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Genome-wide Promoter Analysis of the *SOX4* Transcriptional Network in Prostate Cancer Cells

Christopher D. Scharer[1,2], **Colleen D. McCabe**[2], **Mohamed Ali-Seyed**[2], **Michael F. Berger**[3,4], **Martha L. Bulyk**[3,4,5,6], and **Carlos S. Moreno**[2,7]

[1] *Program in Genetics & Molecular Biology, Emory University*

[2] *Department of Pathology & Laboratory Medicine, Emory University School of Medicine, Atlanta, GA 30322*

[3] *Division of Genetics, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02115*

[4] *Committee on Higher Degrees in Biophysics, Harvard University, Cambridge, MA 02138*

[5] *Harvard/MIT Division of Health Sciences and Technology, Harvard Medical School, Boston, MA 02115*

[6] *Department of Pathology, Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02115*

[7] *Winship Cancer Institute, Emory University School of Medicine, Atlanta, GA 30322*

Abstract

SOX4 is a critical developmental transcription factor in vertebrates and is required for precise differentiation and proliferation in multiple tissues. In addition, *SOX4* is overexpressed in many human malignancies, but the exact role of *SOX4* in cancer progression is not well understood. Here we have identified the direct transcriptional targets of *SOX4* using a combination of genome-wide localization ChIP-chip analysis and transient overexpression followed by expression profiling in a prostate cancer model cell line. We have also used protein-binding microarrays to derive a novel *SOX4*-specific position-weight matrix and determined that *SOX4* binding sites are enriched in *SOX4*-bound promoter regions. Direct transcriptional targets of *SOX4* include several key cellular regulators such as *EGFR*, *HSP70*, *Tenascin C*, *Frizzled-5*, *Patched-1*, and *Delta-like 1* We also show that *SOX4* targets 23 transcription factors such as *MLL*, *FOXA1*, *ZNF281*, and *NKX3-1* In addition, *SOX4* directly regulates expression of three components of the RNA-induced silencing complex (RISC), namely *Dicer*, *Argonaute 1*, and *RNA Helicase A*. These data provide new insights into how *SOX4* impacts developmental signaling pathways and how these changes may influence cancer progression via regulation of gene networks involved in microRNA processing, transcriptional regulation, the *TGFβ*, *Wnt*, *Hedgehog*, and *Notch* pathways, growth factor signaling, and tumor metastasis.

Keywords

Prostate Cancer; *SOX4*; Transcription; Systems Biology; ChIP-chip

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Corresponding Author: Carlos S. Moreno, Assistant Professor, Department of Pathology & Laboratory Medicine, Winship Cancer Institute, Emory University, Whitehead Research Building, Rm. 105J, 615 Michael St., Atlanta GA, 30322, (404)712-2809 (Ph), (404) 727-8538 (Fx), cmoreno@emory.edu.

INTRODUCTION

The sex determining region Y-box 4 (*SOX4*) gene is a developmental transcription factor important for progenitor cell development and *Wnt* signaling (1,2). *SOX4* is a 47 kDa protein that is encoded by a single exon and contains a conserved high-mobility group (HMG) DNA binding domain (DBD) related to the *TCF/LEF* family of transcription factors that mediate transcriptional responses to *Wnt* signals. *SOX4* directly interacts with *β-catenin*, but its precise role in the *Wnt* pathway is unknown (2). In adult mice, *SOX4* is expressed in the gonads, thymus, T- and pro-B-lymphocyte lineages and to a lesser extent in the lungs, lymph nodes and heart (1). Embryonic knock-out of *SOX4* is lethal around day E14 due to cardiac failure and these mice also showed impaired lymphocyte development (3). Tissue specific knock-out of *SOX4* in the pancreas results in failure of normal development of pancreatic islets (4). *SOX4* heterozygous mice have impaired bone development (5), whereas prolonged expression of *SOX4* inhibits correct neuronal differentiation (6). These studies suggest a critical role for *SOX4* in cell fate decisions and differentiation.

While SOX2 is known to be critical for maintenance of stem cells (7), SOX4 may specify transit-amplifying progenitor cells that are the immediate daughters of adult stem cells and have been proposed to be the population that gives rise to cancer stem cells. In humans, *SOX4* is expressed in the developing breast and osteoblasts and is upregulated in response to progestins (8). *SOX4* is upregulated at the mRNA and protein level in prostate cancer cell lines and patient samples and this upregulation is correlated with Gleason score or tumor grade (9). In addition, *SOX4* is overexpressed in many other types of human cancers, including leukemias, melanomas, glioblastomas, medulloblastomas (10), and cancers of the bladder (11) and lung (12). A meta-analysis examining the transcriptional profiles of human cancers found *SOX4* to be one of 64 genes upregulated as a general “Cancer Signature” (12), suggesting that *SOX4* has a role in many malignancies. Furthermore, *SOX4* cooperates with *Evi1* in mouse models of myeloid leukemogenesis (13). Recently, we showed that *SOX4* can induce anchorage-independent growth in prostate cancer cells (9). Consistent with the concept that *SOX4* is an oncogene, three independent studies searching for oncogenes have found *SOX4* to be one of the most common retroviral integration sites, resulting in increased mRNA (14-16).

Despite these findings, the role that *SOX4* plays in carcinogenesis remains poorly defined. While the transactivational properties of *SOX4* have been characterized (17), genuine transcriptional targets remain elusive. To date, three studies have used expression profiling of cells after either siRNA knockdown or overexpression of *SOX4* to identify candidate downstream target genes (9,11,18). Very recently, 31 *SOX4* target genes were confirmed by chromatin immunoprecipitation (ChIP) in a hepatocellular carcinoma cell line (19). While interesting, this study was limited by the fact that it focused on a specific tumor stage transition and did not use a genome-wide localization approach.

Here, we have performed a genome-wide localization analysis using a ChIP-chip approach to identify those genes that have *SOX4* bound at their proximal promoters in human prostate cancer cells. We have identified 282 genes that are high-confidence direct *SOX4* targets, including many genes involved in microRNA processing, transcriptional regulation, developmental pathways, growth factor signaling, and tumor metastasis. We have also utilized unique protein-binding DNA microarrays (PBMs) (20-22) to query the binding of recombinant *SOX4* to every possible 8-mer. The PBM-derived *SOX4* DNA binding data will further facilitate computational analyses of genomic *SOX4* binding sites. These data provide new insights into how *SOX4* impacts key growth factor and developmental pathways and how these changes may influence cancer progression.

MATERIALS AND METHODS

Cell Culture and Stable Cell Line Construction

All cell lines were cultured as described by ATCC except LNCaP cells which were cultured with T-Medium (Invitrogen, Carlsbad, CA). HA tagged SOX4 was cloned into the pHR-UBQ-IRES-eYFP-ΔU3 lentiviral vector (gift from Dr. Hihn Ly, Emory University) and stable cells isolated as previously described (23).

Chromatin Immunoprecipitation

Two 90% confluent P150s of both LNCaP-YFP and LNCaP-YFP/HA-SOX4 or RWPE-1-YFP and RWPE-1-YFP/HA-SOX4 cells were formaldehyde fixed, sonicated and ChIP assay performed as described previously (23). Anti-HA 12CA5 or mouse IgG was used to immunoprecipitate protein-DNA complexes overnight at 4°C and collected using Dynal M280 sheep anti-mouse IgG beads for 2 hours. Dynal beads were washed, protein-DNA complexes eluted and DNA purified as described previously (24). A detailed description of the ChIP-chip protocol can be found in the supplemental methods. Anti-HA 12CA5, Anti-Flag-M2 (Sigma-Aldrich, St. Louis, MI) or mouse IgG was used to immunoprecipitate protein-DNA complexes overnight at 4°C. All PCR primers used in ChIP-PCR can be found in Supplemental Table 7.

ChIP-chip Analysis

To determine the direct *SOX4* target genes on a global scale we performed ChIP assays in triplicate from the LNCaP cell line stably expressing *SOX4* and in duplicate from a control cell line that expressed YFP alone. Immunoprecipitated and input DNA were subjected to whole genome amplification, Cy3/Cy5 fluorescent labeling, and hybridization to the NimbleGen 25K human promoter array set. Input and immunoprecipitated DNA isolated from LNCaP-YFP and LNCaP-YFP/HA-SOX4 cells was amplified using linker-mediated PCR as described previously (25). Amplified DNA was labeled and hybridized in triplicate by NimbleGen Systems, Inc to their human 25K promoter array. This set consists of two microarrays that tile 4 kb of upstream promoter sequence and 750 bp of downstream intronic sequence on average, with a total genomic coverage of 110 Mb. Raw hybridization data was Z-score normalized and ratios of IP to Input DNA were determined for each sample. ChIPOTle software was used to determine enriched peaks using a 500 bp sliding window every 50 bp as previously described (23). NimbleGen microarray data are available from the GEO database, accession number GEO11915.

Luciferase Assays

PCR fragments representing the binding sites in the *EGFR*, *ERBB2* and *TLE1* genes were cloned in front of the pGL3-promoter luciferase construct (Promega, Fitchburg, WI). Primers sequences used can be found in Supplemental Table 7 LNCaP cells were transfected with 100 ng of a TK-Renilla construct, 500 ng of pGL3-promoter vector alone and with cloned inserts, as well as 500 ng of either a *SOX4* or vector expression construct. Dual Luciferase assays were performed 48 hours post transfection according to the manufacturer's guidelines (Promega, Fitchburg, WI). All assays were performed in triplicate on separate days.

qPCR

LNCaP cells were plated in 6-well culture dishes and grown to 90% confluency before transfection with 1 μg of *SOX4* plasmid or vector control using Lipofectamine-2000 (Invitrogen, Carlsbad, CA). 24 hours post-transfection total RNA was harvested using the RNeasy kit (Qiagen, Valencia, CA) and reverse transcription performed using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA). qPCR was performed using SYBR Green I (Invitrogen, Carlsbad, CA) on a Biorad iCycler using 18s or β-actin as a control and data analyzed using the delta-Ct method (26). All primers used in this study are listed in Supplemental Table 7.

Microarray Analysis

Total RNA was isolated from three independent experiments of either vector control or *SOX4* transfected LNCaP cells as described above. Each transfection was performed in triplicate and each sample was hybridized in duplicate creating six data points for each condition. Total RNA was submitted to the Winship Cancer Institute DNA Microarray Core facility (http://microarray.cancer.emory.edu/). All samples demonstrated RNA integrity (RIN) of 8.3 or greater using an Agilent 2100 Bioanalyzer. RNA was hybridized to the Illumina Human6 v2 Expression Beadchip that query roughly 47,000 transcripts with 48,701 probes, and after normalization significantly changed probes were calculated using Significance Analysis of Microarrays (SAM) software (27). Settings for SAM were: two-class unpaired (X4 vs Vector control), Imputation engine – 10 Nearest Neighbor, Permutations – 500, RNG seed – 1234567, Delta – 1.316, Fold Change – 1.5, False discovery rate – 0.749%. Microarray data are available in the GEO database, accession number GEO11915.

Immunoblotting

Cells were lysed in lysis buffer (0.137M NaCl, 0.02M TRIS pH 8.0, 10% Glycerol, and 1% NP-40), 50 μg total lysate separated by SDS-PAGE electrophoresis and transferred to nitrocellulose for immunoblotting. Immunoblots were probed with polyclonal rabbit *SOX4* antisera described previously (9) and *DICER* (Santa Cruz, Santa Cruz, CA). To control for equal loading immunoblots were also probed with a mouse monoclonal antibody to protein phosphatase 2A (*PP2A*) catalytic subunit (BD Biosciences, San Jose, CA).

RESULTS

SOX4 Transcriptionally Activates EGFR

Using expression profiling to determine the genes whose mRNA levels change when *SOX4* is either overexpressed, or eliminated using siRNA (9), we identified *EGFR* as a candidate *SOX4* transcriptional target (Fig. 1A). Analysis of the promoter and first intron of *EGFR* and other family members with CONFAC software (28) revealed the presence of potential *SOX4* binding sites within the first intron of *EGFR* and *ERBB2* (Fig. 1B). CONFAC functions by identifying the conserved sequences in the 3 kb proximal promoter region and first intron of human-mouse ortholog gene pairs and then identifying transcription factor binding sites (TFBS), defined by position weight matrices from the MATCH software (29), that are conserved between the two species (28).

While limited commercial antibodies exist for *SOX4* and show activity in immunoblots, in our hands, none of them have been useful in a ChIP assay. Therefore, we employed epitope-tagged *SOX4* as described in other SOX4 ChIP studies (9,19). While the FLAG epitope tag was not tested directly for activity, a GST-SOX4 construct demonstrated binding to a known *SOX4* motif and not a control motif (Supplemental Fig. 2B), validating that the epitope tag does not interfere with *SOX4* binding. To determine if *SOX4* directly bound the *EGFR* and *ERBB2* enhancers, we performed ChIP analysis on RWPE-1 prostate cancer cells stably infected with FLAG-SOX4 or a control lentiviral vector. DNA representing the predicted SOX4 sites was specifically amplified from the FLAG-SOX4 cell line and not from the control cell line, indicating that *SOX4* binds to intronic sequence of *EGFR* and *ERBB2* (Fig. 1C). EGFR is expressed in RWPE-1 cells, but not in LNCaP cells, and *SOX4* did not bind to these sequences in LNCaP cells (data not shown).

To characterize the transcriptional effect of *SOX4* levels on the regions bound by *SOX4* in ChIP assays, the amplified ChIP fragments were cloned in front of a minimal promoter luciferase reporter plasmid and tested in transient transfections in LNCaP cells. Compared to a vector control, *SOX4* significantly increased transcription of the *EGFR* fragment 3-fold and the *TLE1* positive control fragment roughly 4-fold. While not found significant, *ERBB2* was activated 1.5-fold compared to the vector control (Fig. 1D). Consistent with microarray data, *SOX4* transcriptionally activates the *EGFR* enhancer.

Genome-wide Localization Analysis

To determine the direct *SOX4* target genes on a global scale we performed ChIP assays in triplicate from the LNCaP HA-SOX4 stable cell line and in duplicate from the control LNCaP-YFP cell line. Peaks ($p < 0.001$) that overlapped in at least two of the three data sets and were not present in the LNCaP-YFP cell line were called significant (Fig. 2A). Based on these parameters, we classified 3,600 significant, overlapping peaks as *SOX4* target sequences. Since some transcription start sites (TSS) are quite close to each other (< 3 kb), it was not always possible to assign a unique gene to every peak. In addition, many genes had multiple peaks in their promoters, and thus we mapped the 3,600 peaks to 3,470 different genes (Supplemental Table 1).

To verify the set of 3,600 *SOX4* peaks, 28 candidate *SOX4* target sites representing a range of p-values in promoters of genes of biological interest were chosen, primers were designed around the peaks, and enrichment was verified by conventional ChIP. Ten of these 28 candidates were analyzed by ChIP quantitative real-time PCR (qPCR) and 18 by ChIP-PCR. Overall, 24/28 (86%) of the candidate targets were confirmed, validating our dataset. All 10 of the peaks chosen to validate by qPCR were reproducibly enriched over the *YFP* control in both the LNCaP-HA-SOX4 cell line as well as the RWPE-1 cell line (Fig. 2B). Of the target sites validated by conventional PCR, 14 of 18 genes were confirmed in both the LNCaP and RWPE-1 cell lines while a mock, control PCR was negative (Fig. 2C and 2D and data not shown). The only exception was *ANKRD15*, which was enriched only in the LNCaP cell line and not in the RWPE-1 line.

Target Gene Expression Analysis

To determine whether *SOX4* binding affects transcription of the 3,470 genes that have *SOX4* bound at their promoters, we performed whole genome expression analysis on LNCaP cells after transfection with *SOX4* or a control vector. To increase the likelihood of identifying direct *SOX4* targets, total RNA was isolated at a relatively early timepoint (24 hours post-transfection) and hybridized to Illumina Human 6-v2 whole genome arrays. A total of 1,766 genes were changed at least 1.5-fold with a false discovery rate (FDR) of 0.749% (Fig. 3A, and Supplemental Table 2). Of those 1,766 genes, 244 were also direct *SOX4* targets by ChIP-chip analysis (Fig. 3A, and Supplemental Table 3). Seven of these genes were confirmed by qPCR (Fig. 3B).

Our previous expression profiling of LNCaP cells after *SOX4* siRNA knockdown (9) identified 465 downstream targets, and we confirmed that *SOX4* regulates the expression of *DICER*, *DLL1* and *HES2* in LNCaP cells by qPCR (Fig. 3B). We further confirmed *SOX4*'s regulation of *DICER* at the protein level (Fig. 3C). Out of those 465 candidate targets, 47 genes overlapped with the 3,470 ChIP-chip targets, increasing the number of direct *SOX4* targets to 282 genes (Fig. 3A and Supplemental Table 3). We classified these 282 genes bound by *SOX4* in ChIP-chip and significantly changed by expression profiling as high confidence direct *SOX4* target genes. Nine genes (*PIK4CA*, *DHX9*, *BTN3A3*, *CDK2*, *MVK*, *ADAM10*, *RYK*, *ISG20*, and *DBI*) overlapped in all three datasets. The transcription factor *SON* and purine biosynthetic enzyme *GART*, two genes on chromosome 21 that are transcribed in opposite directions and regulated by a bidirectional promoter, were affected in opposite ways. *SON* was activated by *SOX4* 1.8-fold as detected by *SOX4* overexpression, while *GART* was increased almost 3-fold as determined by *SOX4* siRNA knockdown, suggesting that *SOX4* regulates the directionality of this promoter.

We next analyzed the p-values of the peaks in our ChIP-chip dataset, comparing the p-values of the genes that were altered by transient overexpression of *SOX4* with those that were not (Supplemental Fig. 2). We found no difference in the distributions of the ChIP-chip p-values for those genes that were changed in expression profiling experiments and those that were not. Thus, based on our ChIP-chip validation experiments and the similar p-value distributions, we conclude that *SOX4* is genuinely bound at the promoters of the 3,188 genes that did not change, but that *SOX4* by itself is not limiting or sufficient to generate changes in transcription without corresponding changes in the cellular context, such as activation of co-factors or signaling pathways.

Novel SOX4 Position Weight Matrix (PWM)

To facilitate computational analyses of *SOX4* DNA binding sites we sought to determine the DNA binding preferences of *SOX4* using universal protein-binding microarrays (PBMs) (20). This universal PBM array allows recombinant *SOX4* protein to interact with and bind every possible 8-mer, thus allowing *in vitro* binding site specificities to be calculated.

We generated an N-terminal, GST-SOX4-DBD fusion protein, expressed and purified it from *E. coli, and tested for activity* (Supplemental Fig. 3). The GST-SOX4-DBD was incubated with the protein binding microarray and a novel PWM (RWYAAWRV) was calculated from the PBM data (Supplemental Table 4) using the Seed-and-Wobble algorithm (Fig. 3D) (20). Three groups have previously reported similar binding site sequences for *SOX4*: AACAAAG (30), AACAAT (31) and WWCAAWG (19). Our PWM confirms the *SOX4* core binding sequence of the previously known binding sites, but there are some differences in the specificity at the 1st and 7th positions and we find a bias towards A,C and G at the 8th position. These differences could be due to the fact that earlier reports used no more than 31 sequences to develop the binding motif while our study queried every possible 8-mer.

SOX4 Peaks Contain SOX4 Binding Sites

Using our newly derived PWM, we applied CONFAC software (28) to analyze the enriched sequences for the presence of *SOX4* binding sites. We analyzed the sequences of the peaks in the promoters of our 282 high confidence genes against 10 sets of control promoter sequences to see if *SOX4* sites were enriched in our target gene set. Control promoter peaks, of equal size to SOX4 peaks, were chosen randomly from sequences covered by the NimbleGen array and each control set contained equal total sequence coverage as our 282 high confidence peaks. With stringent criteria (core similarity ≥ 0.85, matrix similarity ≥ 0.75) we find 60% of the peaks contain *SOX4* binding sites. *SOX4* sites were significantly enriched relative to 10 sets of random promoter sequence, by Mann-Whitney U-test using Benjamini correction for multiple hypothesis testing ($q < 0.0019$).

To further characterize the SOX4 binding sites we searched the entire set of 3600 SOX4 peaks, and 10 equal sets of random promoter sequence for the presence of PBM-bound k-mers (here, ungapped 8-mers). The specificity of PBM k-mers can be quantified by the enrichment score (ES), which ranges from -0.5 to 0.5 (32). We analyzed the enrichment of PBM k-mers with $0.45 > ES > 0.40$ (moderate) and $ES > 0.45$ (stringent). While both SOX4-bound peaks and random promoter sequence contained moderate and stringent k-mers, SOX4 peaks contained significantly more stringent ($p = 0.0002$) and moderate ($p = 1.08 \times 10^{-5}$) k-mers by two-tailed Mann-Whitney test (Supplemental Fig. 4).

To investigate interaction with protein partners that may increase *SOX4*'s affinity for 'poor' matching sites *in vivo*, we searched for enrichment of co-occuring TFBS in the *SOX4* peaks. We applied CONFAC software to search the sequences for the presence of co-occurring transcription factor binding sites within the same peak (Table 1). Using the same criteria as above, we determined that the *E2F* family had the most frequently co-occurring motif (similar to TTTCGCGC, q-value $=1.78 \times 10^{-11}$). Interestingly, Ingenuity Pathway Analysis (IPA) identified Cell Cycle as a functionally enriched process in the 3,470 *SOX4* target genes ($p = 0.00916$), suggesting that part of *SOX4*'s function is to control the expression of genes involved in cell-cycle progression.

CONFAC analysis identified other significant TFBS motifs enriched in the *SOX4* peaks (Table 1), including those for transcription factors in the *TGFβ, Wnt*, and *NF-κB* pathways. *SOX4* modulates *Wnt* signaling via interaction with *β-catenin* and the *TCF4* transcription factor (2), suggesting a possible role for *SOX4* in transcriptionally modulating *Wnt* signals. We confirmed the recent report that *SOX4* cooperates with constitutively active *β-catenin* to activate TOP-Flash luciferase reporters (2), and found that *SOX4* synergistically induces activation of these constructs, further highlighting a role for *SOX4* in the *Wnt* pathway (Supplemental Fig. 5).

SOX4 Target Genes

In order to determine the biological processes and functions of the *SOX4* targets we performed a Gene Ontology analysis using DAVID software (33) on the 282 high confidence *SOX4* targets. Among the *SOX4* targets were 23 transcription factors (Table 2), and DAVID analysis determined that the top annotations were transcription ($p = 3.7\times10^{-18}$), transmembrane ($p = 5.59\times10^{-10}$) and protein phoshorylation/dephosphorylation ($p = 3.5\times10^{-18}/6.6\times10^{-7}$). These findings are paralleled by expression profiling of *SOX4* overexpression in HU609 bladder carcinoma cells where top annotated functions were signal transduction and protein phosphorylation (11).

Commercial Ingenuity Pathway Analysis (IPA) software[1] identified biological pathways and functions that are enriched in our 282 high confidence targets, as well as the 1,766 significant genes identified by SAM analysis, and the 3,470 unique genes that had *SOX4* bound at their promoters in ChIP-chip. As anticipated, among the most significant annotations were cell cycle, cancer, and tissue development. In the significant expression data set of 1,766 genes we observed an upregulation of three Frizzled family receptors, *FZD3*, *FZD5* and *FZD8*, as well as the downstream transcription factor *TCF3* Overall, IPA analyses discovered key components of the *EGFR, Notch, AKT-PI3K*, microRNA, and *Wnt-β-catenin* pathways as *SOX4* regulatory targets. Based on these findings, we built *SOX4* regulatory networks found in prostate cancer cells (Fig. 4 and Supplemental Fig. 6). *SOX4* target genes comprise key pathway components such as ligands (*DLL1* and *NGR1*), receptors (*FZD5* and *PTCH1*), an *AKT* regulatory kinase (*PDPK1*), and downstream transcription factors (*FOXO3* and *HES2*). In addition, *SOX4* activates expression of *tenascin C* (*TNC*), an extracellular matrix protein that is a target of *TGFβ* signaling (34) and β-catenin (35). In addition, SOX4 regulates three components of the RISC complex, *DICER*, *AGO1*, and *RHA/DHX9* (Supplemental Table 3). We confirmed these data by qPCR (Fig. 3B) and by western blot for *DICER* (Fig. 3C).

Gene Set Enrichment Analysis (GSEA) (36) and GSEA Leading Edge analysis (37) of these gene sets identified *TGFβ*-induced *SMAD3* direct target genes (Supplemental Table 5) as enriched in SOX4 target genes. *SOX4* is upregulated by *TGFβ-1* treatment (4,38) and we found *SMAD4* sites are significantly enriched in the *SOX4* ChIP-chip peaks (Table 1), suggesting that

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[1] http://www.ingenuity.com

SOX4 impacts key developmental and growth factor signaling pathways in prostate cancer cells at both the transmembrane signaling and transcriptional levels.

DISCUSSION

While many studies have identified SOX4 as a crucial developmental transcription factor that is often overexpressed in many types of malignancies, little is known of what SOX4 regulates in cancer cells. We have utilized a ChIP-chip approach to report the first genome-wide localization analysis of *SOX4 and* mapped 3,600 binding peaks that represent 3,470 unique genes possibly under the transcriptional control of *SOX4* We have also identified 1,766 genes that respond to increased *SOX4* levels by whole genome expression profiling. Integration of these datasets mapped 282 high-confidence direct targets in the *SOX4* transcriptional network. In addition, we have utilized protein-binding microarrays to determine a novel PWM specific for *SOX4* and show that our ChIP-chip predicted peaks are significantly enriched for *SOX4* binding sites. These data provide several new insights into the roles that SOX4 plays in the cell.

SOX4 Direct Target Genes

Although only 10% of the significant differentially expressed genes overlapped with the ChIP-chip data, this is likely a conservative estimate, because the NimbleGen 25K promoter array only queries proximal promoter sequences, and none more than 1 kb downstream of the TSS. We found that *SOX4* binds *EGFR* and *ERBB2* in the first intron over 20 kb downstream of the TSS (Fig. 1D), and unsurprisingly we did not detect *EGFR* or *ERBB2* in our ChIP-chip experiment. Thus, more of the 1,900 genes that responded to changes in *SOX4* mRNA levels (but were not detected by ChIP-chip) could still be direct targets. Excellent candidates would be the 40 genes that responded to *SOX4* on both microarray platforms, such as the *IL6* receptor, *SOX12*, and *NME1* (Supplemental Table 6). While 3,600 is a fairly large number of *SOX4* bound regions, some background can be expected. Nevertheless, we were able to validate 24 out of 28 (86%) candidate binding sites chosen adding confidence to our data set. In fact, an even higher number of over 4,200 genomic binding sites had been previously observed for *c-Myc* in ChIP-PET whole genome studies (39). Whole genome tiling arrays or ChIP-seq could provide additional binding sites that may show more overlap with the Illumina expression data set.

Conversely, many of the bound genes may not respond to changes in *SOX4* mRNA levels alone, but to multiprotein activator complexes of which *SOX4* is only one component. Furthermore, the stability of SOX4 bound to a promoter could be greater than unbound SOX4, limiting the effects observed by siRNA knockdown. In different cell types or cellular contexts, *SOX4* may activate a different subset of these genes. Of the 31 *SOX4* target genes reported by Liao *et al* (19) only six are represented in our NimbleGen data set and three found to be changed in our Illumina expression profiling data set. The small overlap could be due to the fact that those genes were identified in hepatocellular carcinomas, while we have examined prostate cancer cells. Interestingly, *DKK* was one of the six genes that overlapped in both data sets, further implicating *SOX4* in the *Wnt* pathway. Since *SOX4* is known to interact with *β-catenin* and other co-activators, it may be poised at many of these promoters to enable responses to developmental signals from the *Wnt* or *TGFβ* pathway.

Receptor and Signaling Regulation

Our data suggest that *SOX4* regulates cellular differentiation through a variety of transcription factors and receptors. *SOX4* is upregulated in response to numerous external ligands ranging from *TGFβ* (38) and *BMP-6* (40) to parathyroid hormone and progesterone (8). Previous work has shown that *SOX4* directly signals from *IL-5Rα* (41) and here we have shown that *SOX4* directly regulates the *EGFR* receptor (Fig. 1). Membrane receptors in the *SOX4* transcriptional network also include Frizzled family members *FZD3, FZD5, FZD8*; the Hedgehog receptor *PTCH-1*; the Notch ligand *DLL1*; TRAIL decoy receptor *TNFRSF10D*; and other growth factor receptors such as *FGFRL1* and *IGF2R*. DAVID analysis also revealed protein phosphorylation/dephosphorylation ($p = 3.5\times10^{-18}/6.6\times10^{-7}$) and transcription ($p = 3.7\times10^{-18}$) are enriched annotations, identifying 23 transcription factors that are direct targets of *SOX4* This evidence suggests that *SOX4* regulates signaling events both at the external “input” level as well as the internal “output” or transcription level. This regulation could be direct, as with *IL- 5Rα*, or through the transcriptional targets *SOX4* activates.

Transcription Factors and SOX4

Here we have reported DNA binding specificity data for *SOX4*, which will improve computational analyses for *SOX4* specific binding sites. Our data confirm the known SOX family core-binding motif and adds new specificity at the 1st, 7th and 8th positions. While crystal structure evidence from *SOX2* has shown the importance of the core-binding motif, it is possible that the specificity for *SOX4* is enhanced outside of the core motif at the extra positions. A limitation of these data is that we did not assess how other DNA binding proteins influence the sequences to which *SOX4* can bind. The enrichment of *SMAD4* sites is particularly interesting in light of the GSEA results, which suggest that *SOX4* regulates many *TGFβ* target genes, including *Tenascin C*. Thus, we hypothesize that *SOX4* may physically interact with *SMAD4* in response to *TGFβ* signals. Experiments to test this hypothesis are underway. Nevertheless, evidence points to a role for *SOX4* in modulating other transcriptional programs via hierarchical regulation of 23 downstream transcription factors.

SOX4 and Cancer

Based on the target genes we identified, *SOX4* appears to influence cancer progression in several ways. First, it plays a key role in activation of, and response to, developmental pathways such as *Wnt, Notch, Hedgehog,* and *TGFβ*. Second, *SOX4* inhibits differentiation via repression of transcription factors such as *NKX3.1*, and activation of *MLL* and *MLL3*, two histone H3 K4 methyltransferases that induce activation of HOX gene expression (42). *MLL* methyltransferase complexes also facilitate *E2F* activation of S phase promoters, facilitating cell cycle progression. Activation of *MLL* also suggests a mechanism for SOX4’s role in myeloid leukemogenesis, since *MLL* is a critical oncogene that is often translocated or amplified in this disease (43). Thirdly, *SOX4* targets growth factor receptors such as *EGFR, FGFRL1*, and *IGF2R*, enhancing proliferative signals in tumors and potentially activating the *PI3K-AKT* pathway. Mice heterozygous for *NKX3.1* and *PTEN* in the prostate develop prostate adenocarcinomas and metastases to the lymph node (44). Thus, our data suggest that *SOX4* may promote prostate cancer progression directly through *NKX3.1* repression and indirectly through *PI3K-AKT* activation. Finally, *SOX4* appears to promote metastasis via upregulation of *tenascin C*. Recently, both *SOX4* and *tenascin C* were shown to enhance metastasis of breast cancer cells to the lung (45), as has the *TGFβ* pathway which activates their expression (46). Other metastasis-associated *SOX4* target genes include *Integrin αV* and *Rac1 Rac1* was recently shown to control nuclear localization of *β-catenin* in response to *Wnt* signals (47).

SOX4 regulates components of the RISC Complex and small RNA pathway

MicroRNAs (miRNAs) are small noncoding RNA species that regulate the translation and stability of mRNA messages for hundreds of downstream target genes via partial complementarity to short sequences in the 3’UTRs of messenger RNAs. The RNA-induced silencing complex (RISC), which is composed of *Argonaute 1* (*AGO1*) or *Argonaute 2* (*AGO2*), *TRBP*, and *Dicer* processes miRNAs from precursors (pre-miRNAs) to their mature form, cleaves target mRNAs, and participates in translational inhibition. *RNA Helicase A*

(*RHA/DHX9*) interacts with the RISC complex and participates in loading of small RNAs into the RISC complex (48). We observed that three components of the RISC complex, *DICER*, *AGO1*, and *RHA/DHX9*, are high-confidence direct targets of *SOX4* (Supplemental Table 3) and we confirmed these data by qPCR (Fig. 3B). *Dicer* has been independently observed to be overexpressed in prostate cancers (49).

In addition, we observed that *Toll-like Receptor 3* (*TLR3*), which binds to double-stranded RNAs, induces gene silencing, and can induce apoptosis (50), was induced 2.8-fold upon overexpression of *SOX4* This induction may be indirect, since *TLR3* was not detected by ChIP-chip, but we cannot exclude the possibility that *SOX4* may directly regulate *TLR3* from a distal or intronic enhancer.

Our observation that *SOX4* targets three genes important in small RNA processing is of particular interest in light of *SOX4*'s role in development and cancer progression. MicroRNAs have been implicated in numerous physiological processes from development to oncogenesis. MiRNAs can also act as suppressors of breast cancer metastasis via targeting of *tenascin C* and *SOX4* (45), and as promoters of breast cancer metastasis (51). The finding that *SOX4* can affect expression of multiple components of the RISC complex also provides insight into why long-term loss of *SOX4* induces widespread apoptosis (9,18). In summary, these data shed light on the mechanisms and pathways through which *SOX4* may exert its effects during development and cancer progression. Further studies are necessary to elucidate the precise role of SOX4 in the functioning of these pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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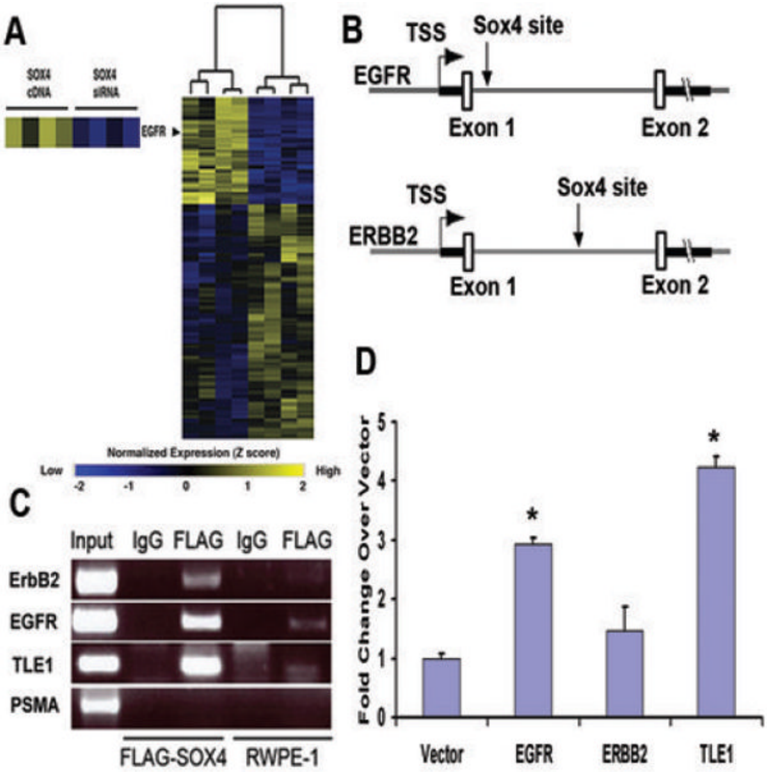


Figure 1.

(A) Affymetrix U133A GeneChip microarray analysis of *SOX4* overexpression and knockdown in LNCaP prostate cancer cells. Overexpression of *SOX4* leads to increased *EGFR* expression while siRNA knockdown of *SOX4* results in decreased *EGFR* expression. **(B)** Schematic showing the location of the *SOX4* binding site in the first intron of the *EGFR* (Top) and *ERBB2* (Bottom) genes. Arrows denote location of the *SOX4* binding site. **(C)** ChIP assay of FLAG-SOX4 bound to the introns of *EGFR, ERBB2* and *TLE1 PSMA* is shown as a negative control. *SOX4* bound DNA is specifically amplified in the FLAG IP lane from FLAG-SOX4 expressing cells (lane 3) and not control cells (lane 5) or with a non-specific antibody (lanes 2 and 4). **(D)** Luciferase reporter assays with *SOX4* binding sites showing activation in the presence of *SOX4* compared to empty vector. * indicates p-value less than 0.01 by students T-test and error bars indicate 1 SD (n = 3 independent biological replicates performed on separate days).

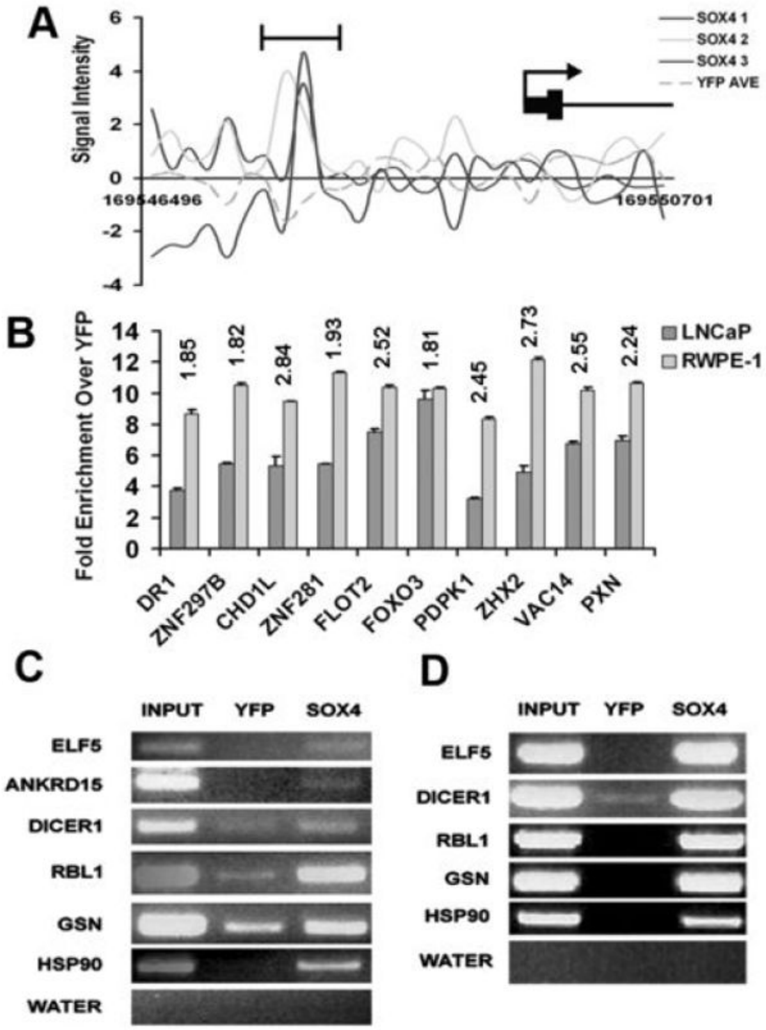
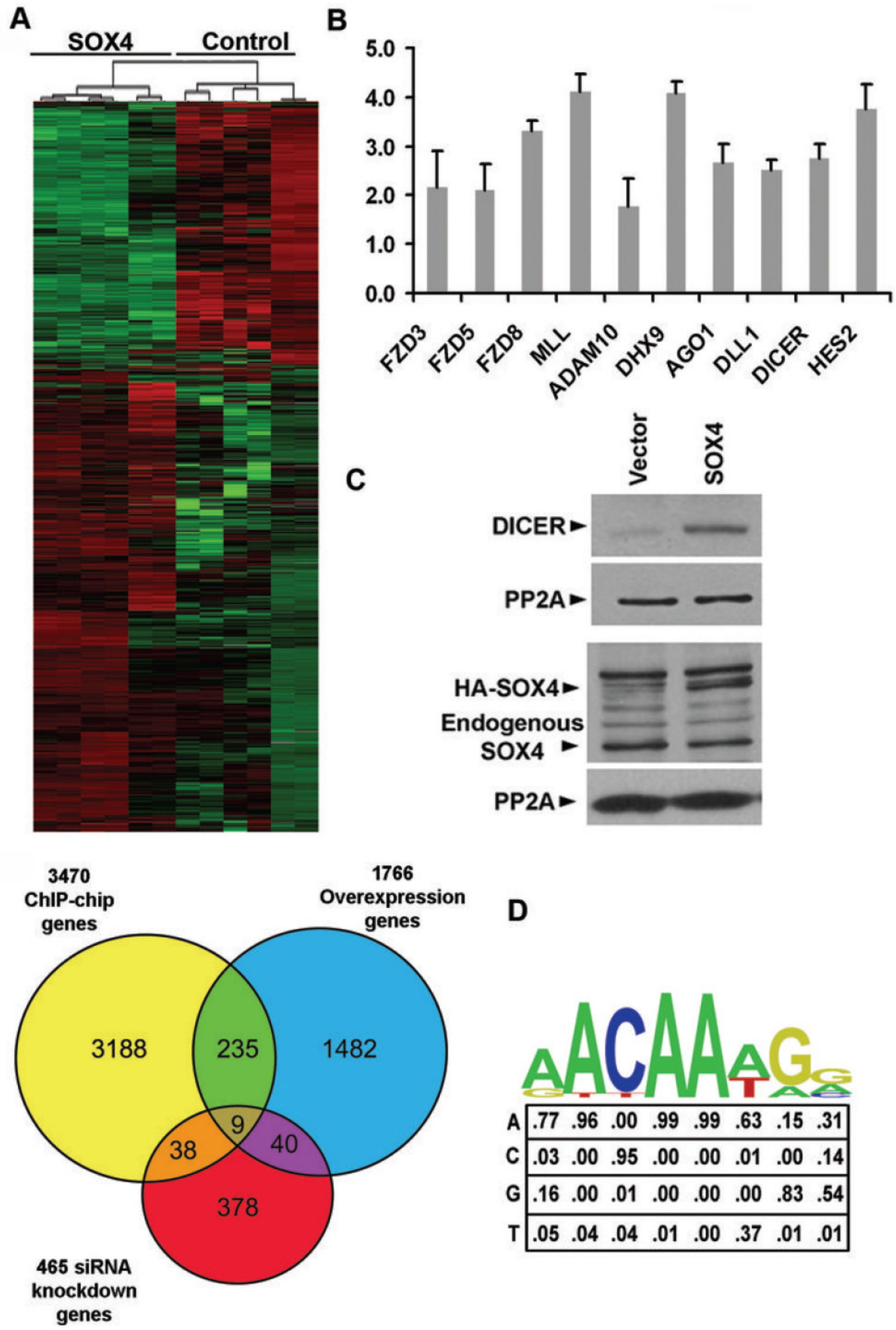


Figure 2.

(A) Graph showing enrichment in the three HA-SOX4 lanes over the average of the two YFP replicates for the *SOX4* target gene *FMO4* Y-axis is the signal intensity across the genomic coordinates on the X-axis. **(B)** qPCR ChIP analysis of 10 randomly selected genes verified in both the RWPE-1 and LNCaP cell lines. Graph shows fold enrichment of the HA-SOX4 IP over the YFP negative control IP. Numbers above the bars represent the mean log2 of fold enrichment of ChIP-chip signal for the probes contained in the peak relative to YFP. Error bars indicate 1 SD (n = 3 technical replicates). **(C) and (D)** Genes that were verified by conventional ChIP assay. HA-SOX4 and YFP cells were subjected to conventional ChIP followed by PCR in both the LNCaP **(C)** and RWPE-1 **(D)** prostate cell lines. Six genes verified in the LNCaP cell lines and five in the RWPE-1 cell lines.



A	.77	.96	.00	.99	.99	.63	.15	.31
C	.03	.00	.95	.00	.00	.01	.00	.14
G	.16	.00	.01	.00	.00	.00	.83	.54
T	.05	.04	.04	.01	.00	.37	.01	.01

Figure 3.

(A) Heat map (top) illustrating Illumina expression data of the 1,766 significant genes as determined by SAM analysis. Red indicates overexpressed and green denotes underexpressed genes. Venn diagram (bottom) depicts the overlap between 3,470 ChIP-chip *SOX4* direct target genes, the Illumina expression data set of 1,766 genes, and the Affymetrix expression dataset of 465 genes. **(B)** qPCR expression analysis of *SOX4* direct target genes after *SOX4* overexpression in LNCaP cells. All ten genes were upregulated over a vector control transfection, similar to values determined by the Illumina array with a p-value less than 0.005 by students T-test. Error bars indicate 1 SD (n = 3 independent biological replicates performed on separate days). **(C)***DICER* protein expression is upregulated by *SOX4* HA-SOX4 or vector control were transfected into LNCaP cells and immunoblots were probed for DICER, SOX4, and PP2Ac as a loading control. **(D)** PBM-derived 8mer PWM for *SOX4* displayed both graphically and numerically for each base position derived from incubation of recombinant GST-SOX4-DBD with a universal ‘all 8-mer’ double-stranded DNA protein-binding microarray. With stringent criteria (core similarity ≥ 0.85, matrix similarity ≥ 0.75) we find 60% of the peaks in the 282 high-confidence promoters contain *SOX4* binding sites.

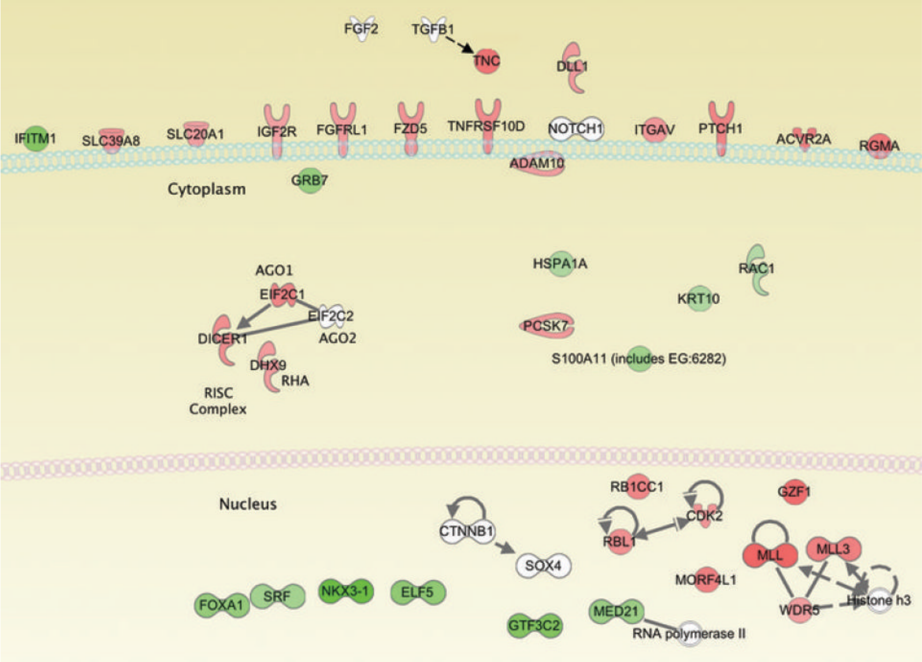


Figure 4.

IPA analysis of direct target genes graphically illustrating the cellular location of the *SOX4* transcriptional target genes. *SOX4* regulates a host of nuclear and membrane localized proteins as well as multiple components of the RISC complex. Red indicates target genes upregulated by *SOX4*, green denotes downregulated genes and white represents genes for which no expression change was detected.

Table 1

Benjamini corrected q-values for co-occurring transcription factor binding sites.

Transcription Factor	Family	Benjamini Corrected q-value
E2F4	E2F	1.78E-11
E2F1	E2F	3.06E-11
PAX5	Paired Box	2.07E-10
WHN	Forkhead	2.94E-10
SMAD3	SMAD	1.82E-09
SMAD4	SMAD	3.33E-09
MYC	MYC	6.25E-09
NFKAPPAB	NF-κB	2.95E-08
LEF1/TCF1	LEF	1.12E-06

Table 2

DAVID analysis identified 23 transcription factors present in our high confidence SOX4 target genes. GO Term: transcription, DNA dependent ($p = 3.7\times10^{-18}$).

Entrez ID	Symbol	Microarray Fold Change
196528	ARID2	1.99
2001	ELF5	-2.65
3169	FOXA1	-2.47
2976	GTF3C2	-3.12
64412	GZF1	2.42
84458	LCOR	2.41
4173	MCM4	1.55
58508	MLL3	2.06
10933	MORF4L1	2.07
8031	NCOA4	2.64
4784	NFIX	-2.83
4824	NKX3-1	-4.53
7799	PRDM2	2.48
5933	RBL1	1.80
55509	SNFT	-2.32
6722	SRF	-2.03
54816	SUHW4	-1.93
9412	SURB7	-2.24
9338	TCEAL1	-1.57
7718	ZNF165	1.53
7738	ZNF184	1.66
23528	ZNF281	1.71
30834	ZNRD1	-1.63