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Supplemental Information

Mechanism and Role of *SOX2* Repression in Seminoma: Relevance to Human Germline Specification

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Supplemental figure legends

Supplemental Fig. 1. Analysis of SOX2 promoter methylation in (A) 8 EC and TCam-2 cell lines along with positive (IVD) and negative (WGA) controls shown as EpiGrams. (B) 5 SEM and 5 EC tumors along with positive (IVD) and negative (WGA) controls shown as EpiGrams. Colored circles indicate level of methylation at each CpG island identified above, red un-methylated, yellow methylated, white not analyzed. Data for 4 primers (SOX_02, SOX_03, SOX_05, SOX_06) are shown. (Related to figure 4)

Supplemental Fig. 2. Heat maps generated by R programming showing expression of statistically significant lineage genes following TCam2 treated RA cells by GEP analysis. a) Neuronal genes, b) Endodermal genes, c) Mesodermal genes, d) Smooth Muscle genes, e) Epithelial genes. (Related to figure 7).

Supplemental tables

Supplemental Table 1. List of upregulated and downregulated genes in SEM in comparison to normal testis by SAM analysis.

Supplemental Table 2. List of upregulated and downregulated genes in EC in comparison to normal testis by SAM analysis.

Supplemental Table 3. List of Primer Sequences used for the analysis of SOX2 promoter.

Supplemental Experimental Procedures

Quantitative DNA methylation analysis

Tumor specimens**:** Tumor tissues and clinical data were collected under IRB-approved protocol at Memorial Sloan-Kettering Cancer Center. Seminoma (n=5) and Embryonal carcinoma (n=5) samples were selected based on clinical information, availability of frozen tumor material, and review of corresponding H&E stained sections from paraffin

blocks. Frozen tumor specimens were grossly microdissected in an attempt to minimize normal cell contamination. The genomic DNA was isolated with DNeasy Blood & Tissue kit (Qiagen, catalogue number 69504, USA) from tumor tissues and cell lines (TCAM2 and NT2/D1) along with WGA-negative control and IVD-positive control. The genomic DNA was isolated with DNeasy Blood & Tissue kit (Qiagen, catalogue number 69504, USA), and genomic DNA was given to core facility MSKCC. DNA methylation analysis was carried out using the Epityper system from Sequenom (San Diego, CA). The EpiTYPER assay is a tool for the detection and quantitative analysis of DNA methylation using base-specific cleavage of bisulfite-treated DNA and Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS). Specific PCR primers for bisulfite-converted DNA were designed using the EpiDesigner software (www.epidesigner.com), for the entire CpG island of the SOX2 gene. T7-promoter tags are added to the reverse primer to obtain a product that can be *in vitro* transcribed, and a 10-mere tag is added to the forward primer to balance the PCR conditions. One µg of tumor DNA was subjected to bisulfite treatment using the EZ-96 DNA methylation Kit, which results in the conversion of unmethylated cytosines into uracil, following the manufacturer's instructions (Zymo Research, Orange, CA). PCR reactions were carried out in duplicate, for each of the 2 selected primer pairs, for a total of 4 replicates per sample. For each replicate, 1 µl of bisulfite-treated DNA was used as template for a 5 µl PCR reaction in a 384-well microtiter PCR plate, using 0.2 units of Kapa2G Fast HotStart DNA polymerase (Kapa Biosystems, Cape Town, South Africa), 200 µM dNTPs, and 400 nM of each primer. Cycling conditions were: 94 ^{0}C for 15 minutes, 45 cycles of 94 ^{0}C for 20 seconds, 56 ^{0}C for 30 seconds, 72 ^{0}C for 1 minute, and 1 final

cycle at 72 ^{0}C for 3 minutes. Unincorporated dNTPs were deactivated using 0.3 U of shrimp alkaline phosphatase (SAP) in 2 µl, at 37 ^{0}C for 20 minutes, followed by heat inactivation at 85 ^{0}C for 5 minutes. Two µl of SAP-treated reaction were transferred into a fresh 384-well PCR plate, and *in vitro* transcription and T cleavage were carried out in a single 5 µl reaction mix, using the MassCleave kit (Sequenom) containing 1 X T7 polymerase buffer, 3 mM DTT, 0.24 µl of T Cleavage mix, 22 units of T7 RNA and DNA polymerase, and 0.09 mg/ml of RNase A. The reaction was incubated at 37 ^{0}C for 3 h. After the addition of a cation exchange resin to remove residual salt from the reactions, 10 nl of Epityper reaction product were loaded onto a 384-element SpectroCHIP II array (Sequenom). SpectroCHIPs were analyzed using a Bruker Biflex III matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF) mass spectrometer (SpectroREADER, Sequenom). Results were analyzed using the Epityper Analyzer software, and manually inspected for spectra quality and peak quantification.

Sequence analysis of SOX2 promoter regions was performed using Epityper Analyzer software Supplemental Table 3). Quantitation of degree of SOX2 methylation using mass spectrometry of amplification product with the Sequenom EpiTYPER assay was performed in 5 Seminoma (Sem) and 5 Embryonal carcinoma (EC) tumor samples along with TCAM2 (Sem cell lines) and NT2/D1 (EC cell lines). Eight primer sets were designed covering entire CpG island of SOX2 gene (1000 bp from upstream and +1000 bp downstream from the transcription site). There was no difference between methylation was observed between Sem and EC tumor or TCAM2 and NT2/D1 cell lines. Standard samples with a known composition (WGA as a negative control and IVD

as a positive control) were used in parallel to acquire standard curves to quantify the level of methylation of each CpG site.

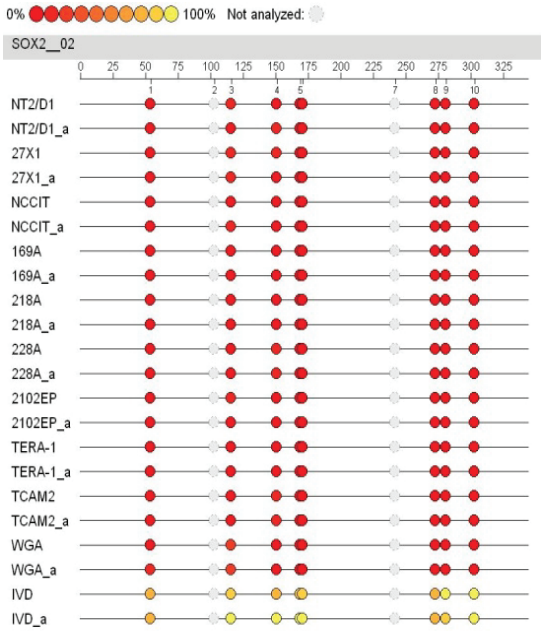
qRT PCR for the neuronal genes

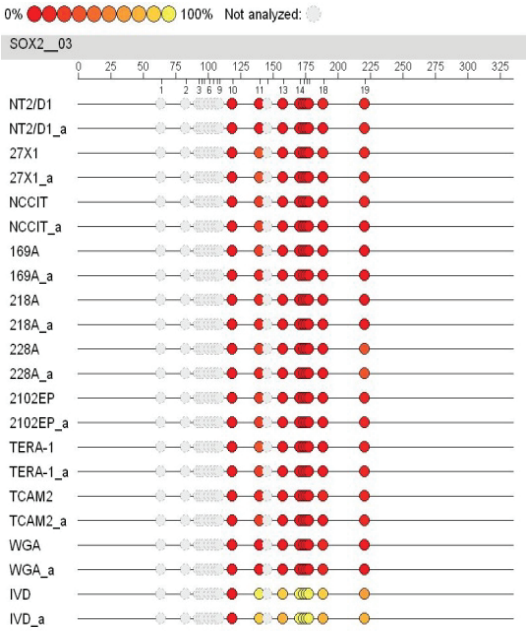
RNA was isolated from UTX-KD and Scramble cells on day6 of silencing. MRNA was prepared using SuperScript® VILO cDNA Synthesis Kit (Life Technologies Grand Island, NY) and was further assayed with Taqman probes in an applied Biosystems 7500 Real Time PCR system (Applied Biosystems, Grand Island, NY according to the manufacturer's instructions. PGK1 mRNA level was used as internal normalization control. Each experiment represented here was repeated three independent times.

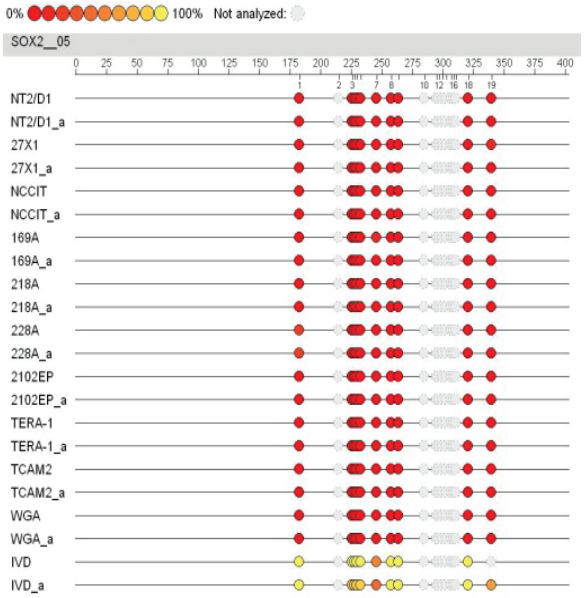
Heat map generation

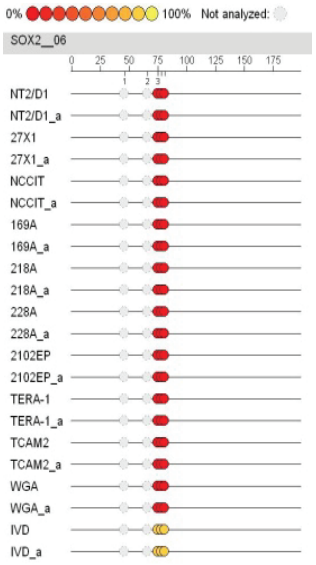
The GNU-R platform was used to generate the heat maps displayed in Appendix A through E, specifically the 'gplots' and the 'RColorBrewer' libraries in R. The displays show expression of lineage markers in 3 TCAM2 Retinoic Acid treated samples when compared to 3 untreated control TCAM2 samples. Red color stands for a higher expression value and blue color stands for lower expression. All color values in a row are row-normalized so the expression differences become apparent. The legend at the bottom of the images shows a histogram of counts of expression values in the plot. The cyan jagged line in each column of the plots shows the real expression value along with the average expression (dotted line) of all the spots in the heat map.

Kushwaha_Sup.Figure 1A.
(Related to Fig.2)

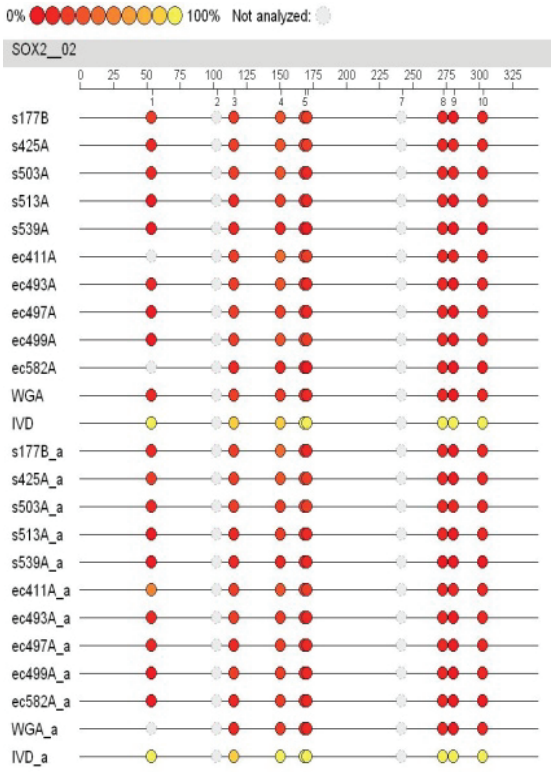








Kushwaha_Sup.Figure 1B.
(Related to Fig.2)



Kushwaha_Sup.Fig 2.

(Related to Fig.7)