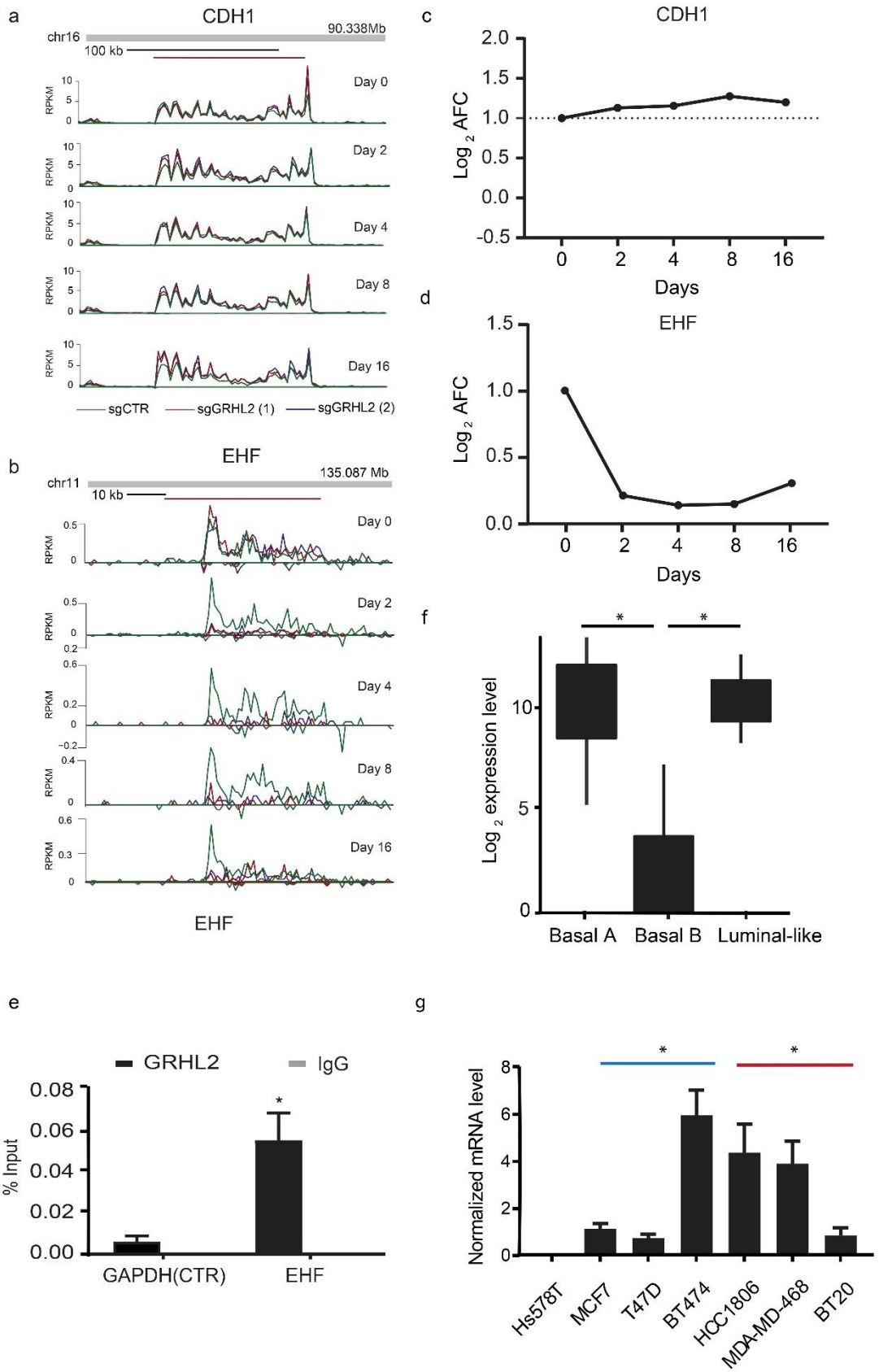
DUSP5
ICAM2
MAP2K7
LOX
SMARCA4
GSTP1
HPGD
PDK1
TMPRSS13
TYRO3
BRD4
HLA-B
EREG
SAA1
ACSL5
CCR1
GPR68
EHF
PLAT

(Last page) Fig. 2 Predicted networks based on direct GRHL2 target genes. **(a-c)** Top 3 networks with the highest scores predicted by IPA based on the 48 direct GRHL2 target genes. Single-way arrows indicate one gene regulating another, two-sided arrows indicate co-regulation, looped arrows indicate self-regulation.

regulation by GRHL2. Rather, *CDH1* RNA levels may be regulated indirectly through other transcriptional regulators [21] or by GRHL2-mediated post-transcriptional modification (e.g., miR200) [2,19,22] at later timepoints.

EHF is co-regulated with GRHL2

EHF was identified as a direct target of GRHL2 that was rapidly and continuously attenuated following GRHL2 loss **(Table S1; Fig. 3b and d)**. ChIP-PCR confirmed the interaction between GRHL2 and the promoter region of the *EHF* gene **(Fig. 3e)**. EHF is a member of the ETS transcription factor subfamily characterized by epithelial-specific expression [4]. Epithelial markers (e.g., GRHL2 and E-cadherin) are specifically reduced in basal B subtype breast cancer [1]. We examined whether *EHF* expression was also low in basal B versus other breast cancer subtypes. Indeed, RNA-seq data for a large panel of breast cancer cell lines showed loss of *EHF* in the basal B subtype **(Fig. 3f)** and qRT-PCR confirmed this finding in a smaller panel of breast cancer cell lines **(Fig. 3g)**.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Basal A
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Luminal-like
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GRHL2
IgG
% Input
GAPDH(CTR)
EHF
g
Normalized mRNA level
Hs578T
MCF7
T47D
BT474
HCC1806
MDA-MD-468
BT20

Fig. 3 EHF but not CDH1 is a direct GRHL2 target gene in luminal breast cancer. (a and b) Bru-seq reads for the *CDH1* and *EHF* gene at the indicated time points after GRHL2 loss. **(c and d)** Line graphs depicting the $\log_2$ AFC of *CDH1* and *EHF* transcription in MCF7 sgGRHL2 (1) and sgGRHL2 (2) cells at the indicated timepoints after doxycycline-induced GRHL2 deletion. The positive y-axis indicates the plus-strand signal of RNA synthesis from left to right and the negative y-axis represents the minus-strand signal of RNA synthesis from right to left. **(e)** ChIP-PCR showing enrichment of GRHL2 binding sites in *EHF* promoter region but not in the control *GAPDH* gene. Graph represents the efficiency of indicated genomic DNA co-precipitation with anti-GRHL2 Ab (black bars) or IgG control Ab (grey bars). Signals for IgG control and GRHL2 antibody pulldown samples are normalized to input DNA and are presented as % input with SEM from 3 technical replicates. Data are statistically analyzed by t-test and * indicates $p < 0.05$. **(f)** *EHF* mRNA expression in a panel of >50 human breast cancer cell lines covering luminal-, basal A-, and basal B-like subtypes extracted from RNA-seq data. Data is statistically analyzed by t-test and * indicates $p < 0.05$. **(g)** qRT-PCR validating downregulation of *EHF* mRNA in basal B-like subtype breast cancer. Blue and red lines represent luminal- and basal A-like subtypes of breast cancer, respectively. Data are presented as mean ± SEM from 2 biological replicates performed in triplicate. Normalized mRNA expression in each cell line is compared to the Hs578T basal B subtype cell line using t-test, * indicates $p < 0.05$.

EHF overexpression does not rescue proliferation in GRHL2 KO cells

We previously found that GRHL2 loss induced inhibition of cell proliferation accompanied by a downregulation of *EHF* in MCF7 cells (Wang et al, manuscript under revision). Our previous work, exploring only Bru-seq analysis, also showed that EHF was connected to cell cycle regulators such as E2F1 **(see Chapter 4 Fig. 3)** and EHF has been implicated in tumor initiation and tumorigenesis [5,23]. To determine whether GRHL2 loss inhibits cell proliferation through reduced expression of its direct target, *EHF*, we investigated the effect of ectopically overexpressed *EHF* on cell proliferation. As shown in **Fig. 4a~d**, overexpression of *EHF* did not rescue GRHL2 loss-induced inhibition of cell proliferation and cell survival. Taken together, these findings indicate that *EHF* is a direct GRHL2-target whose expression is reduced in absence of GRHL2 but is not solely responsible for the proliferation arrest caused by GRHL2 depletion in luminal breast cancer cells.

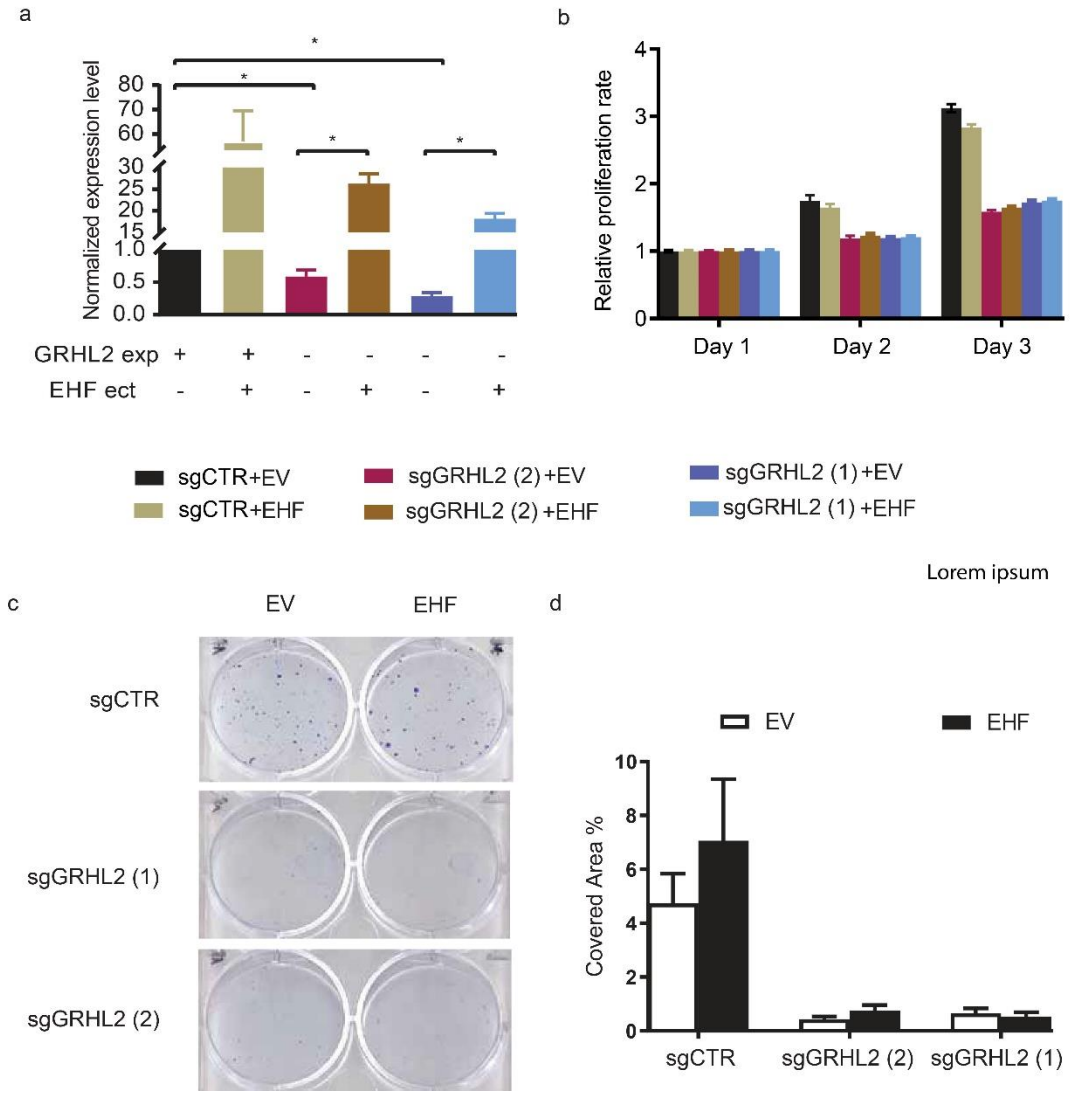

Fig. 4 Ectopic expression of EHF does not rescue proliferation upon GRHL2-loss. (a) Examination of expression level of *EHF* mRNA after 4 days of doxycycline treatment and transfection with *EHF* or EV plasmid by qRT-PCR in MCF7 cells transduced with sgCTR and sgGRHL2. Data are presented as mean ± SEM from three technical replicates. EV, empty vector; EHF, *EHF* cDNA plasmid. Data are statistically analyzed by t-test. * indicates $p < 0.05$. "GRHL2 exp" represents GRHL2 expression; "EHF ect" represents *EHF* ectopic overexpression. **(b)** Graph showing results from SRB assay after 4 days doxycycline-induction and transfection with *EHF*/EV plasmid and subsequent incubation for the indicated time periods. **(c,d)** Representative images of colony formation assay **(c)** and quantification of colony formation potential **(d)** for sgCTR and sgGRHL2 transduced MCF7 cells after 4 days doxycycline-induction and transfection with *EHF* or EV plasmid and subsequent incubation for the indicated time periods. Data are presented as mean ± SEM from 2 biological replicates performed in triplicate. Data are statistically analyzed by t-test.

Discussion

By integrating ChIP-seq analysis with Bru-seq analysis of a conditional KO model, we identify 48 high confidence direct target genes of GRHL2 in luminal breast cancer MCF7 cells. Of 48 direct target genes, 44 genes can encode proteins, whose interactions are investigated. The other 4 encode non-coding RNAs. Interestingly, in the signaling networks identified by IPA using differentially expressed genes identified from Bru-seq, the hub genes are not the direct target genes of GRHL2 **(see Chapter 4 Fig. 3)**. Rather, GRHL2-regulated genes interact with candidate hub genes providing entry points for GRHL2 regulation of these signaling networks. SAPK/JNK and CDK5 signaling pathways associated with cell proliferation are included in the top 10 canonical pathways identified by genes regulated by GRHL2 directly. In addition, the functions of direct GRHL2 target genes are mainly enriched in cell cycle, growth and proliferation. This indicates that in luminal breast cancer MCF7 cells GRHL2 acts predominantly as a regulator of cell proliferation, which is consistent with our previous findings (Wang et al, manuscript under revision).

Notably, we demonstrate that *CDH1* RNA synthesis is not altered following GRHL2 loss, which confirms and extends an earlier report that *CDH1* mRNA has no obvious changes in response to GRHL2 depletion [24]. Our Bru-seq result is in line with the fact that the *CDH1* gene is not identified as a GRHL2 target by ChIP-seq. Together, this demonstrates that E-cadherin downregulation must occur in an indirect manner in our luminal breast cancer model. *CDH1* was identified as a direct target gene of GRHL2 in normal epithelial [3] due to remodeling of the *CDH1* promoter caused by an interaction of GRHL2 at an intron of *CDH1*. However, there was no existence of GRHL2 binding sites in the promoter region of *CDH1*, which means GRHL2 has no direct contact with a

DNA consensus motif from -1000 bp to 100 bp from the transcription start site (TSS) of *CDH1* [3]. To date, ChIP-seq data reveals no GRHL2 binding in the *CDH1* promoter region in human prostate cancer cells [17], ovarian cancer cells [19] and murine kidney cells [15]. Taken together, the *CDH1* gene is not a direct target of GRHL2.

We establish *EHF* as a direct GRHL2 target gene whose expression, like GRHL2, is lost in the basal B subtype of breast cancer. EHF was previously implicated in cell proliferation [5,25,26]. On the other hand, EHF may act as a tumor suppressor, resulting from its role in controlling differentiation, the cancer stem-like phenotype [27] and transcriptional repression of genes positively regulated by MAP kinase signaling cascades [28]. The biological function of EHF may be cell context-dependent. In our study, inhibition of cell proliferation caused by GRHL2 loss in MCF7 cells is accompanied by reduced expression of *EHF* but ectopic expression of *EHF* is not sufficient to rescue proliferation triggered by GRHL2 loss. Altogether, these findings unravel multiple connections between GRHL2 and regulation of proliferation and point to cooperative roles of GRHL2 target genes (e.g., *EHF, E2F2* and *CDCA7L*) in sustaining proliferation.

Taken together, this study identifies direct target genes of GRHL2 and their related signaling pathways. It also explores the co-regulation and function of the epithelial factors GRHL2 and EHF in luminal-like breast cancer.

Acknowledgements

Zi Wang was supported by the China Scholarship Council. This work was supported by the Dutch Cancer Society (KWF Research Grant #10967).

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Table S1. GRHL2 direct target genes.

SPATA18	CADM1
FOXP2	WIPI1
ZBTB20	DUSP10
MIR5087	GPR87
THAP11	RND3
DDX11	TMEM140
PSMB6	SYNPO
UHRF1	MTUS2
TMPRSS13	KIAA0513
E2F2	KIF5C
HAX1	DDX58
EEF2	VMP1
HMGB2	LAMB3
TYRO3	FAM13A
EPN3	DAPP1
PPP1CA	PGLYRP2
RPL41	LINC00885
HIST1H4B	EHF
HIST1H4E	DDX12P
ANKRD1	NPY1R
GPR155	PRSS23
MAPK10	CORO2A
PRICKLE2-AS3	CADPS
LIMCH1	CDCA7L