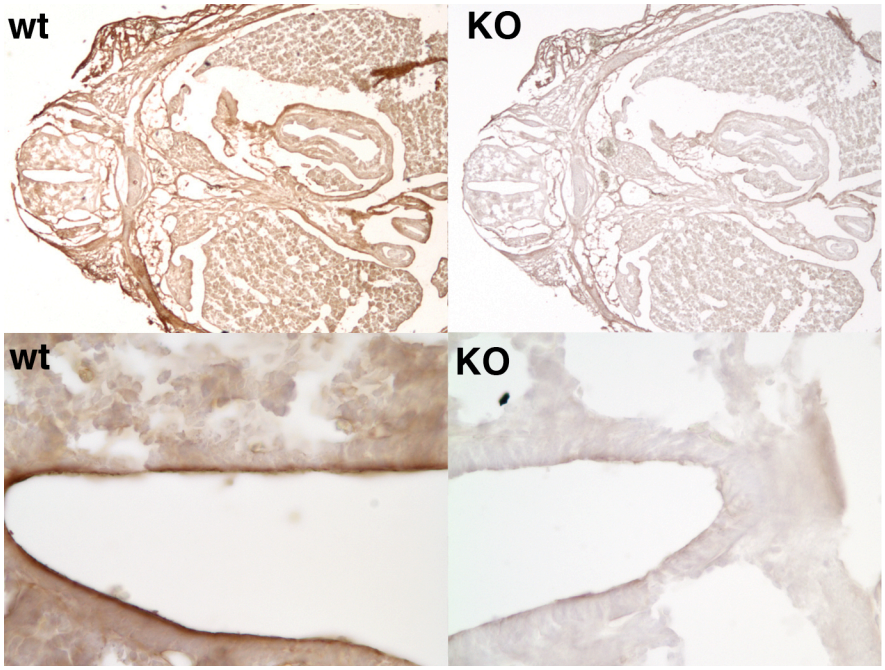
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Supplementary Figure Legends

Supplementary Figure 1:

IHC of frozen sections of wild-type and SOX4 knockout mouse embryos. **(A)** Wild-type mouse embryo (2x). **(B)** SOX4 knockout mouse embryo (2x). **(C)** Wild-type mouse embyo (10x). **(D)** SOX4 knockout mouse embryo (40x). **(E)** Wild-type mouse embyo (10x). **(F)** SOX4 knockout mouse embryo (40x).

**Supplementary Methods for Liu et al, SOX4 is a Transforming Oncogene in Human Prostate Cancer Cells**

*Microarray analysis*

Total RNA was prepared by trizol extraction of serial sections from snap frozen tissues. For each frozen specimen of prostate, one frozen section was stained with hematoxylin and eosin (H&E) to map areas of carcinoma and non-neoplastic prostate, after which 15-μm thick serial sections were cut for isolation of total RNA. Histologic confirmation was obtained in all cases and dissection of cancer foci was performed to assure that at least 90% of cells collected were malignant epithelial cells. Trizol reagent (50 μl) was dispensed onto an area of interest on the frozen section and pipetted up and down several times to solubilize tissue. Surrounding tissue on the frozen section was avoided in order to isolate RNA selectively from either cancer or non-neoplastic tissue. Pooled extracts from 10-15 serial sections typical produced 1-3 μg total RNA after trizol extraction. RNA was labeled and a minimum of 20 μg of labeled cRNA was hybridized to the U133A and U133B GeneChips according to manufacturer's protocols (Affymetrix, Santa Clara, CA), and CEL files were normalized using the GCRMA method using GeneTraffic software (Iobion, La Jolla, CA), and filtered to identify 26,806 expressed probe sets with a maximal expression level above a cutoff threshold log2 signal $\geq$ 5. After data normalization and filtering, Significance Analysis of Microarrays (SAM) analysis was performed as two class, unpaired data (tumor and normal), using the 10-Nearest Neighbor Imputer engine and the following relevant parameters: $\Delta$ = 1.15, fold-change = 1.5, number permutations = 500, RNG seed = 1234567, median FDR = 1%, significant probes = 730, predicted false positives = 7. Significance of correlation of SOX4 with Gleason

Score was determined by two-tailed Spearman's Rho Rank Correlation Coefficient using R Language 2.0 and corrected for multiple hypothesis testing as described (1). Heat maps were generated using Spotfire DecisionSite 7.2 for Functional Genomics. Complete microarray datasets have been deposited in the public ArrayExpress database, accession number E-TABM-26.

*Cell Culture*

The LNCaP prostate cancer cell line was maintained in T-medium containing 10% fetal bovine serum at $37^0$C and 5% $CO_2$. RWPE-1 cells were obtained from the American Tissue Culture Collection (ATCC) and were grown in 100 mm tissue culture dishes (Corning) in K-SF media containing 50 $\mu$g/ml bovine pituitary extract, 5 ng/ml epidermal growth factor (Invitrogen Corporation, Carlsbad, CA). Cultures were incubated at 37°C in a humidified atmosphere containing 5% $CO_2$. Normal human prostate epithelial cells (PrEc cells) were obtained commercially (Cambrex Bio Science, Walkersville, MD) and maintained in PrEGM™ - Prostate Epithelial Cell Medium according to the supplier's protocols.

*Construction of FLAG-SOX4 N-terminal fusion clone*

The pNM16.2 plasmid was a gift from Dr. Christine Farr (University of Cambridge). pcDNA3.1HISA plasmid was obtained from Invitrogen Corporation (Carlsbad, CA). The pcDNA3.1HISA-SOX4 construct was made by excision of the *EcoR1-EcoR1* fragment of SOX4 from pNM16.2 and ligation into the *EcoR1* site of pcDNA3.1HISA to give pcDNA3.1HISA-SOX4. A pcDNA3-Flag-FKHR plasmid (gift of Dr. William Sellers,

Dana Farber Cancer Institute, Harvard University) was restricted with *Bam*HI and *Xba*I, and the *Bam*HI-*Xba*I pcDNA3-Flag vector backbone was gel purified according to QIAquick gel extraction kit protocol (Qiagen, Valencia, CA). pcDNA3.1HISA-SOX4 was restricted with *Sac*I and *Xba*I and the 1.5kb SOX4 fragment, lacking the 5'UTR, was gel purified using the QIAquick kit. Equal molar ratios of oligonucleotides 5'-gatccATGGTGCAGCAAACCAACAATGCCGAGAACACGGAAGCGCTGCTGGCC G G C G A g a g c t - 3 ' a n d 5 ' - cTCGCCGGCCAGCAGCGCTTCCGTGTTCTCGGCATTGTTGGTTTGCTGCACCAT g-3' were phosphorylated in vitro with T4 polynucleotide kinase (New England Biolabs, Cambridge, MA) and annealed to form a *Bam*HI-*Sac*I linker encoding the first 19 amino acids of the SOX4 open reading frame. The SOX4 *Sac*I-*Xba*I fragment was joined with the *Bam*HI-*Sac*I linker in a three-way ligation into the *Bam*HI-*Xba*I pcDNA3-Flag backbone to create a pcDNA3-Flag-SOX4 plasmid. The resulting clone was sequence verified using the T7 forward primer and an internal SOX4 primer (TTGCCGGACTTCACCTTCTTCCT).

*Construction of Stable RWPE-1 Cell Lines*

RWPE-1 cells (80-90% confluency) were washed once with 5ml of HBSS (Hanks' Balanced Salt Solution) and transfected with 10μg of DNA (pcDNA3-Flag-SOX4 or pcDNA3.1HISA empty vector) using DMRIE-C lipid transfection reagent in OptiMEM media (Invitrogen Corporation, Carlsbad, CA). Transfected cells were cultured for two days without selection in K-SF media containing 50 $\mu$g/ml bovine

pituitary extract, 5 ng/ml epidermal growth factor, then cultured for three days in media supplemented with G418 (400 $\mu$g/ml, Mediatech, Herndon, VA). G418 concentration was reduced to 200 $\mu$g/ml for one week for selection and reduced to 100 $\mu$g/ml for maintenance. Individual colonies were transferred to 6-well plates and expanded in the presence of G418 (100 $\mu$g/ml). Nine clonal cell lines and 1 pool of clones were analyzed for SOX4 protein expression by immunoblot analysis using rabbit polyclonal SOX4 antisera.

*siRNA design and transient transfection*

siRNA duplexes targeting SOX4 were designed, synthesized, annealed, and purified (MWG Biotech, NC). Optimal transfection of LNCaP cells was obtained with a combination of 8 different siRNA's to SOX4 used as a cocktail at a final concentration of 100 nM. All siRNA sequences were subjected to BLAST search to confirm the absence of homology to any additional known coding sequences in the human genome. The SOX4 siRNA target sequences are as follows: S1:AAAGACAGCGACAAGATCCCTTT, S2:AATCGCCTCCTCCCCCACGCCCG, S3:AATTTGGCCGATGGCAGATGTTT, S4:AAACTGAAATGGATTTGCACGTT, S5:AAACGCTGGAAGCTGCTCAAA, S6:AACTCCAAACCGGCGCAGAAA, S7:AAATCAGTGAGGTGAGACTTC, S8:AACGAGCTTCCGGACTTGTCT, S9:AAAGGACAGACGAAGAGTTTA, S10:AAACGCGTGATGAAGACAGAA.

Lamin A/C siRNA (AACTGGACTTCCAGAAGAACA) and GFP siRNA (AAGGCTACGTCCAGGAGCGCACC) were standard control siRNA's procured from MWG Biotech. Transient siRNA transfections were performed using DMRIE-C

(Invitrogen, CA) for prostate cancer cells in six well plates. At the end of 48 hrs, cells were harvested for total RNA isolation and protein lysate for immunoblots, flow cytometry and viability assays. Protein lysates were prepared as described (2), and total RNA was prepared using RNeasy kits (Qiagen, CA) according to the manufacturer's instructions.

*Co-transfection experiments*

To validate the specificity of the siRNA-induced apoptosis, LNCaP cells were co-transfected with siRNA specific to the SOX4 3'UTR, and a mammalian expression vector containing the SOX4 open reading frame, but lacking the 3'UTR (3). A clone of the human SOX4 gene was a generous gift of Christine Farr (4). Co-transfection experiments were carried out in LNCaP cells in six well plates with siRNA (100 nM) in combination with and without SOX4-DNA (2 μg) for 48hrs.

*Quantitative Real-Time PCR*

To confirm decrease in SOX4 at the mRNA level, 2 ug of total RNA from SOX4 siRNA post-transfected LNCaP cells was used to prepare cDNA by reverse transcription (PowerScript, ClonTech). Quantitative PCR was performed on iCycler (Bio-Rad, Richmond, CA) with SOX4 specific primers using SYBR Green dye that binds preferentially to double strand DNA. Cycle threshold differences were quantitated relative to beta-actin to control for total starting RNA levels, and experiments were performed on three separate days in duplicate. The primers used to amplify specific gene product for SOX4 sense 5'-CCGAGCTGGTGCAAGACC-3' and SOX4 antisense 5'-

CCACACCATGAAGGCGTTC-3'. Beta-actin primers were sense 5' CTGGAACGGTGAAGGTGACA 3'; antisense 5' AAGGGACTTCCTGTAACAATGCA 3'.

*Western blots*

Immunoblots were probed with monoclonal antibody to Lamin A/C (Santa Cruz Biotech) and our polyclonal antisera to SOX4. We synthesized three synthetic peptides specific to SOX4 (MVQQTNNAENTEALLAGESSDSC, ASSPTPGSTASTGGKADDPSWC, ASGGGANSKPAQKKSC), linked them to keyhole limpet hemocyanin (KLH) with maleimide-activated KLH (Pierce Inc., IL), and raised polyclonal rabbit antisera (Rockland Inc., PA). Blots were then probed using a monoclonal antibody to PP2A catalytic subunit (BD Transduction Labs, CA) to normalize for equal protein loading

*Immunohistochemistry*

The CPCTR prostate TMA2 (Gleason TMA) was used for immunohistochemistry. IHC staining was performed using the EnVision double labeling system, (DAKOCytomation., Carpinteria, CA) on formalin-fixed paraffin-embedded human prostate cancer tissue. Briefly, slides were deparaffinized and hydrated through two series of xylene and ethanol. Endogenous peroxidase activity was quenched with 0.3% $H_2O_2$ in MeOH for 30 minutes. Slides were then placed in 0.1M citrate buffer (pH 6.0) and steamed for 10 min for antigen retrieval. Next, they were blocked with 5% non-fat dried milk (NFDM) for 30 min and followed by peroxidase blocking solution (provided by supplier) for 10 minutes.

Tissue samples were washed three times in 0.1M Citrate buffer and placed in ice for two minutes. Sections were incubated with rabbit polyclonal anti-SOX4 antisera (1:2000) for1hr followed by incubation with anti-rabbit secondary antibodies conjugated to HRP-linked labeled polymers. DAB+ substrate-chromogen was incubated for 5 min for color development. Finally, slides were counterstained with hematoxylin (Richard-Allan Scientific, Kalamazoo, MI) for 5 seconds, dehydrated, and mounted.

*Cell viability assay*

Both the attached and floating cells were harvested at 48 hrs post transfection of SOX4 siRNA, spun and the pellet made up to a known volume with regular media. Cell viability was measured by hemacytometer cell counts using the trypan blue dye. Live cells exclude the dye, and results were expressed as % viability. Results are expressed as mean ± standard deviation. Statistical comparisons are evaluated by Student's T-test and the significance was set as $p < 0.05$.

*Apoptosis assay*

Apoptosis levels in transient siRNA transfections against SOX4 were quantitated by immunoblotting with a monoclonal antibody (Cell Signaling, Inc.) that is specific for the cleaved form of poly-ADP-ribose polymerase (PARP), a substrate of caspase 3. GFP siRNA transfections served as a negative control.

*Flow cytometry*

The percent of cells undergoing apoptosis and the different phases of the cell cycle were determined by flow cytometry as previously described (5). Apoptotic cells were defined as those cells in the sub-G0/G1 (< 2N DNA) peak.

*Soft Agar Assays*

Soft Agar assays were performed essentially as described (6). Briefly, 10,000 cells were seeded in 0.3% Noble Agar, KSF media supplemented with bovine pituitary extract and insulin-like growth factor (Invitrogen, Carlsbad, CA) fungizome, penicillin-streptomycin, L-glutamine. Colony number was determined using MTT dye staining (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) as described (7).

*Chromatin Immunoprecipitations*

Two P150 culture dishes (Corning) each of RWPE-1 FLAG-SOX4 cells and parental RWPE-1 cells were grown to confluency. Cells were fixed, harvested and lysed as described previously (8). Sonicated chromatin was precleared overnight with 50 µl Dynal M-280 Sheep anti-Mouse IgG magnetic beads (Invitrogen), followed by a 5 hour incubation with 50 µl magnetic beads prebound with 15 µg of the M5 anti-Flag monoclonal antibody (Sigma Immunochemicals) or mouse IgG. 400 µl of supernatant from the IgG control was saved as Input. Beads were washed, antibodies were eluted, and DNA purified as described previously (8). Purified DNA was subjected to PCR using the following primers: BBC3/PUMA-fwd: ACACCCACGGACACACATACATCA, rev: GTTTGGCTTGTGTGTCTGGCATCA. TLE1-fwd: GGTAGGACCGTGGATGAGGAA, rev:

ATGTGGCCAGATTCATGGTACAGG. PSMA Intron 3-fwd: CTTGCCTTCTAAAATGAGTTGG, rev: TTGGGAGGCTGAGGTGGAAGAA. Products were visualized on 1.3% agarose gels with 0.4 μg/ml Ethidium Bromide.

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