



Universiteit
Leiden
The Netherlands

Exploring Grainyhead-like 2 target genes in breast cancer

Wang, Z.

Citation

Wang, Z. (2020, October 6). *Exploring Grainyhead-like 2 target genes in breast cancer*. Retrieved from <https://hdl.handle.net/1887/137309>

Version: Publisher's Version

License: [Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden](#)

Downloaded from: <https://hdl.handle.net/1887/137309>

Note: To cite this publication please use the final published version (if applicable).

Cover Page



Universiteit Leiden



The handle <http://hdl.handle.net/1887/137309> holds various files of this Leiden University dissertation.

Author: Wang, Z.

Title: Exploring Grainyhead-like 2 target genes in breast cancer

Issue date: 2020-10-06

Chapter 4

Dynamic changes in nascent RNA after GRHL2 loss in luminal-like breast cancer

Zi Wang¹, Yao-Jun Chen¹, Mats Ljungman², Erik HJ Danen^{1,3}

¹Leiden Academic Center for Drug Research, Leiden University, Leiden, The Netherlands; ²University of Michigan, Ann Arbor, MI 48109; ³correspondence to Erik HJ Danen, e.danen@lacdr.leidenuniv.nl

Abstract

GRHL2 drives expression of key epithelial genes and supports proliferation, survival, and epithelial differentiation. It plays a dual role in cancer by stimulating proliferation and suppressing EMT. GRHL2 has been reported to act as a transcription factor as well as a modulator of gene expression through epigenetic mechanisms. The relevant genetic programs controlled by GRHL2 in cancer are not resolved. In the present study, the response to GRHL2 loss in luminal breast cancer cells was studied by combining an MCF7 conditional knockout model with Bru-seq analysis. The rate of RNA synthesis of 264 and 244 genes was upregulated or downregulated, respectively, for at least one out of four time points following GRHL2 loss ranging from 1-16 days. Five dynamic response patterns were characterized and GRHL2-controlled canonical pathways and signaling networks were identified. Collectively, this study characterizes patterns of RNA synthesis regulated by GRHL2 and identifies signaling pathways regulated by GRHL2.

Introduction

GRHL2 is a mammalian homolog of the *Drosophila* Grainyhead gene. GRHL2 has a crucial role in neural tube closure, epithelial cell morphology, cancer cell proliferation and migration ¹⁻³. It is widely accepted that GRHL2 has dual roles in cancer development ^{4,5}. GRHL2 can inhibit epithelial to mesenchymal transition (EMT) by upregulating E-cadherin and Claudin4 ⁶ and downregulating ZEB1 ^{7,8}. On the other hand, GRHL2 is frequently overexpressed or amplified in breast cancer ⁹, lung cancer ¹⁰, and ovarian cancer ¹¹ and high expression of GRHL2 was associated with histological differentiation and lymphatic metastasis in pancreatic carcinoma ¹².

The relevant genetic programs controlled by GRHL2 in cancer are not resolved. GRHL2 has been reported to act as a transcription factor as well as a modulator of gene expression through epigenetic mechanisms^{13,14}. Gene regulation includes transcriptional initiation, RNA processing, post-transcriptional modification, translation and post-translational modification. Conventional RNA-seq is used for analysis of steady-state RNA levels whereas bromouridine sequencing (Bru-seq) measures nascent RNA, allowing for direct assessment of changes in DNA transcription^{15,16}. Bru is relatively non-toxic as compared to other ribonucleotide analogs and is widely used to label nascent RNA in vitro and in cells^{17,18 19}.

In this study, we used Bru-seq to investigate genome-wide dynamic changes of nascent RNA induced by GRHL2 loss in an MCF7 conditional knockout model. Following identification of differentially expressed genes in response to GRHL2 loss, bioinformatics analysis was performed to predict signaling networks regulated by GRHL2. Thus, GRHL2-controlled gene networks were unraveled.

Materials and methods

Cell culture and lentiviral transduction

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% CO₂. For production of lentiviral particles, VSV, GAG, REV and Cas9 or single guide (sg)RNA plasmids were transfected into HEK293 cells using Polyethylenimine (PEI). After 2 days, lentiviral particles were harvested and filtered. Conditional Cas9 cells were generated by infecting parental cells with lentiviral particles expressing the Edit-R Tre3G promoter-driven Cas9 (Dharmacon) and selected by blasticidin. Limited dilution was used to generate

Cas9 monoclonal cells. Subsequently, Cas9-monoclonal cells were transduced with U6-gRNA:hPGK-puro-2A-tBFP control non-targeting sgRNAs or GRHL2-specific sgRNAs (Sigma) and selected by puromycin.

Western blot

Cells were lysed by radioimmunoprecipitation (RIPA) buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate and 0.1% Tris and 1% protease cocktail inhibitor (Sigma-Aldrich. P8340)). Lysates were sonicated and protein concentration was determined by bicinchoninic acid assay (BCA) assay. Cell lysates were mixed with protein loading buffer, separated by SDS-PAGE, and transferred to a methanol-activated polyvinylidene difluoride (PVDF) membrane (Milipore, The Netherlands). The membrane was blocked with 5% bovine serum albumin (BSA; Sigma-Aldrich) for 1 hour at room temperature (RT). Next, membranes were stained with primary antibody overnight at 4°C and HRP-conjugated secondary antibodies for half hour at room temperature (RT). After staining with Prime ECL Detection Reagent (GE Healthcare Life science), chemoluminescence was detected with an Amersham Imager 600 (GE Healthcare Life science, The Netherlands). The following antibodies were used: GRHL2 (Atlas-Antibodies, hpa004820) Cas9 (Cell Signaling, 14697), and GAPDH (SantaCruz, sc-32233).

Bru-seq

At different timepoints after doxycycline-induced deletion of GRHL2, cells were incubated with a final concentration of 2 mM Bru at 37°C for 30 minutes. Cells were lysed in TRIzol reagent (Sigma) and Bru-labelled nascent RNA was isolated using an anti-BrdU antibody conjugated to magnetic beads ¹⁵. Subsequently, cDNA libraries were generated using the Illumina TruSeq library

kit and sequenced using the Illumina NovaSeq 6000 Sequencing System. Sequencing and read mapping were carried out as previously described^{15,20}

Bioinformatics analysis

To identify GRHL2-regulated genes, an inter-sample comparison analysis was performed comparing RPKM (reads per kilobase per million mapped reads) for each gene in the doxycycline-treated samples compared to the untreated sample, to obtain fold-change (FC) and *p* values. Genes with *p*<0.05 and FC>2 or FC<0.5 in any of the doxycycline-treated samples relative to untreated cells were filtered. Subsequently, genes responding to Cas9 induction in the context of sgGRHL2 (1) as well as sgGRHL2 (2) were selected and genes responding also in the context of sgCTR were eliminated from this list. Canonical pathways and networks analysis was performed with the Ingenuity Pathways Analysis (IPA) software (Ingenuity Systems, USA). A heat map was generated by R. The Database for Annotation, Visualization, and Integrated Discovery (DAVID)^{21,22} was utilized to identify signaling pathways associated with GRHL2 loss. Gene Ontology (GO) terms (biological process, cellular component and protein class) analysis was performed by Protein Analysis Through Evolutionary Relationships (PANTHER) database²³.

ChIP-PCR

Chromatin preparation was described previously²⁴. 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: E2F2 forward, tctggaagaggaatgatg; E2F2 reverse, caggcagcttgggagagtag; CDCA7L forward, ttggggctgttttgttt; CDCA7L reverse: ggtgtggaggcctactgtgt; control (an intergenic region upstream of the GAPDH locus) forward, atgggtgccactgggatct; control reverse, tgccaaagcctaggggaaga. ChIP-PCR data were analyzed using the $2^{-\Delta\Delta Ct}$ method ²⁵.

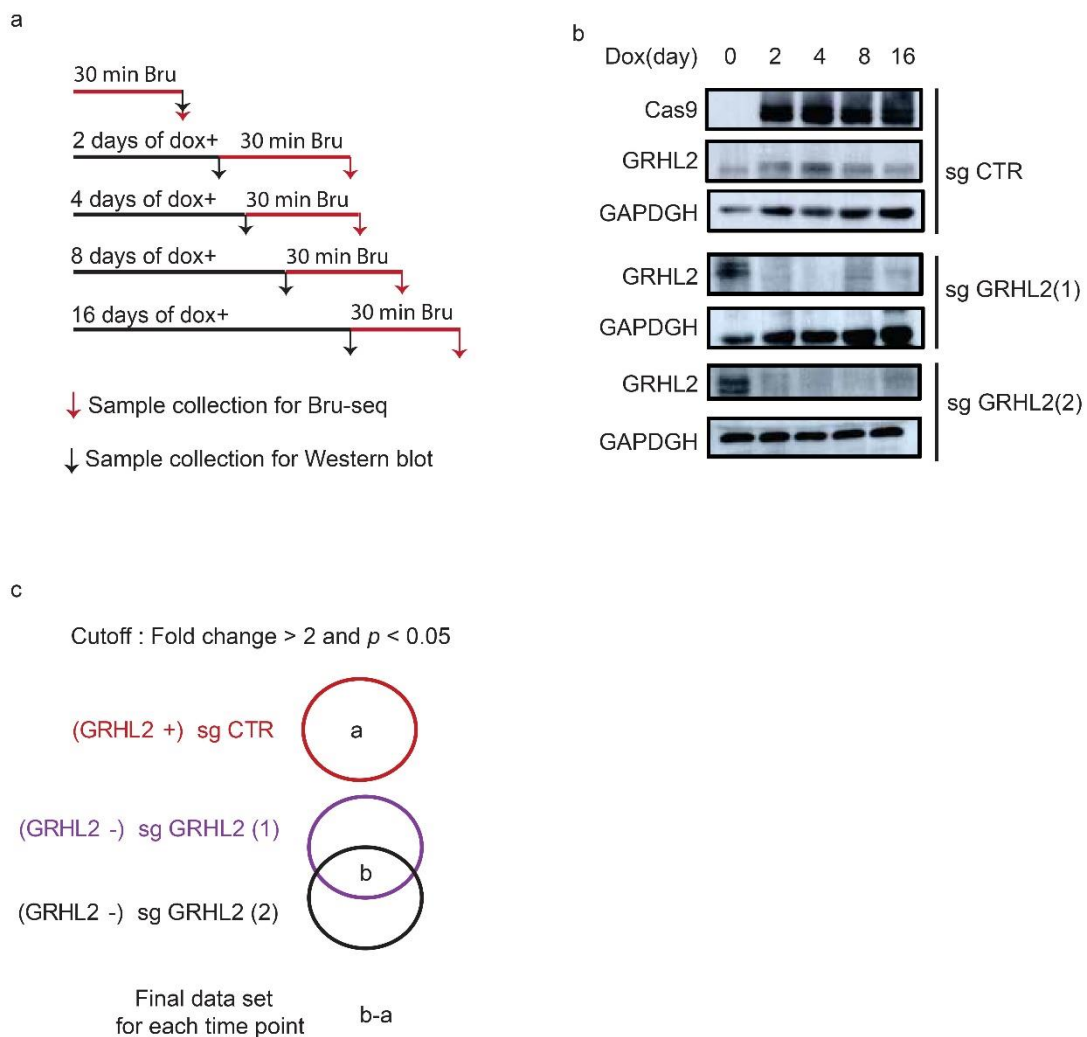


Fig. 1 Bru-seq sample preparation and Bru-seq data analysis strategy. (a) Bromouridine (Bru) labeling of nascent RNA was carried out for 30 minutes at the indicated time points after doxycycline (dox)-induced GRHL2 deletion. **(b)** Western blot analysis of GRHL2 expression levels at the indicated time points in sgCTR and sgGRHL2 transduced MCF7 cells. GAPDH serves as loading control. **(c)** Strategy for Bru-

seq data analysis. Each circle represents a gene set with differential transcription relative to the condition where no doxycycline was added.

Results

Dynamic regulation of RNA synthesis in response to GRHL2 loss

Using conditional CRISPR-Cas9 MCF7 cells, Bru-seq was carried out to investigate the dynamic changes in DNA transcription triggered by GRHL2 loss. At 0, 2, 4, 8, or 16 days after doxycycline-induced GRHL2 knockout, cells were incubated with Bru for 30 minutes to label nascent RNA (Fig. 1a) or they were analyzed by Western blot to examine the expression of GRHL2 protein (Fig. 1b).

To identify GRHL2-regulated genes, for each time point, the log₂ average fold change (AFC) of transcription induced by doxycycline treatment in the two sgGRHL2 and the control sgRNA sample was determined. A list of genes was generated whose transcription was altered in both sgGRHL2 samples ($FC > 2$; $p < 0.05$ or $FC < 0.5$; $p < 0.05$) but not in the sgCTR sample (Fig. 1c). Using these criteria, 264 genes were upregulated and 244 genes were downregulated in at least one time point after GRHL2 loss (Table S1).

Distinct dynamic patterns of response to GRHL2 depletion

GRHL2-regulated genes were clustered in a heat map using the AFC at each time point in sgGRHL2 (1) and sgGRHL2 (2) cells (Fig. 2a). In response to GRHL2 loss, one cluster of genes exhibited rapid and continuing upregulation in RNA synthesis. For instance, *LAMB3* encoding the $\beta 2$ unit of the trimeric basement membrane protein laminin-332²⁶ was rapidly induced after GRHL2 loss (Fig. 2b). Another cluster showed rapid and sustained downregulation of RNA synthesis following GRHL2 deletion. This cluster included *UBB*, encoding

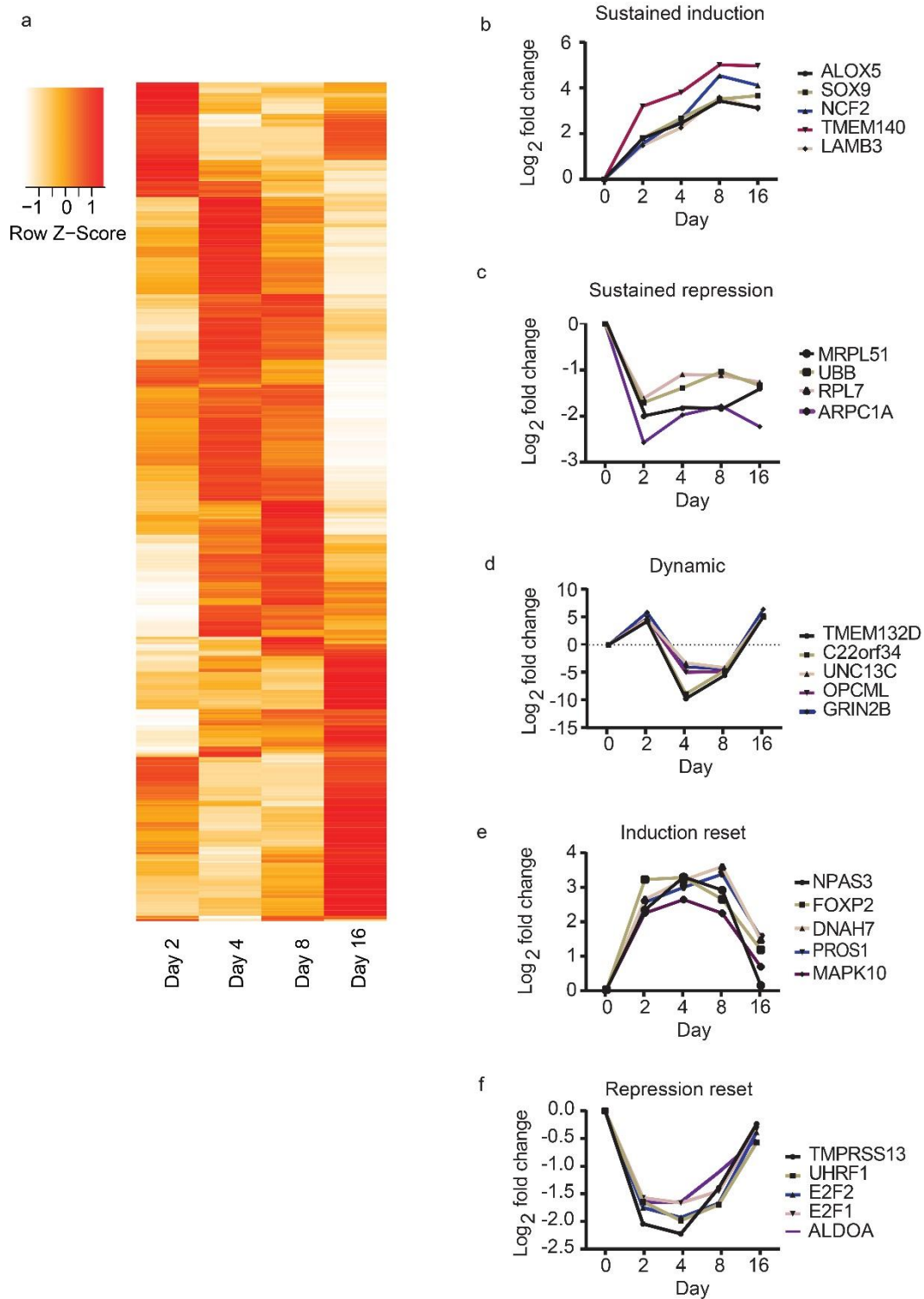


Fig. 2 Dynamic changes in RNA synthesis following GRHL2 loss. (a) Heat map for GRHL2 loss response genes. **(b-f)** After GRHL2 abrogation, genes are categorized according to RNA synthesis patterns. The line graph depicts the log₂ average fold change (AFC) of transcription in sgGRHL2 (1) and sgGRHL2 (2) cells. **Dynamic:** genes

with $AFC > 2$; $p < 0.05$ at some and $AFC < 0.5$; $p < 0.05$ at other time points. **Sustained induction:** genes with $AFC > 2$; $p < 0.05$ at all time points. **Sustained repression:** genes with $AFC < 0.5$; $p < 0.05$ at all time points. **Induction reset:** genes with $AFC > 2$ at early time points followed by a return to $1 < AFC < 2$ at day 16. **Repression reset:** genes with $AFC < 0.5$ at early time points followed by a return to $0.5 < AFC < 1$ at day 16.

the highly conserved ubiquitin protein that is involved in the regulation of protein degradation, signaling, and gene expression ²⁷. The downregulation in RNA synthesis of *UBB* was observed at each labeling period (Fig. 2c). Another cluster of genes displayed a dynamic transcriptional response following GRHL2 loss. For example, GRHL2 loss enhanced transcription of the *GRIN2B* gene (encoding GluN2B, a subunit of NMDA-type glutamate-gated ion channels ²⁸) within 2 days, followed by a repression at day 4 and 8, and followed by another peak of enhanced transcription at day 16 after GRHL2 deletion (Fig. 2d). The “induction reset” cluster included genes whose transcription was transiently induced initially followed a repression phase where transcription returned to baseline. This cluster included *FOXP2* encoding a forkhead transcription factor (Fig. 2e). The “repression reset” cluster showed an opposite pattern with an initial repression that returned to baseline at later timepoints, and included the *E2F1* gene encoding the E2F1 transcription factor involved in cell survival and proliferation ²⁹ (Fig. 2f).

Predicted signaling networks regulated by GRHL2

IPA software was utilized to elucidate GRHL2-regulated signaling pathways and networks from the differently expressed genes at the different time points following GRHL2 depletion. Canonical pathway results predicted changes in Granzyme A signaling, remodeling of epithelial adherens junctions, mTOR signaling, and DNA methylation and transcriptional repression signaling at each time point after GRHL2 loss (Table 1). At early time points (day 2, 4 and 8), significantly enriched canonical pathways included EIF2 signaling, germ cell-

sertoli cell junction signaling, pancreatic adenocarcinoma signaling, and a transcriptional regulatory network in embryonic stem cells (Table 1).

Next, using IPA, networks associated with multiple biological functions and diseases were identified and ranked according to the score. The top 10 networks scored >22, indicating that the likelihood that genes in these networks were not connected was $<10^{-22}$ (Table 2). Overall, networks associated with

Table 1. Top 10 canonical pathways responding to GRHL2 loss at the indicated time points generated by IPA.

| Canonical pathway | -log(p-value) | Downregulated | Upregulated |
|--|---------------|---------------|-------------|
| Day 2 | | | |
| EIF2 Signaling | 6.33 | 16/224 (7%) | 0/224 (0%) |
| Granzyme A Signaling | 4.96 | 5/20 (25%) | 0/20 (0%) |
| Remodeling of Epithelial Adherens Junctions | 4.95 | 7/69 (10%) | 1/69 (1%) |
| DNA Methylation and Transcriptional Repression Signaling | 4.91 | 6/34 (18%) | 0/34 (0%) |
| Germ Cell-Sertoli Cell Junction Signaling | 4.10 | 7/172 (4%) | 4/172 (2%) |
| Transcriptional Regulatory Network in Embryonic Stem Cells | 3.74 | 6/54 (11%) | 0/54 (0%) |
| Pancreatic Adenocarcinoma Signaling | 3.53 | 3/109 (3%) | 5/109 (5%) |
| Epithelial Adherens Junction Signaling | 3.19 | 7/153 (5%) | 2/153 (1%) |
| Signaling by Rho Family GTPases | 2.82 | 5/243 (2%) | 6/243 (2%) |
| mTOR Signaling | 2.77 | 6/210 (3%) | 4/210 (2%) |
| Day 4 | | | |
| EIF2 Signaling | 5.77 | 16/224 (7%) | 0/224 (0%) |
| Remodeling of Epithelial Adherens Junctions | 5.59 | 6/69 (9%) | 3/69 (4%) |
| Granzyme A Signaling | 4.75 | 5/20 (25%) | 0/20 (0%) |
| DNA Methylation and Transcriptional Repression Signaling | 4.67 | 6/34 (18%) | 0/34 (0%) |
| Transcriptional Regulatory Network in Embryonic Stem Cells | 3.51 | 6/54 (11%) | 0/54 (0%) |
| Pancreatic Adenocarcinoma Signaling | 3.24 | 3/109 (3%) | 5/109 (5%) |
| Germ Cell-Sertoli Cell Junction Signaling | 3.11 | 6/172 (3%) | 4/172 (2%) |
| Breast Cancer Regulation by Stathmin1 | 2.62 | 7/200 (4%) | 3/200 (2%) |
| Inhibition of Angiogenesis by TSP1 | 2.59 | 1/34 (3%) | 3/34 (9%) |
| mTOR Signaling | 2.47 | 6/210 (3%) | 4/210 (2%) |
| Day 8 | | | |
| Remodeling of Epithelial Adherens Junctions | 5.60 | 6/69 (9%) | 3/69 (4%) |
| EIF2 Signaling | 5.12 | 15/224 (7%) | 0/224 (0%) |
| Granzyme A Signaling | 4.75 | 5/20 (25%) | 0/20 (0%) |
| DNA Methylation and Transcriptional Repression Signaling | 4.67 | 6/34 (18%) | 0/34 (0%) |
| Transcriptional Regulatory Network in Embryonic Stem Cells | 3.52 | 6/54 (11%) | 0/54 (0%) |
| Pancreatic Adenocarcinoma Signaling | 3.25 | 3/109 (3%) | 5/109 (5%) |
| Germ Cell-Sertoli Cell Junction Signaling | 3.12 | 6/172 (3%) | 4/172 (2%) |
| Inhibition of Angiogenesis by TSP1 | 2.59 | 1/34 (3%) | 3/34 (9%) |
| Synaptogenesis Signaling Pathway | 2.54 | 6/312 (2%) | 7/312 (2%) |
| mTOR Signaling | 2.47 | 6/210 (3%) | 4/210 (2%) |
| Day 16 | | | |
| Granzyme A Signaling | 5.68 | 5/20 (25%) | 0/20 (0%) |
| mTOR Signaling | 3.26 | 5/210 (2%) | 4/210 (2%) |
| Synaptogenesis Signaling Pathway | 3.14 | 2/312 (1%) | 9/312 (3%) |
| Remodeling of Epithelial Adherens Junctions | 3.01 | 3/69 (4%) | 2/69 (3%) |
| GP6 Signaling Pathway | 2.69 | 0/119 (0%) | 6/119 (5%) |
| DNA Methylation and Transcriptional Repression Signaling | 2.21 | 3/34 (9%) | 0/34 (0%) |
| Opioid Signaling Pathway | 2.18 | 0/250 (0%) | 8/250 (3%) |
| Axonal Guidance Signaling | 2.10 | 2/486 (0%) | 10/486 (2%) |
| T Helper Cell Differentiation | 2.06 | 0/73 (0%) | 4/73 (5%) |
| Glioma Invasiveness Signaling | 2.04 | 1/74 (1%) | 3/74 (4%) |

diseases including cancer, networks associated with cell cycle, DNA, and RNA regulation, and networks associated with cell-to-cell signaling and interaction

were predicted at most timepoints tested. At day 2, the most enriched networks were associated with cancer, protein synthesis and RNA damage and repair. At day 4, the top enriched networks were linked to developmental disorder, embryonic development and organismal development, in which *AURKB* and *E2F1* represented core genes that were most interconnected with other genes (Fig. 3b). The top enriched networks at day 8 were associated with cell cycle, cellular assembly, DNA replication, recombination and repair. At day 16, the top molecular networks predicted by IPA were closely related to cell to cell signaling and interaction, nervous system development, RNA damage and repair (Fig. 3d).

Table 2. Top 10 networks responding to GRHL2 loss at the indicated time points generated by IPA.

| Top Diseases and Functions | Score | Focus Molecules |
|---|-------|-----------------|
| Day 2 | | |
| Cancer, Protein Synthesis, RNA Damage and Repair | 62 | 33 |
| Cellular Assembly and Organization, Developmental Disorder, DNA Replication, Recombination, and Repair | 59 | 32 |
| Cancer, Organismal Injury and Abnormalities, Respiratory Disease | 59 | 32 |
| Connective Tissue Disorders, Developmental Disorder, Hereditary Disorder | 48 | 28 |
| Cellular Development, Connective Tissue Development and Function, Tissue Development | 34 | 22 |
| Cell Cycle, Hereditary Disorder, Organismal Injury and Abnormalities | 30 | 20 |
| Cell Death and Survival, Neurological Disease, Organismal Injury and Abnormalities | 26 | 18 |
| Cell Cycle, Cellular Assembly and Organization, DNA Replication, Recombination, and Repair | 24 | 17 |
| Cell-To-Cell Signaling and Interaction, Cellular Assembly and Organization, Cellular Function and Maintenance | 24 | 17 |
| Endocrine System Development and Function, Protein Synthesis, Small Molecule Biochemistry | 22 | 16 |
| Day 4 | | |
| Developmental Disorder, Embryonic Development, Organismal Development | 67 | 35 |
| Cellular Assembly and Organization, DNA Replication, Recombination, and Repair, Post-Translational Modification | 58 | 32 |
| Cancer, Protein Synthesis, RNA Damage and Repair | 55 | 31 |
| Cellular Assembly and Organization, Cellular Function and Maintenance, Hematological Disease | 55 | 31 |
| Developmental Disorder, Hereditary Disorder, Metabolic Disease | 49 | 29 |
| Cell Death and Survival, Cellular Movement, Post-Translational Modification | 37 | 24 |
| Cell Cycle, Cellular Assembly and Organization, DNA Replication, Recombination, and Repair | 37 | 24 |
| Cellular Compromise, Energy Production, Nucleic Acid Metabolism | 31 | 21 |
| Connective Tissue Disorders, Dermatological Diseases and Conditions, Developmental Disorder | 31 | 21 |
| Cell-To-Cell Signaling and Interaction, Cellular Assembly and Organization, Nervous System Development and Function | 25 | 18 |

| | | |
|---|----|----|
| Day 8 | | |
| Cell Cycle, Cellular Assembly and Organization, DNA Replication, Recombination, and Repair | 64 | 34 |
| Cancer, Protein Synthesis, RNA Damage and Repair | 58 | 32 |
| Nervous System Development and Function, Neurological Disease, Organ Morphology | 58 | 32 |
| Cancer, Hematological Disease, Immunological Disease | 52 | 30 |
| Energy Production, Nucleic Acid Metabolism, Small Molecule Biochemistry | 42 | 26 |
| Cellular Assembly and Organization, DNA Replication, Recombination, and Repair, Post-Translational Modification | 33 | 22 |
| Connective Tissue Disorders, Dermatological Diseases and Conditions, Developmental Disorder | 31 | 21 |
| Cancer, Hematological Disease, Organismal Injury and Abnormalities | 31 | 21 |
| Cell Cycle, Cellular Assembly and Organization, DNA Replication, Recombination, and Repair | 29 | 20 |
| Cardiovascular System Development and Function, Cell Morphology, Organ Development | 25 | 18 |
| Day 16 | | |
| Cell-To-Cell Signaling and Interaction, Nervous System Development and Function, RNA Damage and Repair | 58 | 30 |
| Cellular Development, Cellular Growth and Proliferation, Embryonic Development | 45 | 25 |
| Connective Tissue Disorders, Dermatological Diseases and Conditions, Developmental Disorder | 37 | 22 |
| Gastrointestinal Disease, Inflammatory Disease, Inflammatory Response | 33 | 20 |
| Carbohydrate Metabolism, Lipid Metabolism, Small Molecule Biochemistry | 31 | 19 |
| Cardiovascular Disease, Cellular Assembly and Organization, DNA Replication, Recombination, and Repair | 26 | 17 |
| Cancer, Organismal Injury and Abnormalities, Reproductive System Disease | 26 | 17 |
| Cell Cycle, Protein Synthesis, RNA Damage and Repair | 24 | 16 |
| Cancer, Organismal Injury and Abnormalities, Reproductive System Disease | 24 | 16 |
| Hereditary Disorder, Neurological Disease, Organismal Injury and Abnormalities | 22 | 15 |

Predicted biological processes and functional pathways regulated by GRHL2

The PANTHER classification system was utilized to identify biological process, cellular component and protein classification predicted to be associated with the genes regulated in response to GRHL2 depletion. Biological processes at all 4 timepoints included cellular process, metabolic process, and biological regulation (Fig. 4a). For cellular component, differentially transcribed genes induced by GRHL2 loss were predominantly involved in organelle, extracellular region, protein-containing complex, membrane and cell junction for each time point (Fig. 4b). In terms of protein classification, genes transcriptionally affected by GRHL2 depletion were enriched in nucleic acid binding, hydrolase, transcription factor, signaling molecule and enzyme modulator for all time points (Fig. 4c). Subsequently, DAVID was utilized to investigate whether GRHL2-regulated genes identified by Bru-seq were enriched for known functional pathways. At each time point, GRHL2-regulated genes were significantly enriched in pathways associated with cancer, focal adhesion and ECM receptor interaction, and several signaling pathways (Fig. 5a). DAVID also identified enrichment of signaling pathways including those involved in viral carcinogenesis, ribosome, alcoholism and systemic lupus erythematosus

signaling at each time point whereas pathways involved in biosynthesis of amino acids, carbon metabolism, transcriptional misregulation in cancer, DNA replication and cell cycle signaling were identified at early timepoints (Fig. 5b).

We and others have reported that GRHL2 loss is associated with growth arrest^{11,30}. Consistent with this notion, the RNA synthesis rate of several genes involved in cell cycle progression and DNA replication were rapidly suppressed in response to GRHL2 loss (i.e., *E2F2*, *CDCA7L*, *SFN* and *MCM2*)³¹⁻³⁴ (Fig. 6a-d). Our previous ChIP-seq data revealed that GRHL2 binding sites were observed at the promoter regions of *E2F2* and *CDCA7L*²⁴ and this finding was corroborated by ChIP-PCR analysis (Fig. 6e). These results suggested that *E2F2* and *CDCA7L* are directly regulated by GRHL2 and inhibition of cell proliferation mediated by GRHL2 loss may be associated with repression of *E2F2* and/or *CDCA7L*.

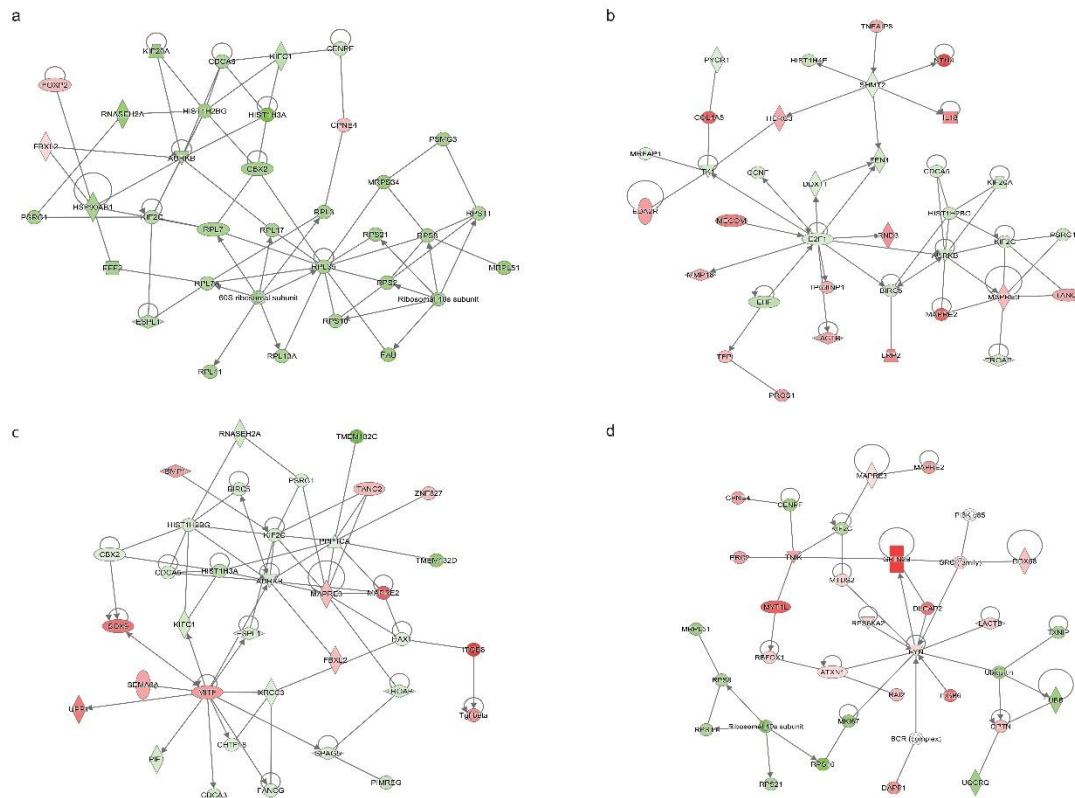


Fig. 3 Networks with the highest scores according to differentially transcribed genes after GRHL2 loss by IPA. (a-d) Networks for day 2, 4, 8 and 16 respectively. The

intensity of the node color indicates up- (red) and down regulation (green). Single-way arrows indicate one gene regulating another, two-sided arrows indicate co-regulation, looped arrows indicate self-regulation.

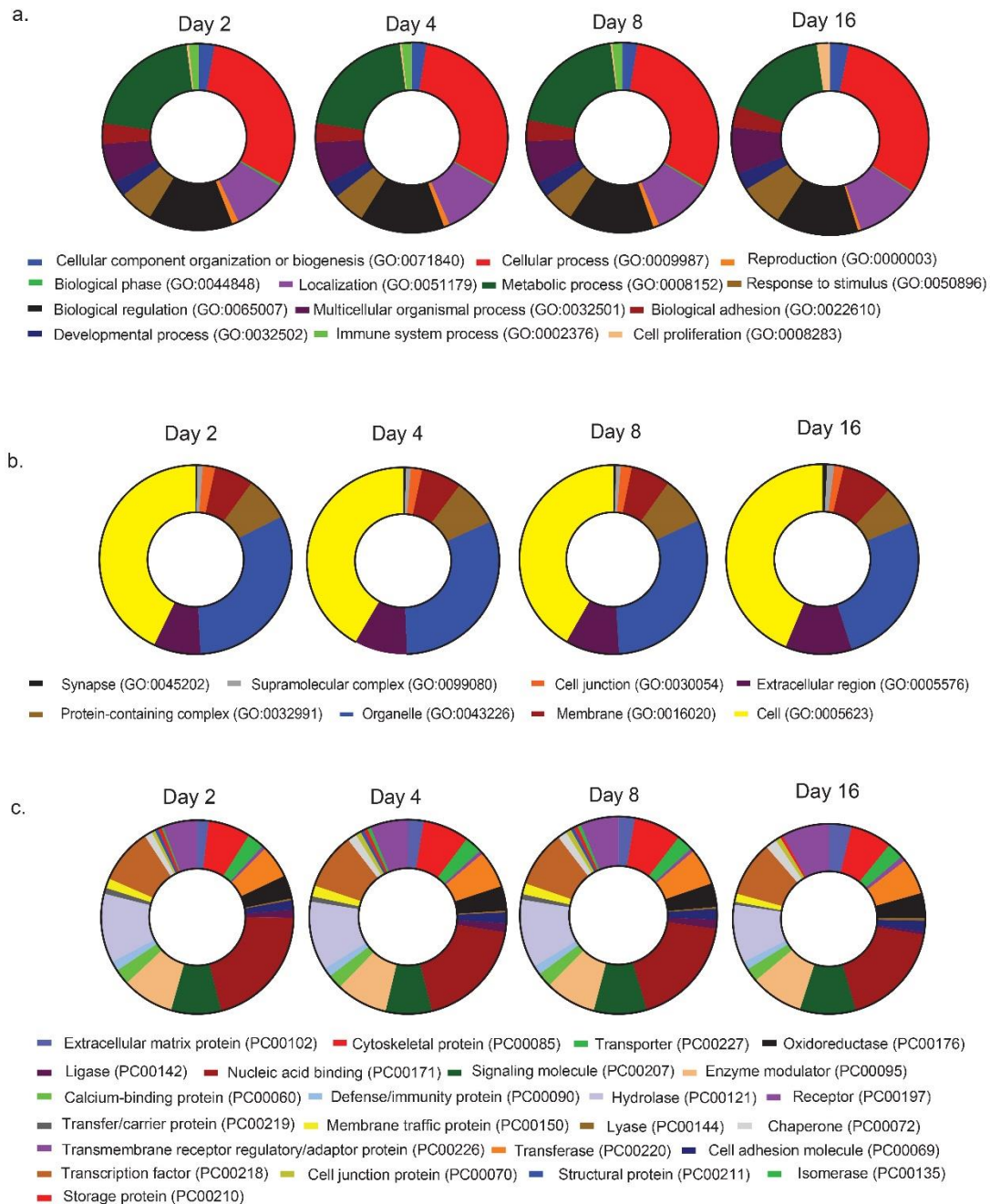
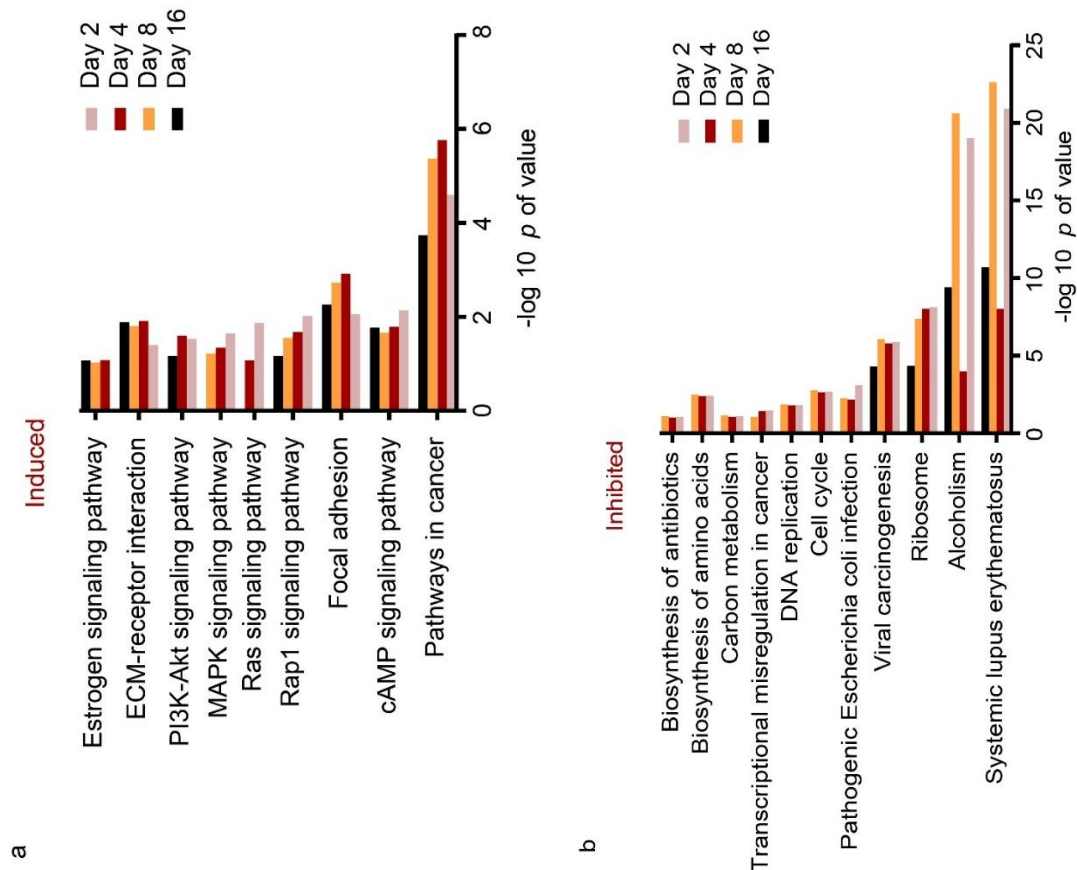


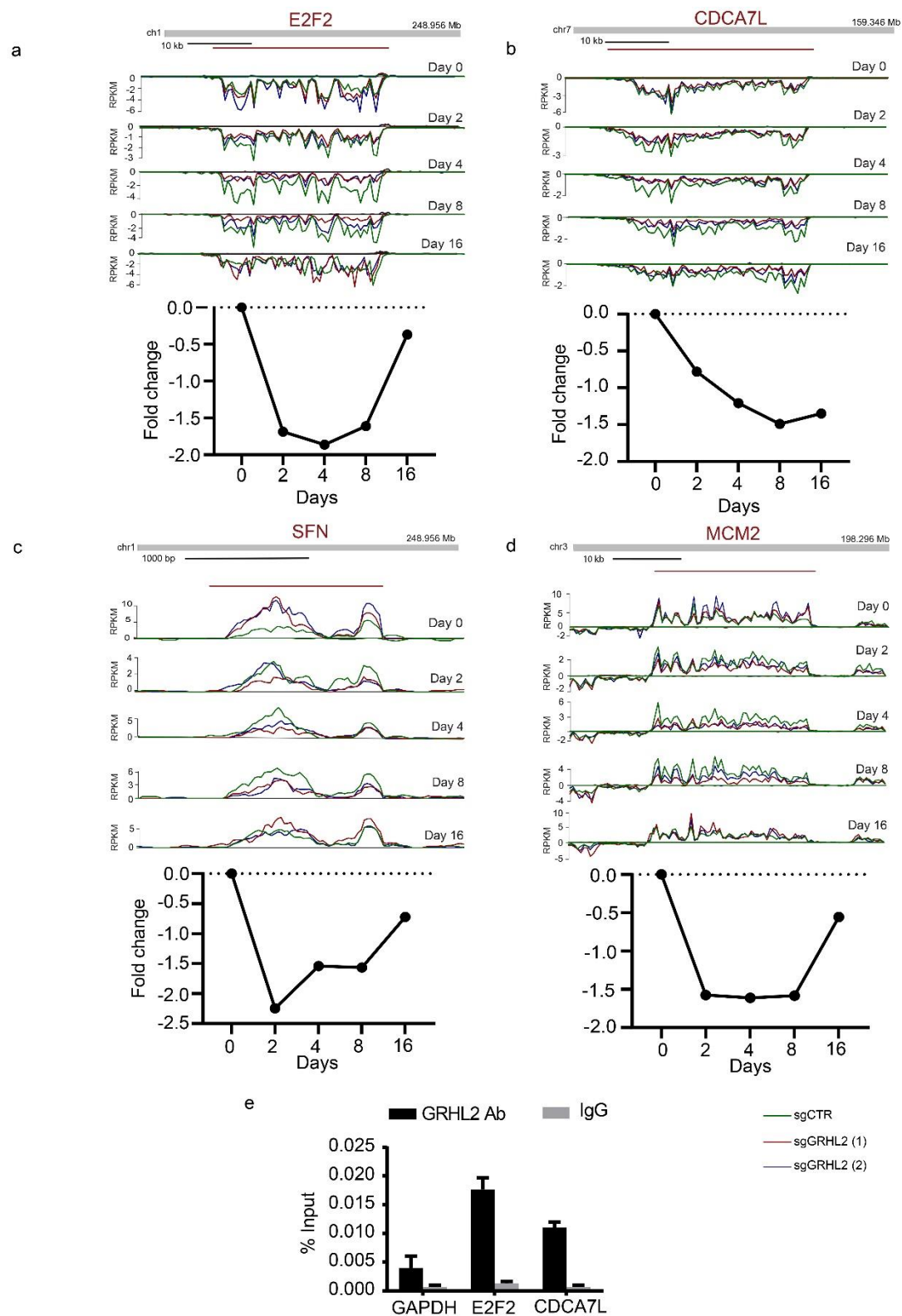
Fig. 4 PANTHER gene ontology enrichment analysis of differentially transcribed genes after GRHL2 loss. Enrichment analyses were carried out for biological process (a), cellular component (b) and protein classification (c).

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 phenotype³⁵. *CDH1* has been proposed to represent a direct target gene of GRHL2^{8,36,37}. Other studies^{2,8,38}, 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 cells⁸. However, our previous ChIP-seq data²⁴ revealed that GRHL2 binding sites were not observed at the *CDH1* promoter region, consistent with other findings^{8,13,39}. Moreover, we did not observe any downregulation of *CDH1* nascent RNA synthesis in the first 16 days after GRHL2 loss (Fig. 8a and b). Together, these findings indicate that the *CDH1* gene is not a direct target for transcriptional regulation by GRHL2. Rather, *CDH1* may be regulated indirectly through other transcriptional regulators⁴⁰ or by GRHL2-mediated post-transcriptional modification (e.g., miR200)^{7,13,41} at later timepoints.



(Last page) Fig. 5 DAVID analysis of differentially transcribed genes after GRHL2 loss. Enriched pathways identified by DAVID for induced (a) and suppressed (b) gene sets at indicated time points.



(Last page) Fig. 6 Downregulation of RNA synthesis for genes involved in cell cycle progression after GRHL2 loss. (a-d) Top: Bru-seq reads for indicated genes at indicated time point in response to GRHL2 deletion. **Bottom:** Line graphs depicting the \log_2 AFC of transcription in sgGRHL2 (1) and sgGRHL2 (2) cells. 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)** Validation of interaction of GRHL2 binding sites with the promoter regions of indicated genes by CHIP-PCR. 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 $p < 0.05$.

Discussion

The expression level of individual mRNAs is determined by the RNA synthesis and degradation rates. Characterization of global RNA dynamics provides insight into mechanisms of cell signaling⁴². In this study, we examined genome-wide time-resolved responses of RNA synthesis after GRHL2 loss in luminal-like breast cancer cells. We used Bru-seq to capture changes in RNA synthesis¹⁶ in a conditional GRHL2 knockout model. We identified 264 induced and 244 repressed genes in at least one time point following GRHL2 loss. These genes exhibit diverse patterns of RNA synthesis that are divided into sustained induction, sustained repression, induction reset, dynamic and repression reset. Genes with similar patterns of RNA synthesis may be regulated by similar means and the fact that patterns of transcription induction are similar to the patterns of transcription repression, suggests that transcriptional induction and repression may involve similar mechanisms¹⁶.

Bioinformatics analysis identifies several signaling pathways that are enriched at each time point analyzed after GRHL2 deletion (i.e., Granzyme A signaling, remodeling of epithelial adherens junctions, mTOR signaling and DNA methylation, and transcriptional repression signaling). Granzyme A induces caspase-independent apoptosis by dysregulation of mitochondrial metabolism

and generation of reactive oxygen species (ROS) in the mitochondrion ⁴³. Some repressed genes caused by GRHL2 loss (i.e., *HIST1H1C*, *HIST1H1D*, *HIST1H1E*, *HMGB2*, and *NME1*) are linked to Granzyme A signaling, of which HMGB2 is a positive regulator of proliferation and negative mediator of apoptosis ^{44,45}. The adherens junctions are specialized structures that encircle epithelial cells and maintain the architectural integrity of epithelial tissues ^{46,47}. A total of 11 genes, including *HIST1H1C*, *HIST1H1D*, *HIST1H1E*, *HMGB2ACTB*, *ACTG1*, *ARPC1A*, *MAPRE2*, *NME1*, *TUBA1B*, *TUBB*, and *TUBB4B*, are identified to be involved in remodeling of epithelial adherens junctions caused by GRHL2 loss. mTOR is a protein kinase that is involved in cell metabolism, proliferation and survival ⁴⁸. A cluster of GRHL2 loss responsive genes (*FAU*, *PLD1*, *PRKD1*, *RND3*, *RPS10*, *RPS11*, *RPS2*, *RPS21*, *RPS6KA2*, and *RPS8*) are associated with mTOR signaling. The activation of the AKT/mTOR pathway can trigger EMT through upregulation of ZEB1 ⁴⁹, which has a negative feedback with GRHL2 ⁷.

Notably, we demonstrate that *CDH1* RNA synthesis is not altered following GRHL2 loss. This is in agreement with our previous report that CDH1 is not identified as a GRHL2 target by CHIP-seq in breast cancer cells ²⁴, demonstrating that E-cadherin downregulation must occur in an indirect manner in our luminal breast cancer model. Others have identified *CDH1* as a direct GRHL2 target in normal epithelia ⁸ suggesting that the mechanism of E-cadherin regulation significantly differs between non-transformed epithelial cells and cancer cells that retain epithelial characteristics.

IPA network analysis shows that signaling networks exhibit numerous similarities among different time points but the most enriched networks vary over time. PANTHER analysis reveals that biological processes, cellular component, and protein classifications associated with networks regulated by

GRHL2 loss are conserved over time. DAVID analysis shows that the genes whose transcription is attenuated after GRHL2 loss are associated with important functions, including DNA replication, which is consistent with our previous finding that GRHL2 loss leads to a G0/1 arrest (Wang et al, manuscript under revision). A group of repressed genes are enriched for cell cycle and DNA replication including *E2F1*, *E2F2*, *MCM7*, *CDC20*, *ESPL1*, *MCM2*, *PTTG1*, *SFN*, *RNASEH2A* and *FEN1*. *E2F2* is a member of E2F transcription factor family that has a crucial role in the control of cell cycle and DNA replication⁵⁰. Our ChIP-PCR validates the presence of GRHL2 binding sites in the *E2F2* promoter region. Additionally, previous studies show that cell division cycle associated 7 like (*CDCA7L*) is a positive regulator of cell proliferation in prostate cancer and glioma^{51,52}. The existence of GRHL2 binding sites in the *CDCA7L* promoter region is also verified by ChIP-PCR. These findings suggest that GRHL2 may regulate DNA replication and cell cycle by multiple mechanisms, including direct transcriptional modulation of *E2F2* and *CDCA7L*. Moreover, we establish *EHF* as a direct GRHL2 target gene.

Taken together, in this study we identify GRHL2-regulated genes, we find five main patterns by which RNA synthesis is altered in response to depletion of GRHL2, and we provide new insights into the dynamics of GRHL2-mediated signaling networks. Additionally, our findings reveal how regulation of epithelial genes such as *CDH1* can be strikingly different in normal and cancer cells involving direct GRHL2-binding or indirect mechanisms.

Acknowledgements

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

Reference

- 1 Ma, L., Yan, H., Zhao, H. & Sun, J. Grainyhead-like 2 in development and cancer. *Tumour Biol* **39**, 1010428317698375, doi:10.1177/1010428317698375 (2017).
- 2 Xiang, X. *et al.* Grhl2 determines the epithelial phenotype of breast cancers and promotes tumor progression. *PLoS One* **7**, e50781, doi:10.1371/journal.pone.0050781 (2012).
- 3 Pyrgaki, C., Liu, A. & Niswander, L. Grainyhead-like 2 regulates neural tube closure and adhesion molecule expression during neural fold fusion. *Developmental biology* **353**, 38-49, doi:10.1016/j.ydbio.2011.02.027 (2011).
- 4 Werner, S. *et al.* Dual roles of the transcription factor grainyhead-like 2 (GRHL2) in breast cancer. *The Journal of biological chemistry* **288**, 22993-23008, doi:10.1074/jbc.M113.456293 (2013).
- 5 Reese, R. M., Harrison, M. M. & Alarid, E. T. Grainyhead-like Protein 2: The Emerging Role in Hormone-Dependent Cancers and Epigenetics. *Endocrinology* **160**, 1275-1288, doi:10.1210/en.2019-00213 (2019).
- 6 Cieply, B. *et al.* Suppression of the epithelial-mesenchymal transition by Grainyhead-like-2. *Cancer Res* **72**, 2440-2453, doi:10.1158/0008-5472.CAN-11-4038 (2012).
- 7 Cieply, B., Farris, J., Denvir, J., Ford, H. L. & Frisch, S. M. Epithelial-mesenchymal transition and tumor suppression are controlled by a reciprocal feedback loop between ZEB1 and Grainyhead-like-2. *Cancer Res* **73**, 6299-6309, doi:10.1158/0008-5472.CAN-12-4082 (2013).
- 8 Werth, M. *et al.* The transcription factor grainyhead-like 2 regulates the molecular composition of the epithelial apical junctional complex. *Development* **137**, 3835-3845, doi:10.1242/dev.055483 (2010).
- 9 Dompe, N. *et al.* A whole-genome RNAi screen identifies an 8q22 gene cluster that inhibits death receptor-mediated apoptosis. *Proceedings of the National Academy of Sciences of the United States of America* **108**, E943-951, doi:10.1073/pnas.1100132108 (2011).
- 10 Pan, X. *et al.* GRHL2 suppresses tumor metastasis via regulation of transcriptional activity of RhoG in non-small cell lung cancer. *Am J Transl Res* **9**, 4217-4226 (2017).
- 11 Faddaoui, A. *et al.* Suppression of the grainyhead transcription factor 2 gene (GRHL2) inhibits the proliferation, migration, invasion and mediates cell cycle arrest of ovarian cancer cells. *Cell Cycle* **16**, 693-706, doi:10.1080/15384101.2017.1295181 (2017).
- 12 Wang, G., Pan, J., Zhang, L. & Wang, C. Overexpression of grainyhead-like transcription factor 2 is associated with poor prognosis in human pancreatic carcinoma. *Oncology letters* **17**, 1491-1496, doi:10.3892/ol.2018.9741 (2019).
- 13 Chung, V. Y. *et al.* GRHL2-miR-200-ZEB1 maintains the epithelial status of ovarian cancer through transcriptional regulation and histone modification. *Sci Rep* **6**, 19943, doi:10.1038/srep19943 (2016).
- 14 Chung, V. Y. *et al.* The role of GRHL2 and epigenetic remodeling in epithelial-mesenchymal plasticity in ovarian cancer cells. *Commun Biol* **2**, 272, doi:10.1038/s42003-019-0506-3 (2019).

- 15 Paulsen, M. T. *et al.* Use of Bru-Seq and BruChase-Seq for genome-wide assessment of the synthesis and stability of RNA. *Methods* **67**, 45-54, doi:10.1016/j.ymeth.2013.08.015 (2014).
- 16 Kirkconnell, K. S., Paulsen, M. T., Magnuson, B., Bedi, K. & Ljungman, M. Capturing the dynamic nascent transcriptome during acute cellular responses: The serum response. *Biol Open* **5**, 837-847, doi:10.1242/bio.019323 (2016).
- 17 Ohtsu, M. *et al.* Novel DNA microarray system for analysis of nascent mRNAs. *DNA Res* **15**, 241-251, doi:10.1093/dnares/dsn015 (2008).
- 18 Haider, S. R., Juan, G., Traganos, F. & Darzynkiewicz, Z. Immunoseparation and immunodetection of nucleic acids labeled with halogenated nucleotides. *Experimental cell research* **234**, 498-506, doi:10.1006/excr.1997.3644 (1997).
- 19 Core, L. J., Waterfall, J. J. & Lis, J. T. Nascent RNA sequencing reveals widespread pausing and divergent initiation at human promoters. *Science* **322**, 1845-1848, doi:10.1126/science.1162228 (2008).
- 20 Paulsen, M. T. *et al.* Coordinated regulation of synthesis and stability of RNA during the acute TNF-induced proinflammatory response. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 2240-2245, doi:10.1073/pnas.1219192110 (2013).
- 21 Huang da, W., Sherman, B. T. & Lempicki, R. A. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic acids research* **37**, 1-13, doi:10.1093/nar/gkn923 (2009).
- 22 Huang da, W., Sherman, B. T. & Lempicki, R. A. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nature protocols* **4**, 44-57, doi:10.1038/nprot.2008.211 (2009).
- 23 Mi, H., Muruganujan, A., Ebert, D., Huang, X. & Thomas, P. D. PANTHER version 14: more genomes, a new PANTHER GO-slim and improvements in enrichment analysis tools. *Nucleic acids research* **47**, D419-D426, doi:10.1093/nar/gky1038 (2019).
- 24 Wang, Z., Wu, H., Daxinger, L. & Danen, E. H. J. Genome-wide identification of binding sites of GRHL2 in luminal-like and basal A subtypes of breast cancer. *bioRxiv*, 2020.2002.2013.946947, doi:10.1101/2020.02.13.946947 (2020).
- 25 Haring, M. *et al.* Chromatin immunoprecipitation: optimization, quantitative analysis and data normalization. *Plant Methods* **3**, 11, doi:10.1186/1746-4811-3-11 (2007).
- 26 Marinkovich, M. P. Tumour microenvironment: laminin 332 in squamous-cell carcinoma. *Nat Rev Cancer* **7**, 370-380, doi:10.1038/nrc2089 (2007).
- 27 Popovic, D., Vucic, D. & Dikic, I. Ubiquitination in disease pathogenesis and treatment. *Nature medicine* **20**, 1242-1253, doi:10.1038/nm.3739 (2014).
- 28 Stroebel, D., Casado, M. & Paoletti, P. Triheteromeric NMDA receptors: from structure to synaptic physiology. *Current opinion in physiology* **2**, 1-12, doi:10.1016/j.cophys.2017.12.004 (2018).
- 29 Trimarchi, J. M. & Lees, J. A. Sibling rivalry in the E2F family. *Nature reviews. Molecular cell biology* **3**, 11-20, doi:10.1038/nrm714 (2002).
- 30 Quan, Y. *et al.* Downregulation of GRHL2 inhibits the proliferation of colorectal cancer cells by targeting ZEB1. *Cancer Biol Ther* **15**, 878-887, doi:10.4161/cbt.28877 (2014).

- 31 Tian, Y. *et al.* CDCA7L promotes hepatocellular carcinoma progression by regulating the cell cycle. *Int J Oncol* **43**, 2082-2090, doi:10.3892/ijo.2013.2142 (2013).
- 32 Miao, B. *et al.* The transcription factor FLI1 promotes cancer progression by affecting cell cycle regulation. *International journal of cancer*, doi:10.1002/ijc.32831 (2019).
- 33 Cheng, A. C., Shen, C. J., Hung, C. M. & Hsu, Y. C. Sulforaphane Decrease of SERTAD1 Expression Triggers G1/S Arrest in Breast Cancer Cells. *J Med Food* **22**, 444-450, doi:10.1089/jmf.2018.4195 (2019).
- 34 Cheung, C. H. Y. *et al.* MCM2-regulated functional networks in lung cancer by multi-dimensional proteomic approach. *Sci Rep* **7**, 13302, doi:10.1038/s41598-017-13440-x (2017).
- 35 Mendonsa, A. M., Na, T. Y. & Gumbiner, B. M. E-cadherin in contact inhibition and cancer. *Oncogene* **37**, 4769-4780, doi:10.1038/s41388-018-0304-2 (2018).
- 36 Aue, A. *et al.* A Grainyhead-Like 2/Ovo-Like 2 Pathway Regulates Renal Epithelial Barrier Function and Lumen Expansion. *J Am Soc Nephrol* **26**, 2704-2715, doi:10.1681/ASN.2014080759 (2015).
- 37 Jolly, M. K. *et al.* E-Cadherin Represses Anchorage-Independent Growth in Sarcomas through Both Signaling and Mechanical Mechanisms. *Mol Cancer Res* **17**, 1391-1402, doi:10.1158/1541-7786.MCR-18-0763 (2019).
- 38 Paltoglou, S. *et al.* Novel Androgen Receptor Coregulator GRHL2 Exerts Both Oncogenic and Antimetastatic Functions in Prostate Cancer. *Cancer Res* **77**, 3417-3430, doi:10.1158/0008-5472.CAN-16-1616 (2017).
- 39 Varma, S. *et al.* The transcription factors Grainyhead-like 2 and NK2-homeobox 1 form a regulatory loop that coordinates lung epithelial cell morphogenesis and differentiation. *The Journal of biological chemistry* **287**, 37282-37295, doi:10.1074/jbc.M112.408401 (2012).
- 40 Goossens, S., Vandamme, N., Van Vlierberghe, P. & Berx, G. EMT transcription factors in cancer development re-evaluated: Beyond EMT and MET. *Biochim Biophys Acta Rev Cancer* **1868**, 584-591, doi:10.1016/j.bbcan.2017.06.006 (2017).
- 41 Park, S. M., Gaur, A. B., Lengyel, E. & Peter, M. E. The miR-200 family determines the epithelial phenotype of cancer cells by targeting the E-cadherin repressors ZEB1 and ZEB2. *Genes & development* **22**, 894-907, doi:10.1101/gad.1640608 (2008).
- 42 Yamada, T. *et al.* 5'-Bromouridine IP Chase (BRIC)-Seq to Determine RNA Half-Lives. *Methods Mol Biol* **1720**, 1-13, doi:10.1007/978-1-4939-7540-2_1 (2018).
- 43 Lieberman, J. Granzyme A activates another way to die. *Immunol Rev* **235**, 93-104, doi:10.1111/j.0105-2896.2010.00902.x (2010).
- 44 Fu, D. *et al.* HMGB2 is associated with malignancy and regulates Warburg effect by targeting LDHB and FBP1 in breast cancer. *Cell Commun Signal* **16**, 8, doi:10.1186/s12964-018-0219-0 (2018).
- 45 Kwon, J. H. *et al.* Overexpression of high-mobility group box 2 is associated with tumor aggressiveness and prognosis of hepatocellular carcinoma. *Clin Cancer Res* **16**, 5511-5521, doi:10.1158/1078-0432.CCR-10-0825 (2010).
- 46 D'Souza-Schorey, C. Disassembling adherens junctions: breaking up is hard to do. *Trends Cell Biol* **15**, 19-26, doi:10.1016/j.tcb.2004.11.002 (2005).

- 47 Rudini, N. & Dejana, E. Adherens junctions. *Curr Biol* **18**, R1080-1082, doi:10.1016/j.cub.2008.09.018 (2008).
- 48 Laplante, M. & Sabatini, D. M. mTOR signaling in growth control and disease. *Cell* **149**, 274-293, doi:10.1016/j.cell.2012.03.017 (2012).
- 49 Lau, M. T., So, W. K. & Leung, P. C. Fibroblast growth factor 2 induces E-cadherin down-regulation via PI3K/Akt/mTOR and MAPK/ERK signaling in ovarian cancer cells. *PLoS One* **8**, e59083, doi:10.1371/journal.pone.0059083 (2013).
- 50 Lafta, I. J. E2F6 is essential for cell viability in breast cancer cells during replication stress. *Turk J Biol* **43**, 293-304, doi:10.3906/biy-1905-6 (2019).
- 51 Lin, T. P. *et al.* R1 Regulates Prostate Tumor Growth and Progression By Transcriptional Suppression of the E3 Ligase HUWE1 to Stabilize c-Myc. *Mol Cancer Res* **16**, 1940-1951, doi:10.1158/1541-7786.MCR-16-0346 (2018).
- 52 Ji, Q. K. *et al.* CDCA7L promotes glioma proliferation by targeting CCND1 and predicts an unfavorable prognosis. *Mol Med Rep* **20**, 1149-1156, doi:10.3892/mmr.2019.10349 (2019).

Supplemental data

Table S1. List of genes whose transcription is altered in response to GRHL2 deletion and their classification into subgroups according to their dynamic pattern of regulation.

| Gene | D2 AFC | D4 AFC | D8 AFC | D16 AFC | Cluster |
|------------|--------|--------|--------|---------|----------------------|
| ABCA4 | 0.93 | 2.23 | 5.48 | 3.87 | |
| AC005821.1 | 2.79 | 6.46 | 12.24 | 6.54 | Sustained induction |
| AC005972.4 | 3.53 | 4.29 | 6.38 | 2.59 | Sustained induction |
| AC007952.4 | 0.17 | 0.19 | 0.32 | 0.61 | Repression reset |
| AC008703.1 | 5.56 | 6.60 | 7.51 | 3.23 | Sustained induction |
| AC009262.1 | 3.58 | 4.70 | 5.98 | 3.37 | Sustained induction |
| AC010653.3 | 0.21 | 0.48 | 0.55 | 0.67 | Repression reset |
| AC013652.1 | 2.43 | 3.59 | 4.10 | 1.57 | Induction reset |
| AC019209.3 | 1.43 | 3.61 | 4.91 | 4.29 | Sustained induction |
| AC022166.1 | 0.00 | 0.00 | 0.00 | 35.23 | |
| AC027277.2 | 0.26 | 0.23 | 0.38 | 0.68 | Repression reset |
| AC027288.3 | 2.30 | 3.95 | 3.23 | 0.94 | Induction reset |
| AC051619.5 | 5.12 | 4.51 | 3.69 | 2.64 | Sustained induction |
| AC055854.1 | 0.50 | 0.32 | 0.27 | 0.45 | Sustained repression |
| AC068633.1 | 7.14 | 0.00 | 0.00 | 12.59 | Dynamic |
| AC083967.1 | 0.52 | 0.32 | 0.37 | 0.40 | |
| AC087762.1 | 4.99 | 13.04 | 20.78 | 6.49 | Sustained induction |
| AC092167.1 | 8.40 | 7.04 | 5.45 | 2.93 | Sustained induction |
| AC092422.1 | 85.82 | 0.02 | 0.04 | 63.37 | Dynamic |
| AC098934.1 | 0.22 | 0.17 | 0.25 | 0.59 | Repression reset |
| AC099520.1 | 2.93 | 4.54 | 5.11 | 2.30 | Sustained induction |
| AC099753.1 | 91.52 | 0.00 | 0.00 | 211.15 | Dynamic |
| AC103770.1 | 2.34 | 3.65 | 3.32 | 3.27 | Sustained induction |
| AC109326.1 | 0.29 | 0.27 | 0.37 | 0.70 | Repression reset |
| AC245014.3 | 0.26 | 0.23 | 0.32 | 0.56 | Repression reset |
| ACKR3 | 0.70 | 0.61 | 1.36 | 2.68 | |
| ACOXL | 4.98 | 9.44 | 26.98 | 10.20 | Sustained induction |
| ACTB | 0.34 | 0.44 | 0.42 | 0.51 | Repression reset |
| ACTG1 | 0.34 | 0.55 | 0.62 | 0.67 | |
| ADCY5 | 1.23 | 2.33 | 4.74 | 6.93 | |
| ADGRE3 | 3.38 | 0.05 | 0.08 | 11.85 | Dynamic |
| AFF3 | 1.89 | 3.18 | 3.47 | 2.41 | |
| AGPAT4 | 4.16 | 8.04 | 15.78 | 7.77 | Sustained induction |
| AL049839.2 | 2.22 | 4.43 | 9.16 | 6.73 | Sustained induction |
| AL132708.1 | 2.08 | 1.97 | 3.83 | 2.12 | |
| AL137003.2 | 3.61 | 5.82 | 6.54 | 2.45 | Sustained induction |

| Gene | D2 AFC | D4 AFC | D8 AFC | D16 AFC | Cluster |
|------------|--------|--------|--------|---------|----------------------|
| AL137145.2 | 3.01 | 3.87 | 5.79 | 3.08 | Sustained induction |
| AL139383.1 | 2.80 | 3.34 | 2.39 | 1.15 | Induction reset |
| AL158066.1 | 0.53 | 0.14 | 0.15 | 0.29 | |
| AL158847.1 | 1.50 | 2.01 | 3.17 | 3.33 | |
| AL354740.1 | 2.61 | 4.43 | 4.57 | 2.31 | Sustained induction |
| AL359976.1 | 12.40 | 13.35 | 31.68 | 3.11 | Sustained induction |
| AL390726.6 | 8.20 | 8.75 | 9.71 | 4.52 | Sustained induction |
| AL590004.4 | 3.58 | 6.52 | 15.14 | 6.33 | Sustained induction |
| ALDH1A3 | 1.89 | 6.70 | 12.97 | 5.21 | |
| ALDOA | 0.33 | 0.33 | 0.48 | 0.71 | Repression reset |
| ALOX5 | 3.48 | 5.47 | 10.74 | 8.74 | Sustained induction |
| AMPH | 1.75 | 2.73 | 6.38 | 4.16 | |
| ANKRD1 | 1.39 | 4.68 | 5.06 | 1.90 | Induction reset |
| ANKRD29 | 3.32 | 6.49 | 8.05 | 6.63 | Sustained induction |
| ANOS1 | 1.93 | 3.05 | 3.15 | 2.13 | |
| ANXA3 | 5.19 | 8.86 | 6.80 | 2.41 | Sustained induction |
| AP000880.1 | 0.16 | 0.12 | 0.12 | 0.41 | Sustained repression |
| AP000924.1 | 1.55 | 4.57 | 11.84 | 8.49 | |
| AP002761.4 | 0.23 | 0.26 | 0.40 | 1.11 | |
| APRT | 0.26 | 0.38 | 0.41 | 0.84 | Repression reset |
| ARHGAP18 | 2.78 | 4.04 | 3.91 | 1.80 | Induction reset |
| ARHGAP22 | 1.88 | 4.43 | 7.13 | 3.45 | |
| ARHGAP42 | 2.55 | 4.45 | 4.23 | 1.84 | Induction reset |
| ARHGEF28 | 0.68 | 0.57 | 0.44 | 0.42 | |
| ARHGEF39 | 0.36 | 0.30 | 0.29 | 0.64 | Repression reset |
| ARPC1A | 0.17 | 0.25 | 0.29 | 0.21 | Sustained repression |
| ARSJ | 4.10 | 11.15 | 10.85 | 2.69 | Sustained induction |
| ASB9 | 10.25 | 13.53 | 11.41 | 4.43 | Sustained induction |
| ATP10D | 3.42 | 10.07 | 11.67 | 5.55 | Sustained induction |
| ATP5O | 0.26 | 0.37 | 0.33 | 0.29 | Sustained repression |
| ATP8A2 | 0.23 | 0.24 | 0.61 | 0.64 | |
| ATXN1 | 2.17 | 3.38 | 3.79 | 2.18 | Sustained induction |
| AURKB | 0.32 | 0.20 | 0.26 | 0.50 | Repression reset |
| BBC3 | 1.47 | 1.98 | 2.07 | 3.27 | |
| BIRC5 | 0.38 | 0.25 | 0.25 | 0.49 | Sustained repression |
| BMP1 | 1.99 | 3.51 | 5.20 | 3.49 | |
| BOC | 1.73 | 2.50 | 3.81 | 1.98 | Induction reset |
| C14orf80 | 0.28 | 0.34 | 0.36 | 1.28 | |
| C1orf105 | 2.83 | 3.30 | 7.20 | 2.74 | Sustained induction |
| C21orf58 | 0.42 | 0.27 | 0.32 | 0.73 | Repression reset |
| C22orf34 | 19.17 | 0.00 | 0.04 | 33.04 | Dynamic |
| CADM1 | 1.89 | 2.93 | 3.51 | 1.49 | Induction reset |

| Gene | D2 AFC | D4 AFC | D8 AFC | D16 AFC | Cluster |
|--------|--------|--------|--------|---------|----------------------|
| CADPS | 53.78 | 0.06 | 0.03 | 51.71 | Dynamic |
| CAMK1D | 1.92 | 3.14 | 3.93 | 1.98 | Induction reset |
| CAPN8 | 4.32 | 5.39 | 11.47 | 5.66 | Sustained induction |
| CBX2 | 0.30 | 0.33 | 0.47 | 0.89 | Repression reset |
| CCNF | 0.38 | 0.28 | 0.31 | 0.72 | Repression reset |
| CD109 | 2.27 | 2.56 | 4.55 | 2.54 | Sustained induction |
| CDC20 | 0.27 | 0.30 | 0.30 | 0.67 | Repression reset |
| CDCA3 | 0.33 | 0.26 | 0.27 | 0.51 | Repression reset |
| CDCA5 | 0.36 | 0.25 | 0.29 | 0.64 | Repression reset |
| CDCA7L | 0.58 | 0.43 | 0.36 | 0.39 | |
| CDH18 | 3.27 | 6.40 | 7.10 | 5.15 | Sustained induction |
| CDKN2B | 2.93 | 9.08 | 13.19 | 6.17 | Sustained induction |
| CEMIP | 2.07 | 1.74 | 4.07 | 3.24 | |
| CENPF | 0.50 | 0.29 | 0.28 | 0.40 | Sustained repression |
| CFL1 | 0.32 | 0.42 | 0.45 | 0.63 | Repression reset |
| CHTF18 | 0.33 | 0.34 | 0.43 | 1.12 | |
| CLIP4 | 1.91 | 3.35 | 4.85 | 3.69 | |
| CNTN4 | 22.17 | 0.05 | 5.10 | 26.44 | |
| COL4A5 | 3.79 | 7.76 | 10.60 | 5.61 | Sustained induction |
| COLQ | 2.50 | 2.83 | 3.71 | 1.81 | Induction reset |
| CORO2A | 1.50 | 2.23 | 2.49 | 2.76 | |
| CPNE4 | 3.77 | 3.01 | 6.43 | 8.86 | Sustained induction |
| CPQ | 2.74 | 4.86 | 4.35 | 2.33 | Sustained induction |
| CPXM2 | 0.40 | 0.27 | 0.29 | 0.62 | Repression reset |
| CREB5 | 2.58 | 8.80 | 12.08 | 5.22 | Sustained induction |
| CTNNA3 | 9.27 | 16.04 | 14.13 | 4.17 | Sustained induction |
| CTNND2 | 2.53 | 3.46 | 3.60 | 1.57 | Induction reset |
| CYB561 | 0.35 | 0.46 | 0.56 | 0.92 | |
| CYC1 | 0.27 | 0.34 | 0.43 | 0.95 | Repression reset |
| DAPP1 | 4.40 | 9.94 | 23.48 | 11.76 | Sustained induction |
| DDX11 | 0.37 | 0.37 | 0.41 | 0.67 | Repression reset |
| DDX12P | 0.33 | 0.27 | 0.30 | 0.58 | Repression reset |
| DDX41 | 0.33 | 0.48 | 0.52 | 0.77 | |
| DDX58 | 2.25 | 2.17 | 3.26 | 4.00 | Sustained induction |
| DDX60L | 2.17 | 3.23 | 3.85 | 3.82 | Sustained induction |
| DISC1 | 2.25 | 3.20 | 3.52 | 1.43 | Induction reset |
| DLGAP2 | 20.26 | 0.01 | 0.02 | 24.32 | Dynamic |
| DNAH5 | 3.07 | 4.84 | 6.54 | 3.08 | Sustained induction |
| DNAH7 | 3.11 | 3.96 | 4.70 | 1.87 | Induction reset |
| DNM3 | 1.78 | 2.03 | 3.69 | 1.55 | Induction reset |
| DOCK4 | 2.36 | 3.98 | 5.25 | 1.92 | Induction reset |
| DOCK8 | 2.24 | 3.15 | 3.16 | 2.68 | Sustained induction |

| Gene | D2 AFC | D4 AFC | D8 AFC | D16 AFC | Cluster |
|----------|--------|--------|--------|---------|----------------------|
| DOK5 | 24.10 | 0.01 | 0.03 | 18.16 | Dynamic |
| DUSP10 | 2.30 | 2.61 | 4.07 | 2.72 | Sustained induction |
| E2F1 | 0.35 | 0.33 | 0.38 | 0.80 | Repression reset |
| E2F2 | 0.31 | 0.28 | 0.33 | 0.77 | Repression reset |
| EDA2R | 3.81 | 3.40 | 2.15 | 1.75 | Induction reset |
| EEF1A1 | 0.36 | 0.45 | 0.50 | 0.56 | Repression reset |
| EEF1B2 | 0.44 | 0.47 | 0.43 | 0.39 | Sustained repression |
| EEF2 | 0.31 | 0.47 | 0.51 | 0.75 | |
| EFNB2 | 1.18 | 1.44 | 2.17 | 3.08 | |
| EHF | 0.21 | 0.14 | 0.15 | 0.30 | Sustained repression |
| ELL2 | 2.04 | 2.30 | 3.50 | 1.47 | Induction reset |
| EPAS1 | 2.35 | 3.41 | 6.43 | 3.06 | Sustained induction |
| EPB41L4A | 2.23 | 3.14 | 3.26 | 2.28 | Sustained induction |
| EPN3 | 0.22 | 0.30 | 0.46 | 1.10 | |
| ERC2 | 2.15 | 4.80 | 8.43 | 8.33 | Sustained induction |
| ESPL1 | 0.36 | 0.30 | 0.31 | 0.70 | Repression reset |
| F2R | 2.29 | 6.41 | 8.42 | 5.27 | Sustained induction |
| FAM13A | 2.62 | 2.74 | 5.96 | 3.59 | Sustained induction |
| FAM83D | 0.40 | 0.30 | 0.29 | 0.55 | Repression reset |
| FANCG | 0.29 | 0.30 | 0.37 | 0.72 | Repression reset |
| FAU | 0.26 | 0.29 | 0.33 | 0.38 | Sustained repression |
| FBN2 | 1.94 | 2.24 | 5.55 | 2.60 | |
| FBXL2 | 2.16 | 3.08 | 3.13 | 1.79 | Induction reset |
| FEN1 | 0.30 | 0.26 | 0.30 | 0.50 | Repression reset |
| FGF12 | 2.39 | 4.68 | 3.75 | 1.65 | Induction reset |
| FHL2 | 2.47 | 3.00 | 4.81 | 3.10 | Sustained induction |
| FLT1 | 5.25 | 7.58 | 9.04 | 3.34 | Sustained induction |
| FLT3 | 3.73 | 5.93 | 5.81 | 3.09 | Sustained induction |
| FOXP2 | 3.99 | 4.09 | 3.12 | 1.65 | Induction reset |
| FRY | 4.41 | 7.10 | 10.36 | 4.92 | Sustained induction |
| FSTL4 | 2.31 | 4.03 | 7.89 | 4.83 | Sustained induction |
| FTL | 0.20 | 0.27 | 0.35 | 0.42 | Sustained repression |
| FYN | 1.58 | 3.13 | 4.03 | 2.52 | |
| GALNT17 | 7.35 | 0.03 | 0.03 | 6.59 | Dynamic |
| GAPDH | 0.19 | 0.30 | 0.38 | 0.53 | Repression reset |
| GBP2 | 9.04 | 5.34 | 5.68 | 3.34 | Sustained induction |
| GLDN | 2.12 | 2.36 | 3.32 | 1.39 | Induction reset |
| GPR155 | 2.74 | 4.26 | 3.92 | 2.03 | Sustained induction |
| GPR87 | 3.32 | 3.32 | 6.20 | 3.14 | Sustained induction |
| GRIN2B | 59.19 | 0.06 | 0.05 | 86.59 | Dynamic |
| GRK5 | 2.15 | 3.92 | 3.74 | 2.42 | Sustained induction |
| GULP1 | 2.50 | 4.50 | 6.29 | 3.19 | Sustained induction |

| Gene | D2 AFC | D4 AFC | D8 AFC | D16 AFC | Cluster |
|-----------|--------|--------|--------|---------|----------------------|
| H2AFZ | 0.17 | 0.20 | 0.24 | 0.36 | Sustained repression |
| HAX1 | 0.28 | 0.38 | 0.44 | 0.61 | Repression reset |
| HDX | 3.74 | 6.57 | 15.96 | 7.88 | Sustained induction |
| HERC3 | 1.59 | 2.96 | 4.04 | 1.55 | Induction reset |
| HIST1H1C | 0.15 | 0.10 | 0.14 | 0.44 | Sustained repression |
| HIST1H1D | 0.14 | 0.09 | 0.14 | 0.50 | Sustained repression |
| HIST1H1E | 0.12 | 0.11 | 0.14 | 0.37 | Sustained repression |
| HIST1H2AB | 0.16 | 0.08 | 0.12 | 0.47 | Sustained repression |
| HIST1H2AE | 0.20 | 0.10 | 0.15 | 0.40 | Sustained repression |
| HIST1H2AJ | 0.09 | 0.08 | 0.09 | 0.33 | Sustained repression |
| HIST1H2AL | 0.25 | 0.10 | 0.11 | 1.18 | |
| HIST1H2AM | 0.14 | 0.08 | 0.11 | 0.51 | Repression reset |
| HIST1H2BF | 0.24 | 0.13 | 0.19 | 0.46 | Sustained repression |
| HIST1H2BG | 0.27 | 0.21 | 0.31 | 0.72 | Repression reset |
| HIST1H2BH | 0.26 | 0.15 | 0.21 | 0.61 | Repression reset |
| HIST1H2BI | 0.13 | 0.10 | 0.11 | 0.31 | Sustained repression |
| HIST1H2BK | 0.14 | 0.10 | 0.13 | 0.30 | Sustained repression |
| HIST1H2BM | 0.16 | 0.10 | 0.11 | 0.29 | Sustained repression |
| HIST1H2BO | 0.14 | 0.09 | 0.14 | 0.41 | Sustained repression |
| HIST1H3A | 0.14 | 0.06 | 0.13 | 0.57 | Repression reset |
| HIST1H3G | 0.17 | 0.10 | 0.15 | 0.55 | Repression reset |
| HIST1H3H | 0.24 | 0.16 | 0.25 | 0.66 | Repression reset |
| HIST1H3I | 0.35 | 0.23 | 0.12 | 1.52 | |
| HIST1H3J | 0.16 | 0.09 | 0.11 | 0.60 | Repression reset |
| HIST1H4A | 0.12 | 0.12 | 0.11 | 0.49 | Sustained repression |
| HIST1H4B | 0.20 | 0.11 | 0.17 | 0.52 | Repression reset |
| HIST1H4D | 0.19 | 0.11 | 0.17 | 0.40 | Sustained repression |
| HIST1H4E | 0.19 | 0.17 | 0.25 | 0.54 | Repression reset |
| HIST1H4F | 0.21 | 0.11 | 0.13 | 0.41 | Sustained repression |
| HIST1H4H | 0.36 | 0.26 | 0.38 | 0.58 | Repression reset |
| HIST2H3D | 0.19 | 0.17 | 0.16 | 2.15 | |
| HIST4H4 | 0.28 | 0.23 | 0.34 | 0.77 | Repression reset |
| HJURP | 0.36 | 0.28 | 0.28 | 0.53 | Repression reset |
| HLA-DQB1 | 3.02 | 3.67 | 8.50 | 3.86 | Sustained induction |
| HMGB2 | 0.25 | 0.23 | 0.29 | 0.46 | Sustained repression |
| HMMR | 0.54 | 0.32 | 0.25 | 0.39 | |
| HR | 0.22 | 0.25 | 0.29 | 0.82 | Repression reset |
| HSD17B11 | 4.28 | 5.50 | 13.27 | 3.41 | Sustained induction |
| HSP90AA1 | 0.19 | 0.41 | 0.33 | 0.24 | Sustained repression |
| HSP90AB1 | 0.33 | 0.57 | 0.57 | 0.59 | |
| HSPA8 | 0.26 | 0.35 | 0.37 | 0.41 | Sustained repression |
| HSPE1 | 0.31 | 0.36 | 0.28 | 0.22 | Sustained repression |

| Gene | D2 AFC | D4 AFC | D8 AFC | D16 AFC | Cluster |
|-------------|--------|--------|--------|---------|----------------------|
| IGSF21 | 3.81 | 0.01 | 0.01 | 7.50 | Dynamic |
| IL18 | 1.84 | 4.63 | 8.54 | 3.08 | |
| INCENP | 0.30 | 0.25 | 0.28 | 0.58 | Repression reset |
| IQCJ-SCHIP1 | 2.79 | 3.41 | 5.34 | 1.83 | Induction reset |
| ISM1 | 1.75 | 3.28 | 4.45 | 1.57 | Induction reset |
| ITGB6 | 3.58 | 7.80 | 41.07 | 26.91 | Sustained induction |
| JAZF1 | 2.15 | 5.12 | 5.89 | 2.88 | Sustained induction |
| KC6 | 6.12 | 8.94 | 12.80 | 6.63 | Sustained induction |
| KCNJ3 | 2.03 | 3.56 | 6.40 | 4.08 | Sustained induction |
| KCNK5 | 0.32 | 0.17 | 0.19 | 0.63 | Repression reset |
| KCNMA1 | 1.33 | 1.96 | 4.48 | 4.79 | |
| KIAA0513 | 2.04 | 3.14 | 3.92 | 2.80 | Sustained induction |
| KIAA2012 | 3.22 | 9.67 | 16.82 | 6.56 | Sustained induction |
| KIF20A | 0.26 | 0.23 | 0.20 | 0.37 | Sustained repression |
| KIF2C | 0.41 | 0.25 | 0.28 | 0.48 | Sustained repression |
| KIF5C | 1.26 | 2.82 | 3.91 | 3.14 | |
| KIFC1 | 0.46 | 0.29 | 0.29 | 0.55 | Repression reset |
| LACTB | 1.58 | 3.33 | 3.83 | 3.25 | |
| LAD1 | 0.32 | 0.41 | 0.56 | 1.13 | |
| LAMA3 | 2.00 | 3.34 | 3.66 | 2.74 | |
| LAMB3 | 2.80 | 4.79 | 12.02 | 8.49 | Sustained induction |
| LAMC2 | 2.16 | 5.02 | 9.71 | 4.78 | Sustained induction |
| LHFPL2 | 1.33 | 2.47 | 3.55 | 2.16 | |
| LIMCH1 | 1.86 | 2.56 | 4.45 | 2.02 | |
| LINC00473 | 7.90 | 6.05 | 6.28 | 2.90 | Sustained induction |
| LINC00871 | 6.51 | 4.62 | 1.99 | 2.96 | |
| LINC00885 | 0.30 | 0.17 | 0.19 | 0.42 | Sustained repression |
| LINC01191 | 2.10 | 5.99 | 7.73 | 3.26 | Sustained induction |
| LINC01214 | 9.20 | 17.88 | 43.41 | 27.34 | Sustained induction |
| LINC01239 | 4.14 | 12.86 | 31.60 | 7.78 | Sustained induction |
| LINC01619 | 0.83 | 0.51 | 0.51 | 0.42 | |
| LIPH | 2.58 | 3.13 | 3.99 | 1.76 | Induction reset |
| LOXL2 | 2.08 | 4.69 | 7.79 | 4.69 | Sustained induction |
| LRP2 | 3.27 | 4.71 | 4.54 | 2.99 | Sustained induction |
| LUCAT1 | 2.68 | 6.05 | 8.36 | 3.28 | Sustained induction |
| LYPD1 | 3.00 | 5.93 | 9.53 | 3.41 | Sustained induction |
| LYPD3 | 0.17 | 0.17 | 0.31 | 0.73 | Repression reset |
| MAF | 0.00 | 0.00 | 19.71 | 31.48 | |
| MAP1B | 2.55 | 9.39 | 16.96 | 6.44 | Sustained induction |
| MAPK10 | 2.62 | 3.10 | 2.61 | 1.33 | Induction reset |
| MAPRE2 | 3.71 | 8.07 | 16.19 | 10.23 | Sustained induction |
| MAPRE3 | 1.78 | 2.44 | 3.22 | 2.17 | |

| Gene | D2 AFC | D4 AFC | D8 AFC | D16 AFC | Cluster |
|-----------|--------|--------|--------|---------|----------------------|
| MCF2L2 | 2.37 | 3.05 | 3.15 | 1.64 | Induction reset |
| MCM2 | 0.34 | 0.33 | 0.33 | 0.68 | Repression reset |
| MCM7 | 0.36 | 0.30 | 0.37 | 0.58 | Repression reset |
| MCTP1 | 2.92 | 5.77 | 5.33 | 4.05 | Sustained induction |
| MDGA2 | 3.42 | 6.23 | 7.58 | 3.42 | Sustained induction |
| MECOM | 3.20 | 5.13 | 5.05 | 2.54 | Sustained induction |
| MIR222HG | 1.63 | 3.09 | 5.04 | 3.01 | |
| MIR3681HG | 4.80 | 0.07 | 0.06 | 6.43 | Dynamic |
| MIR5087 | 0.19 | 0.24 | 0.33 | 0.59 | Repression reset |
| MIR9-3HG | 0.35 | 0.31 | 0.43 | 0.78 | Repression reset |
| MITF | 1.61 | 3.58 | 5.49 | 2.22 | |
| MKI67 | 0.42 | 0.28 | 0.27 | 0.38 | Sustained repression |
| MMP16 | 2.17 | 2.97 | 3.27 | 2.91 | Sustained induction |
| MPPED2 | 0.75 | 0.59 | 0.41 | 0.35 | |
| MRFAP1 | 0.33 | 0.47 | 0.50 | 0.59 | |
| MRPL17 | 0.33 | 0.40 | 0.48 | 0.74 | Repression reset |
| MRPL51 | 0.25 | 0.28 | 0.28 | 0.38 | Sustained repression |
| MRPS34 | 0.25 | 0.28 | 0.29 | 0.65 | Repression reset |
| MSMB | 0.21 | 0.11 | 0.15 | 0.33 | Sustained repression |
| MTUS2 | 2.69 | 2.31 | 5.20 | 2.93 | Sustained induction |
| MYT1L | 32.55 | 0.05 | 0.00 | 37.70 | Dynamic |
| NBEA | 2.41 | 3.23 | 3.26 | 1.63 | Induction reset |
| NCF2 | 2.94 | 6.23 | 23.18 | 17.27 | Sustained induction |
| NECTIN4 | 0.26 | 0.30 | 0.48 | 0.96 | Repression reset |
| NEK10 | 1.50 | 2.78 | 6.78 | 2.59 | |
| NHS | 1.47 | 2.47 | 3.20 | 1.40 | Induction reset |
| NHSL2 | 3.09 | 4.61 | 6.21 | 3.48 | Sustained induction |
| NLGN1 | 7.50 | 0.02 | 0.00 | 17.12 | Dynamic |
| NME1 | 0.28 | 0.38 | 0.37 | 0.39 | Sustained repression |
| NPAS3 | 2.72 | 4.13 | 3.49 | 1.06 | Induction reset |
| NPM1P27 | 0.27 | 0.40 | 0.50 | 0.33 | Sustained repression |
| NPY1R | 0.81 | 0.33 | 0.20 | 0.19 | |
| NR2C2AP | 0.23 | 0.24 | 0.34 | 0.58 | Repression reset |
| NRG2 | 3.76 | 8.84 | 10.44 | 3.37 | Sustained induction |
| NRP1 | 2.18 | 3.57 | 3.13 | 1.58 | Induction reset |
| NRXN3 | 10.82 | 0.94 | 1.45 | 11.75 | |
| NT5DC2 | 0.28 | 0.42 | 0.46 | 0.85 | Repression reset |
| NTN4 | 5.48 | 13.04 | 18.44 | 9.78 | Sustained induction |
| NUDT1 | 0.26 | 0.23 | 0.28 | 0.67 | Repression reset |
| OPCML | 27.66 | 0.03 | 0.04 | 38.49 | Dynamic |
| OPTN | 1.69 | 3.76 | 6.50 | 5.12 | |
| P2RY2 | 0.22 | 0.35 | 0.54 | 1.53 | |

| Gene | D2 AFC | D4 AFC | D8 AFC | D16 AFC | Cluster |
|--------------|--------|--------|--------|---------|----------------------|
| PACIN3 | 0.31 | 0.37 | 0.36 | 0.91 | Repression reset |
| PALM2 | 2.42 | 4.65 | 4.73 | 1.72 | Induction reset |
| PALM2-AKAP2 | 3.43 | 4.12 | 5.24 | 2.77 | Sustained induction |
| PAPSS2 | 2.57 | 6.14 | 7.88 | 3.34 | Sustained induction |
| PAQR5 | 1.21 | 1.66 | 1.91 | 3.05 | |
| PCAT29 | 4.40 | 5.18 | 7.17 | 4.08 | Sustained induction |
| PCSK2 | 63.00 | 0.05 | 0.00 | 99.27 | Dynamic |
| PGLYRP2 | 0.18 | 0.09 | 0.03 | 0.27 | Sustained repression |
| PGM2L1 | 2.30 | 2.73 | 4.43 | 2.62 | Sustained induction |
| PHACTR3 | 40.57 | 0.04 | 0.06 | 47.82 | Dynamic |
| PHGDH | 0.22 | 0.29 | 0.31 | 0.65 | Repression reset |
| PHLDB2 | 3.97 | 8.37 | 11.66 | 5.28 | Sustained induction |
| PID1 | 1.84 | 3.60 | 10.27 | 7.22 | |
| PIF1 | 0.45 | 0.28 | 0.28 | 0.55 | Repression reset |
| PIK3IP1-AS1 | 5.12 | 6.17 | 7.66 | 3.53 | Sustained induction |
| PIMREG | 0.24 | 0.24 | 0.21 | 0.62 | Repression reset |
| PKP1 | 0.27 | 0.18 | 0.19 | 0.73 | Repression reset |
| PLCE1 | 2.78 | 9.19 | 9.46 | 2.97 | Sustained induction |
| PLCXD2 | 4.35 | 9.62 | 12.79 | 5.07 | Sustained induction |
| PLD1 | 3.98 | 7.92 | 7.69 | 2.78 | Sustained induction |
| PLEKHH2 | 3.60 | 4.43 | 4.97 | 2.15 | Sustained induction |
| PLIN4 | 0.11 | 0.03 | 0.09 | 0.34 | Sustained repression |
| PLIN5 | 0.19 | 0.04 | 0.12 | 0.41 | Sustained repression |
| PMP22 | 3.24 | 4.41 | 4.84 | 3.59 | Sustained induction |
| POP7 | 0.30 | 0.30 | 0.40 | 0.71 | Repression reset |
| PPARG | 2.63 | 4.37 | 10.14 | 3.87 | Sustained induction |
| PPIAP22 | 0.11 | 0.18 | 0.20 | 0.18 | Sustained repression |
| PPP1CA | 0.27 | 0.32 | 0.42 | 0.63 | Repression reset |
| PPP1R14B | 0.28 | 0.38 | 0.54 | 0.85 | |
| PRELID1 | 0.33 | 0.36 | 0.41 | 0.60 | Repression reset |
| PRICKLE2-AS1 | 2.03 | 3.23 | 3.48 | 1.32 | Induction reset |
| PRICKLE2-AS3 | 4.11 | 9.01 | 9.17 | 4.29 | Sustained induction |
| PRKD1 | 3.24 | 5.01 | 7.05 | 2.39 | Sustained induction |
| PROS1 | 2.99 | 3.62 | 4.27 | 1.93 | Induction reset |
| PRSS23 | 2.39 | 4.01 | 5.50 | 5.31 | Sustained induction |
| PSG5 | 2.07 | 7.09 | 9.24 | 7.10 | Sustained induction |
| PSMB6 | 0.31 | 0.37 | 0.45 | 0.47 | Sustained repression |
| PSMC3 | 0.34 | 0.45 | 0.48 | 0.67 | Repression reset |
| PSMD2 | 0.36 | 0.52 | 0.61 | 0.63 | |
| PSMG3 | 0.31 | 0.33 | 0.39 | 0.79 | Repression reset |
| PSRC1 | 0.30 | 0.23 | 0.33 | 0.63 | Repression reset |
| PTTG1 | 0.33 | 0.26 | 0.23 | 0.45 | Sustained repression |

| Gene | D2 AFC | D4 AFC | D8 AFC | D16 AFC | Cluster |
|------------|--------|--------|--------|---------|----------------------|
| PYCR1 | 0.24 | 0.37 | 0.37 | 0.90 | Repression reset |
| QARS | 0.30 | 0.44 | 0.43 | 0.66 | Repression reset |
| RAB7B | 3.72 | 4.73 | 14.45 | 7.71 | Sustained induction |
| RAI2 | 2.11 | 3.70 | 8.48 | 4.74 | Sustained induction |
| RBFOX1 | 2.27 | 0.74 | 0.52 | 3.17 | |
| RBFOX3 | 34.53 | 0.01 | 0.02 | 39.26 | Dynamic |
| RCAN2 | 9.68 | 1.28 | 20.29 | 16.55 | |
| RECQL4 | 0.28 | 0.33 | 0.30 | 0.89 | Repression reset |
| REEP4 | 0.24 | 0.27 | 0.34 | 0.99 | Repression reset |
| RETREG1 | 2.37 | 3.64 | 3.37 | 2.27 | Sustained induction |
| RFTN1 | 3.06 | 4.72 | 6.29 | 4.49 | Sustained induction |
| RN7SL2 | 0.22 | 0.34 | 0.42 | 0.71 | Repression reset |
| RN7SL3 | 0.29 | 0.45 | 0.54 | 0.86 | |
| RN7SL4P | 0.18 | 0.29 | 0.36 | 0.74 | Repression reset |
| RNASEH2A | 0.25 | 0.23 | 0.27 | 0.61 | Repression reset |
| RND3 | 2.35 | 3.96 | 5.16 | 3.64 | Sustained induction |
| RNF150 | 3.46 | 6.36 | 7.42 | 2.48 | Sustained induction |
| RNF219-AS1 | 54.69 | 0.00 | 0.00 | 71.81 | Dynamic |
| RNU1-120P | 0.17 | 0.16 | 0.27 | 0.62 | Repression reset |
| RNU1-122P | 0.15 | 0.15 | 0.27 | 0.62 | Repression reset |
| RNU2-63P | 0.18 | 0.20 | 0.34 | 0.79 | Repression reset |
| RNU4-1 | 0.17 | 0.18 | 0.30 | 0.91 | Repression reset |
| RNU5D-1 | 0.14 | 0.29 | 0.43 | 0.53 | Repression reset |
| RNU5E-4P | 0.19 | 0.16 | 0.31 | 0.59 | Repression reset |
| RNVU1-6 | 0.17 | 0.15 | 0.21 | 0.67 | Repression reset |
| RNVU1-7 | 0.20 | 0.22 | 0.24 | 0.59 | Repression reset |
| RPL13A | 0.32 | 0.41 | 0.49 | 0.64 | Repression reset |
| RPL17 | 0.33 | 0.40 | 0.48 | 0.57 | Repression reset |
| RPL3 | 0.30 | 0.48 | 0.55 | 0.72 | |
| RPL35 | 0.32 | 0.38 | 0.41 | 0.58 | Repression reset |
| RPL41 | 0.30 | 0.38 | 0.49 | 0.69 | Repression reset |
| RPL7 | 0.33 | 0.47 | 0.46 | 0.42 | Sustained repression |
| RPL7A | 0.36 | 0.44 | 0.47 | 0.48 | Sustained repression |
| RPL8 | 0.30 | 0.37 | 0.37 | 0.50 | Repression reset |
| RPL9P9 | 0.15 | 0.27 | 0.18 | 0.25 | Sustained repression |
| RPS10 | 0.32 | 0.37 | 0.34 | 0.28 | Sustained repression |
| RPS11 | 0.35 | 0.37 | 0.40 | 0.46 | Sustained repression |
| RPS2 | 0.28 | 0.34 | 0.35 | 0.61 | Repression reset |
| RPS21 | 0.28 | 0.32 | 0.36 | 0.48 | Sustained repression |
| RPS6KA2 | 2.48 | 2.79 | 3.13 | 2.78 | Sustained induction |
| RPS8 | 0.33 | 0.41 | 0.38 | 0.47 | Sustained repression |

| Gene | D2 AFC | D4 AFC | D8 AFC | D16 AFC | Cluster |
|------------|--------|--------|--------|---------|----------------------|
| RTN1 | 2.33 | 5.02 | 4.89 | 2.28 | Sustained induction |
| S100A14 | 0.34 | 0.52 | 0.50 | 0.57 | |
| SAMD12 | 2.42 | 3.61 | 4.11 | 2.57 | Sustained induction |
| SAMD12-AS1 | 3.99 | 6.35 | 7.19 | 4.12 | Sustained induction |
| SAMD9 | 0.00 | 0.00 | 19.92 | 37.58 | |
| SAPCD2 | 0.24 | 0.27 | 0.28 | 0.76 | Repression reset |
| SCARNA12 | 0.14 | 0.20 | 0.26 | 0.26 | Sustained repression |
| SCARNA13 | 0.22 | 0.32 | 0.48 | 0.47 | Sustained repression |
| SCARNA21 | 0.08 | 0.10 | 0.10 | 0.22 | Sustained repression |
| SCARNA7 | 0.17 | 0.31 | 0.55 | 0.31 | |
| SDC1 | 0.28 | 0.35 | 0.48 | 0.97 | Repression reset |
| SEMA6A | 2.34 | 3.48 | 4.69 | 3.74 | Sustained induction |
| SEPT8 | 0.35 | 0.66 | 0.82 | 1.11 | |
| SESN3 | 6.90 | 10.94 | 12.91 | 7.02 | Sustained induction |
| SFN | 0.21 | 0.34 | 0.34 | 0.61 | Repression reset |
| SH3TC2 | 0.16 | 0.22 | 0.26 | 0.26 | Sustained repression |
| SHC4 | 2.49 | 2.64 | 5.43 | 2.78 | Sustained induction |
| SHMT2 | 0.22 | 0.39 | 0.42 | 0.82 | Repression reset |
| SLC12A4 | 1.98 | 3.93 | 4.88 | 5.01 | |
| SLC16A3 | 0.16 | 0.17 | 0.28 | 0.91 | Repression reset |
| SLC1A1 | 6.72 | 11.29 | 17.89 | 10.80 | Sustained induction |
| SLC22A1 | 1.68 | 4.62 | 6.00 | 3.78 | |
| SLC22A15 | 2.05 | 2.94 | 3.12 | 1.68 | Induction reset |
| SLC25A5 | 0.24 | 0.27 | 0.33 | 0.40 | Sustained repression |
| SLC9A3 | 0.31 | 0.00 | 0.00 | 103.49 | |
| SLIT3 | 4.89 | 0.03 | 0.63 | 3.96 | |
| SNORD3A | 0.10 | 0.13 | 0.19 | 0.27 | Sustained repression |
| SNORD3B-1 | 0.19 | 0.23 | 0.32 | 0.67 | Repression reset |
| SOCS2-AS1 | 6.58 | 8.39 | 9.17 | 3.76 | Sustained induction |
| SORCS2 | 1.61 | 2.38 | 3.41 | 2.64 | |
| SOX9 | 3.49 | 6.38 | 11.38 | 12.66 | Sustained induction |
| SOX9-AS1 | 3.93 | 3.43 | 2.82 | 2.14 | Sustained induction |
| SPAG5 | 0.34 | 0.30 | 0.31 | 0.45 | Sustained repression |
| SPATA18 | 4.04 | 4.13 | 2.47 | 1.69 | Induction reset |
| SPEG | 1.99 | 2.92 | 4.04 | 4.71 | |
| SPOCK1 | 2.23 | 3.00 | 16.72 | 4.48 | Sustained induction |
| SSNA1 | 0.22 | 0.27 | 0.39 | 0.74 | Repression reset |
| SSRP1 | 0.33 | 0.44 | 0.45 | 0.58 | Repression reset |
| ST3GAL5 | 1.49 | 2.64 | 4.09 | 3.44 | |
| STAT4 | 2.50 | 6.67 | 5.66 | 2.00 | Sustained induction |
| STUM | 1.75 | 2.22 | 10.66 | 13.14 | |
| SULF1 | 0.60 | 0.31 | 0.26 | 0.17 | |

| Gene | D2 AFC | D4 AFC | D8 AFC | D16 AFC | Cluster |
|----------|--------|--------|--------|---------|----------------------|
| SUN2 | 0.35 | 0.33 | 0.43 | 0.67 | Repression reset |
| SYNPO | 3.53 | 6.07 | 6.99 | 4.11 | Sustained induction |
| SYNPR | 8.34 | 0.01 | 0.01 | 10.33 | Dynamic |
| SYT7 | 0.34 | 0.30 | 0.40 | 0.97 | Repression reset |
| TACSTD2 | 0.32 | 0.30 | 0.43 | 0.64 | Repression reset |
| TANC2 | 2.06 | 3.16 | 3.40 | 1.56 | Induction reset |
| TENM2 | 25.34 | 0.03 | 6.08 | 23.79 | |
| TFPI | 1.65 | 2.39 | 3.30 | 2.04 | |
| TGFB2 | 2.27 | 6.07 | 5.70 | 1.71 | Induction reset |
| TGFB1 | 2.92 | 6.30 | 9.58 | 4.64 | Sustained induction |
| TGFBR2 | 3.05 | 6.81 | 8.02 | 2.99 | Sustained induction |
| THAP11 | 0.20 | 0.23 | 0.29 | 0.81 | Repression reset |
| THEG | 0.23 | 0.07 | 0.15 | 0.19 | Sustained repression |
| TIMP3 | 2.61 | 5.50 | 11.16 | 10.20 | Sustained induction |
| TK1 | 0.32 | 0.26 | 0.31 | 0.70 | Repression reset |
| TMC7 | 2.24 | 3.32 | 4.00 | 2.10 | Sustained induction |
| TMEM107 | 0.22 | 0.20 | 0.31 | 0.47 | Sustained repression |
| TMEM132C | 38.52 | 0.00 | 0.00 | 50.13 | Dynamic |
| TMEM132D | 19.00 | 0.00 | 0.02 | 34.41 | Dynamic |
| TMEM140 | 9.19 | 13.96 | 32.13 | 31.21 | Sustained induction |
| TMEM156 | 4.33 | 4.22 | 20.11 | 4.15 | Sustained induction |
| TMEM54 | 0.12 | 0.20 | 0.29 | 0.95 | Repression reset |
| TMPRSS13 | 0.25 | 0.23 | 0.39 | 0.85 | Repression reset |
| TNFAIP8 | 2.41 | 2.92 | 3.91 | 1.62 | Induction reset |
| TNIK | 3.56 | 8.40 | 17.63 | 7.90 | Sustained induction |
| TONSL | 0.33 | 0.36 | 0.39 | 1.10 | |
| TP53INP1 | 3.11 | 3.01 | 3.30 | 2.22 | Sustained induction |
| TP63 | 1.61 | 4.51 | 34.52 | 16.43 | Sustained induction |
| TPI1 | 0.23 | 0.34 | 0.41 | 0.52 | Repression reset |
| TROAP | 0.36 | 0.28 | 0.27 | 0.56 | Repression reset |
| TSPAN5 | 2.38 | 3.86 | 6.41 | 2.92 | Sustained induction |
| TUBA1B | 0.19 | 0.22 | 0.25 | 0.45 | Sustained repression |
| TUBB | 0.30 | 0.33 | 0.36 | 0.56 | Repression reset |
| TUBB4B | 0.23 | 0.29 | 0.36 | 0.58 | Repression reset |
| TXNIP | 0.31 | 0.22 | 0.36 | 0.44 | Sustained repression |
| TYRO3 | 0.20 | 0.28 | 0.33 | 0.50 | Repression reset |
| U1 | 0.24 | 0.18 | 0.33 | 0.79 | Repression reset |
| U3 | 1.24 | 2.17 | 1.95 | 1.80 | |
| UBB | 0.31 | 0.38 | 0.49 | 0.40 | Sustained repression |
| UBE2C | 0.28 | 0.21 | 0.22 | 0.58 | Repression reset |
| UBE2QL1 | 2.07 | 4.53 | 5.32 | 4.16 | Sustained induction |
| UHRF1 | 0.33 | 0.27 | 0.32 | 0.68 | Repression reset |

| Gene | D2 AFC | D4 AFC | D8 AFC | D16 AFC | Cluster |
|-----------------|--------|--------|--------|---------|----------------------|
| UNC13C | 24.62 | 0.10 | 0.06 | 38.12 | Dynamic |
| UPP1 | 2.23 | 5.94 | 9.75 | 5.93 | Sustained induction |
| UQCRQ | 0.30 | 0.37 | 0.42 | 0.41 | Sustained repression |
| USH2A | 13.62 | 0.02 | 0.03 | 16.11 | Dynamic |
| USP35 | 0.70 | 1.09 | 1.54 | 2.77 | |
| VMP1 | 2.60 | 2.37 | 3.02 | 1.93 | Induction reset |
| VSTM2B | 7.96 | 0.00 | 0.00 | 36.91 | Dynamic |
| WIPF1 | 3.54 | 5.91 | 6.80 | 4.04 | Sustained induction |
| WIPI1 | 2.38 | 3.25 | 4.19 | 2.56 | Sustained induction |
| WLS | 2.59 | 3.48 | 3.91 | 2.16 | Sustained induction |
| XRCC3 | 0.35 | 0.40 | 0.44 | 0.93 | Repression reset |
| YPEL2 | 3.21 | 2.79 | 4.50 | 3.36 | Sustained induction |
| Z93241.1 | 0.24 | 0.21 | 0.31 | 0.63 | Repression reset |
| ZBTB20 | 3.17 | 2.79 | 5.25 | 2.21 | Sustained induction |
| ZMAT4 | 1.72 | 5.72 | 14.92 | 5.98 | Sustained induction |
| ZNF365 | 3.77 | 6.06 | 11.52 | 6.65 | Sustained induction |
| ZNF385B | 2.87 | 4.23 | 3.30 | 1.93 | Induction reset |
| ZNF462 | 1.97 | 3.33 | 3.52 | 1.65 | Induction reset |
| ZNF827 | 2.41 | 3.25 | 3.03 | 1.78 | Induction reset |
| ZWINT | 0.29 | 0.26 | 0.33 | 0.64 | Repression reset |