Exploring Grainyhead-like 2 target genes in breast cancer

Wang, Z.

Chapter 4

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

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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% CO_2. 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 promotor-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^{o}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) database [23].

ChIP-PCR

Chromatin preparation was described previously 24. 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, tcctgggaagaggaatgatg; E2F2 reverse, caggcagcttgggagagtag; CDCA7L forward, tttggggcttgttttgtttt; CDCA7L reverse: ggtgtggaggcctactgtgt; 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 [25].

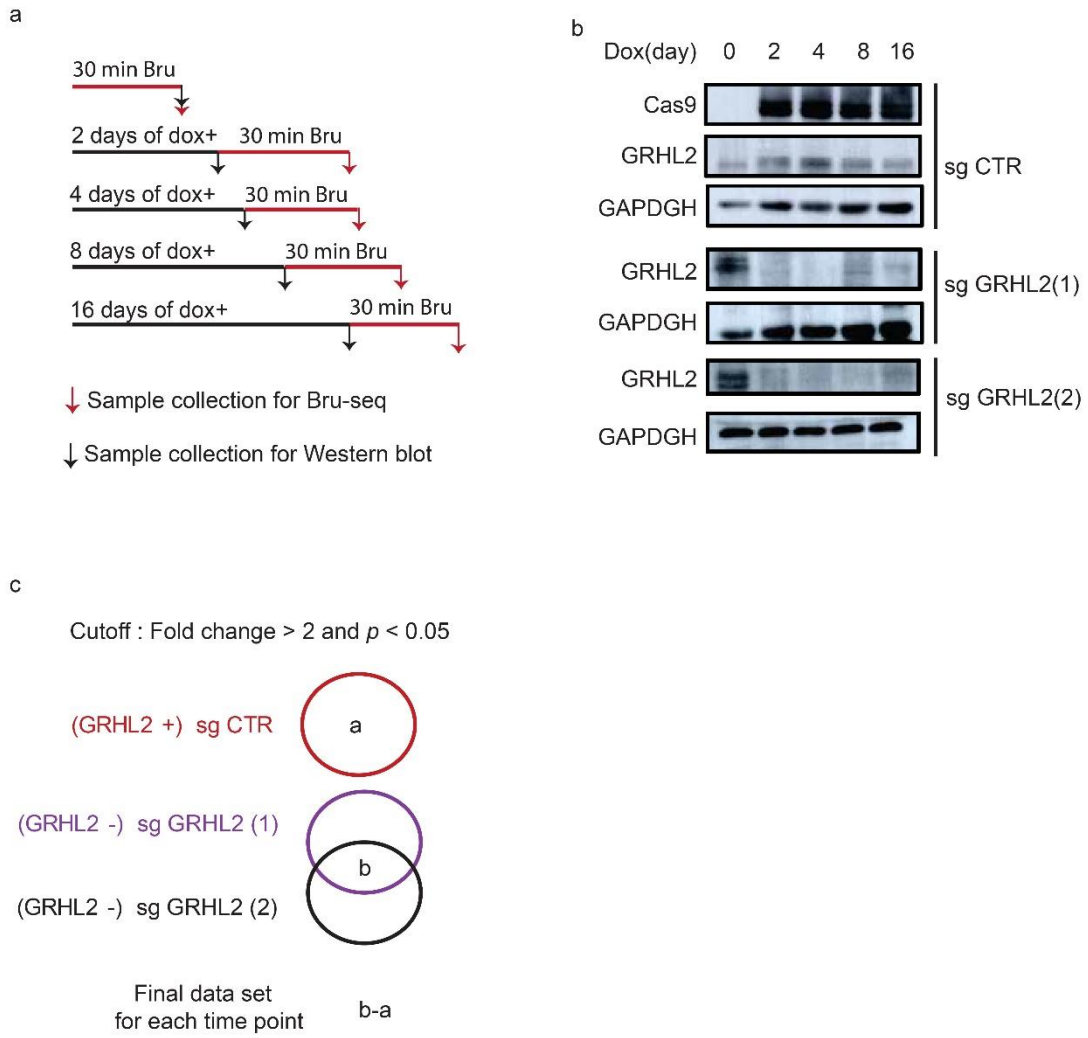


Fig. 1 Bru-seq sample preparation and Bru-seq data analysis strategy. (a) Bromouridine (Bru) labeling of nascent RNA was carried out for 30 minutes at the indicated time points after doxycycline (dox)-induced GRHL2 deletion. **(b)** Western blot analysis of GRHL2 expression levels at the indicated time 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 $\log_2$ 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 β2 unit of the trimeric basement membrane protein laminin-332 [26] 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

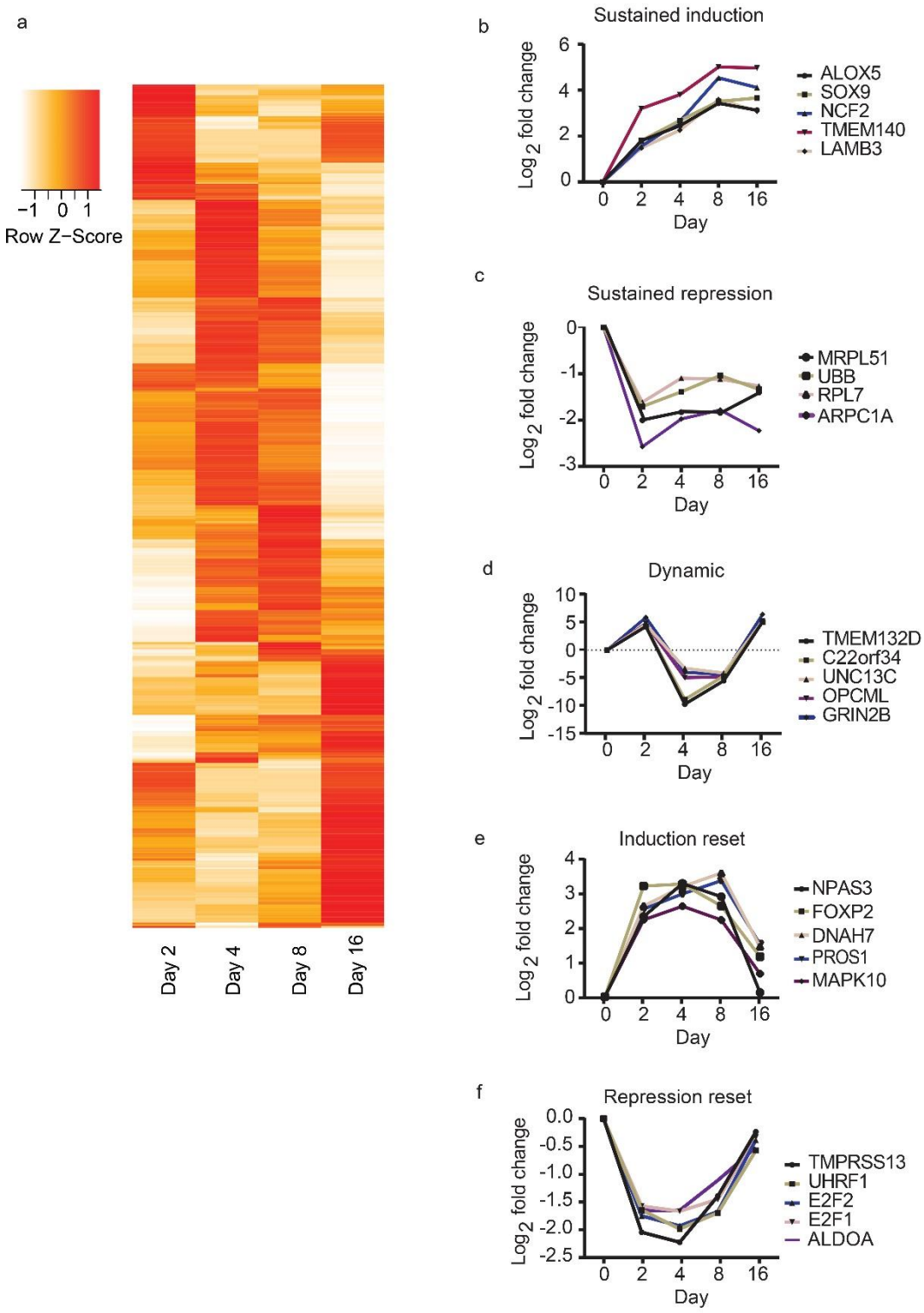


Fig. 2 Dynamic changes in RNA synthesis following GRHL2 loss. (a) Heat map for GRHL2 loss response genes. **(b-f)** After GRHL2 abrogation, genes are categorized according to RNA synthesis patterns. The line graph depicts the $\log_2$ average fold change (AFC) of transcription in sgGRHL2 (1) and sgGRHL2 (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 day16. **Repression reset:** genes with AFC<0.5 at early time points followed by a return to 0.5<AFC<1 at day16.

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*.

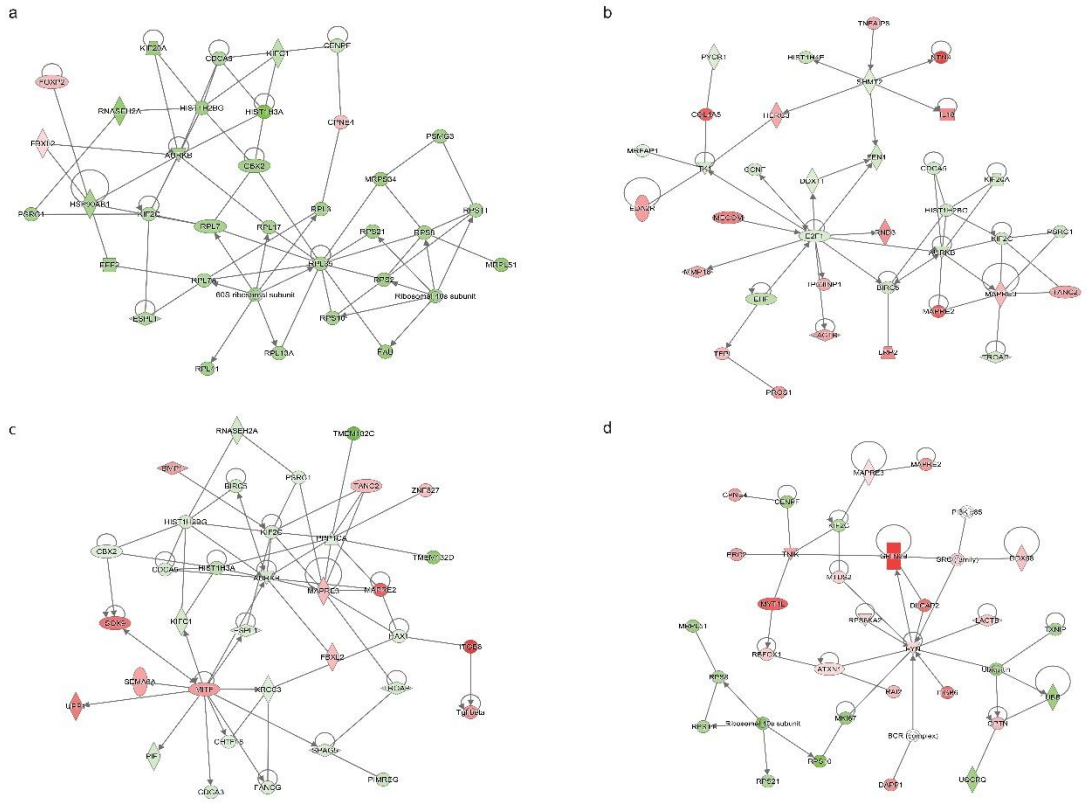


Fig. 3 Networks with the highest scores according to differentially transcribed genes after GRHL2 loss by IPA. (a-d) Networks for day 2, 4, 8 and 16 respectively. The

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.

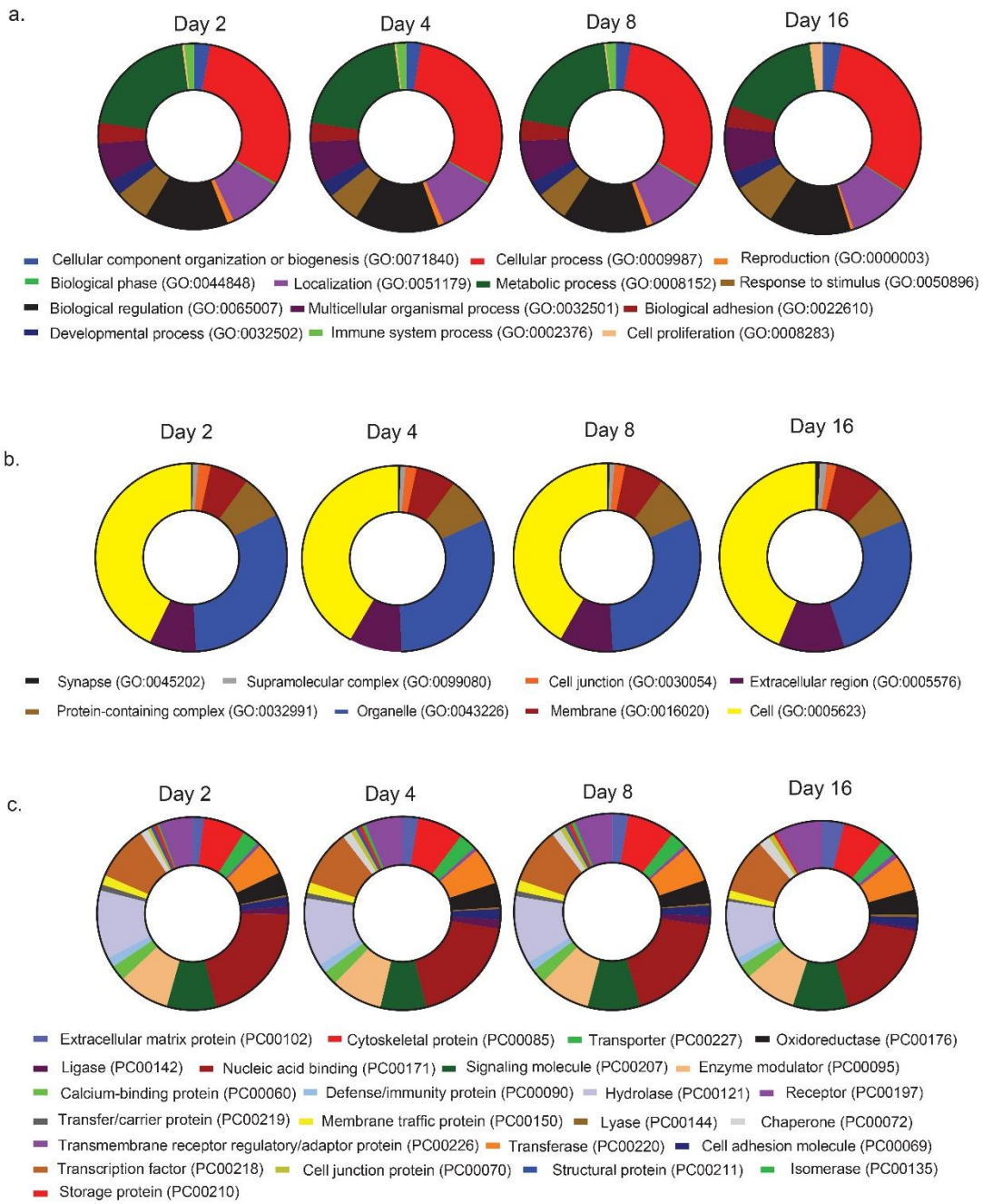
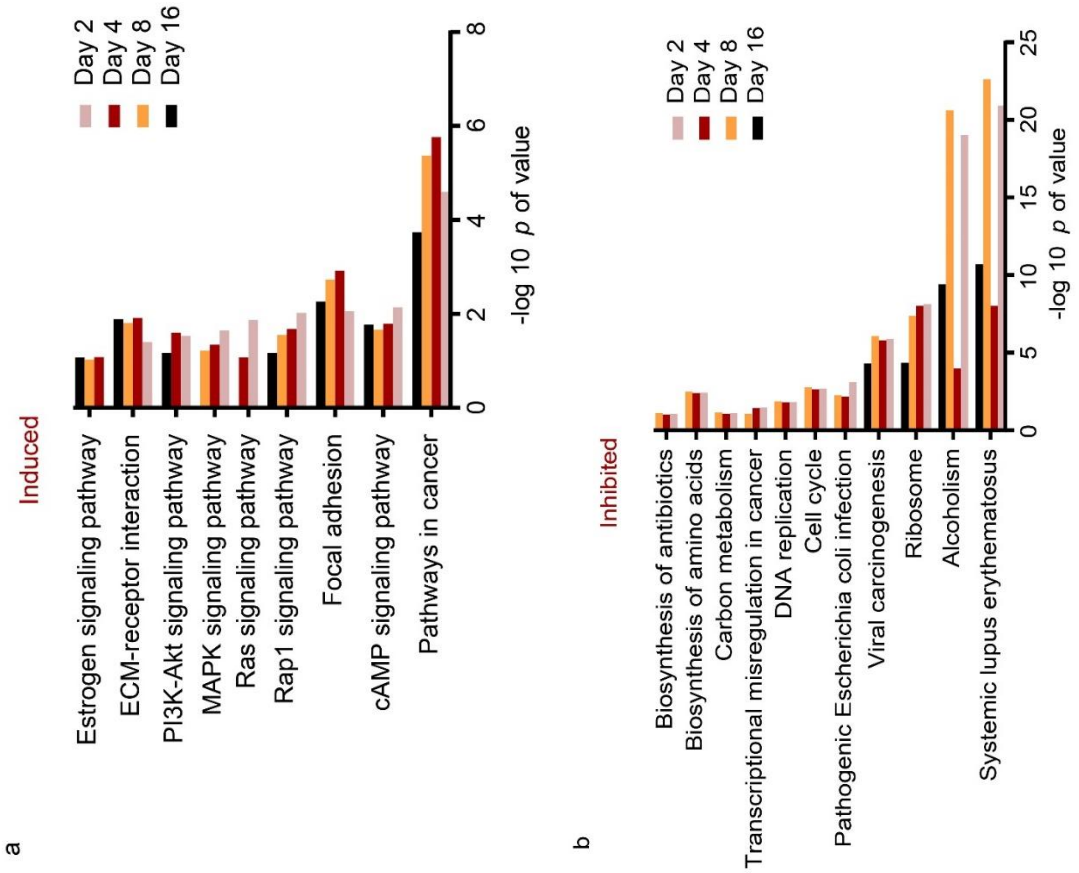


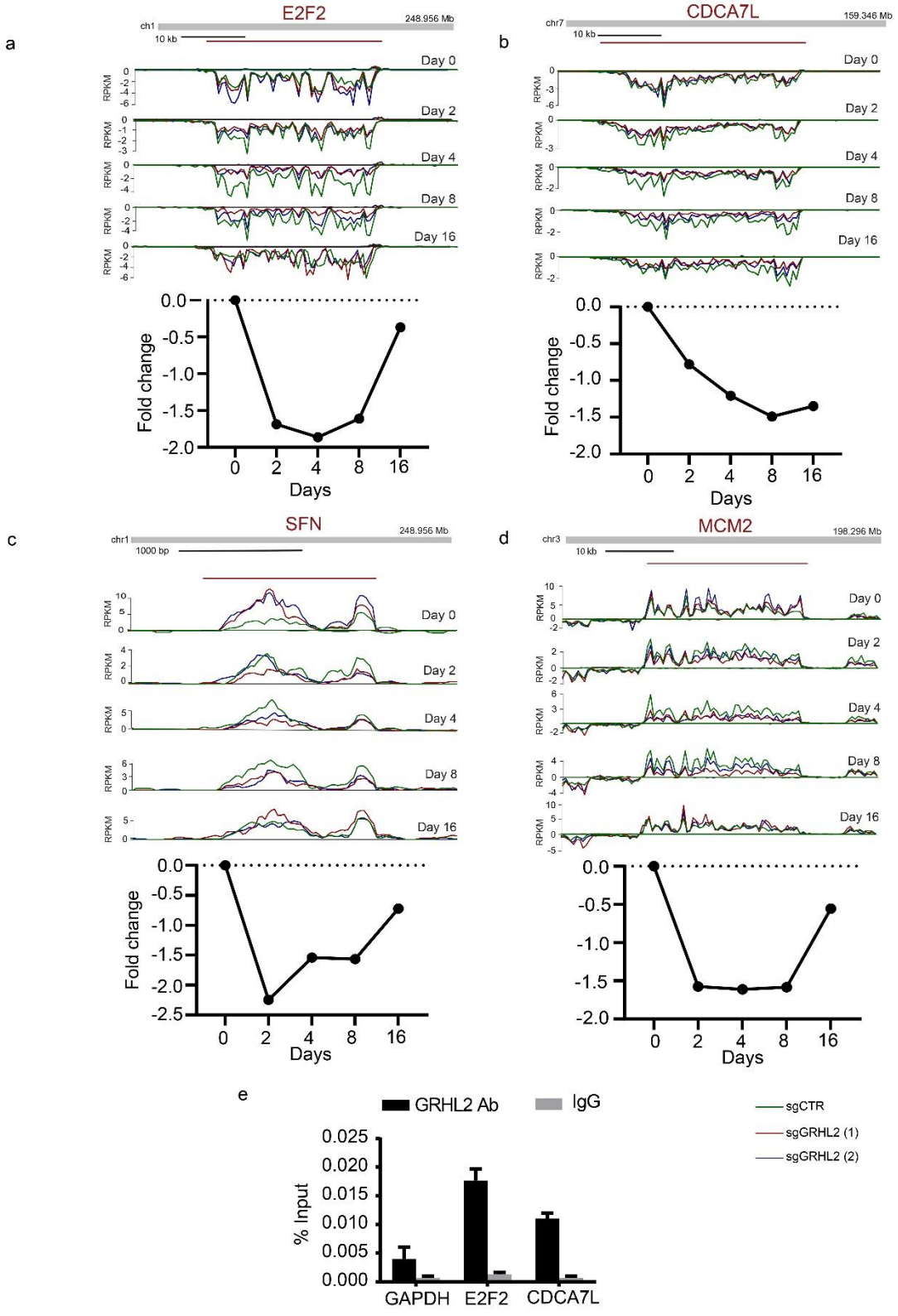
Fig. 4 PANTHER gene ontology enrichment analysis of differentially transcribed genes after GRHL2 loss. Enrichment analyses were carried out for biological process **(a)**, cellular component **(b)** and protein classification **(c).**

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 phenotype [35]. *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 cells [8]. However, our previous ChIP-seq data [24] 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 regulators [40] 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 signaling [42]. 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 synthesis [16] 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 mechanisms [16].

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 replication [50].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.

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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
PACSIN3	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
TGFBI	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