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Supplemental Information

Purification of GFRα1+ and GFRα1– Spermatogonial Stem Cells Reveals a Niche-Dependent Mechanism for Fate Determination

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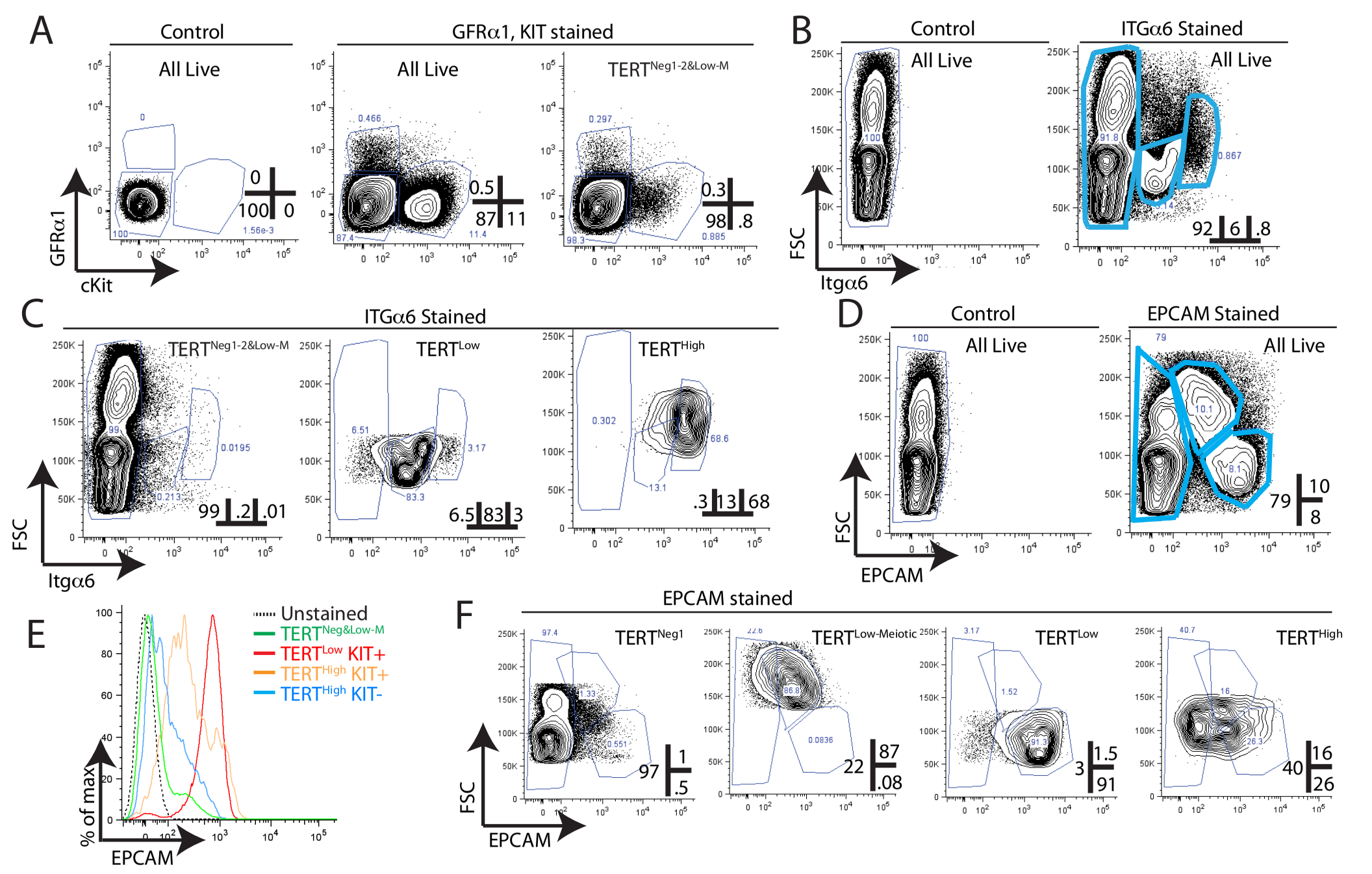


G

Cell Type	Tert-Tomato	Itga6	EPCAM	KIT	THY1	GFRa1
A-undiff	++++	+++	-/+	-	+or -	+or -
Early A-diff	+++	++	-/+	++	-	-
Late A-diff	++	+	+++	+	-	-
Meiosis I	+	-	++	-	-	-
Spermatids	-	-	-	-	-	-

Fig. S1

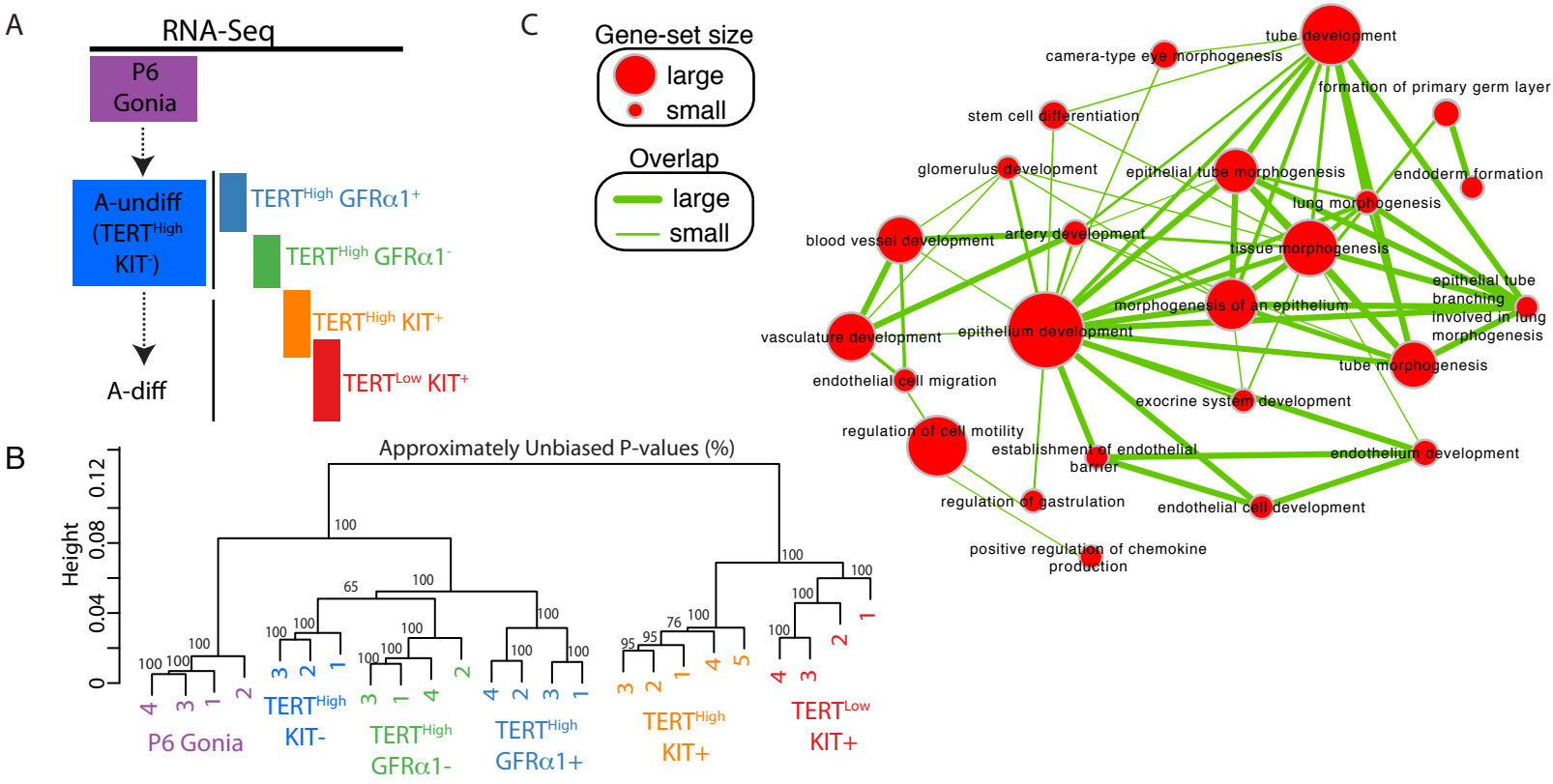

A
RNA-Seq
P6 Gonia
A-undiff (TERTHigh KIT-)
A-diff
TERTHigh GFRα1+
TERTHigh GFRα1-
TERTHigh KIT+
TERTLow KIT+
B
Approximately Unbiased P-values (%)
Height
0
0.04
0.08
0.12
P6 Gonia
TERTHigh KIT-
TERTHigh GFRα1-
TERTHigh GFRα1+
TERTHigh KIT+
TERTLow KIT+
C
Gene-set size
large
small
Overlap
large
small
tube development
camera-type eye morphogenesis
formation of primary germ layer
stem cell differentiation
glomerulus development
epithelial tube morphogenesis
endoderm formation
lung morphogenesis
blood vessel development
artery development
tissue morphogenesis
epithelial tube branching involved in lung morphogenesis
morphogenesis of an epithelium
vasculature development
epithelium development
tube morphogenesis
endothelial cell migration
exocrine system development
regulation of cell motility
establishment of endothelial barrier
endothelium development
regulation of gastrulation
endothelial cell development
positive regulation of chemokine production

Fig. S2

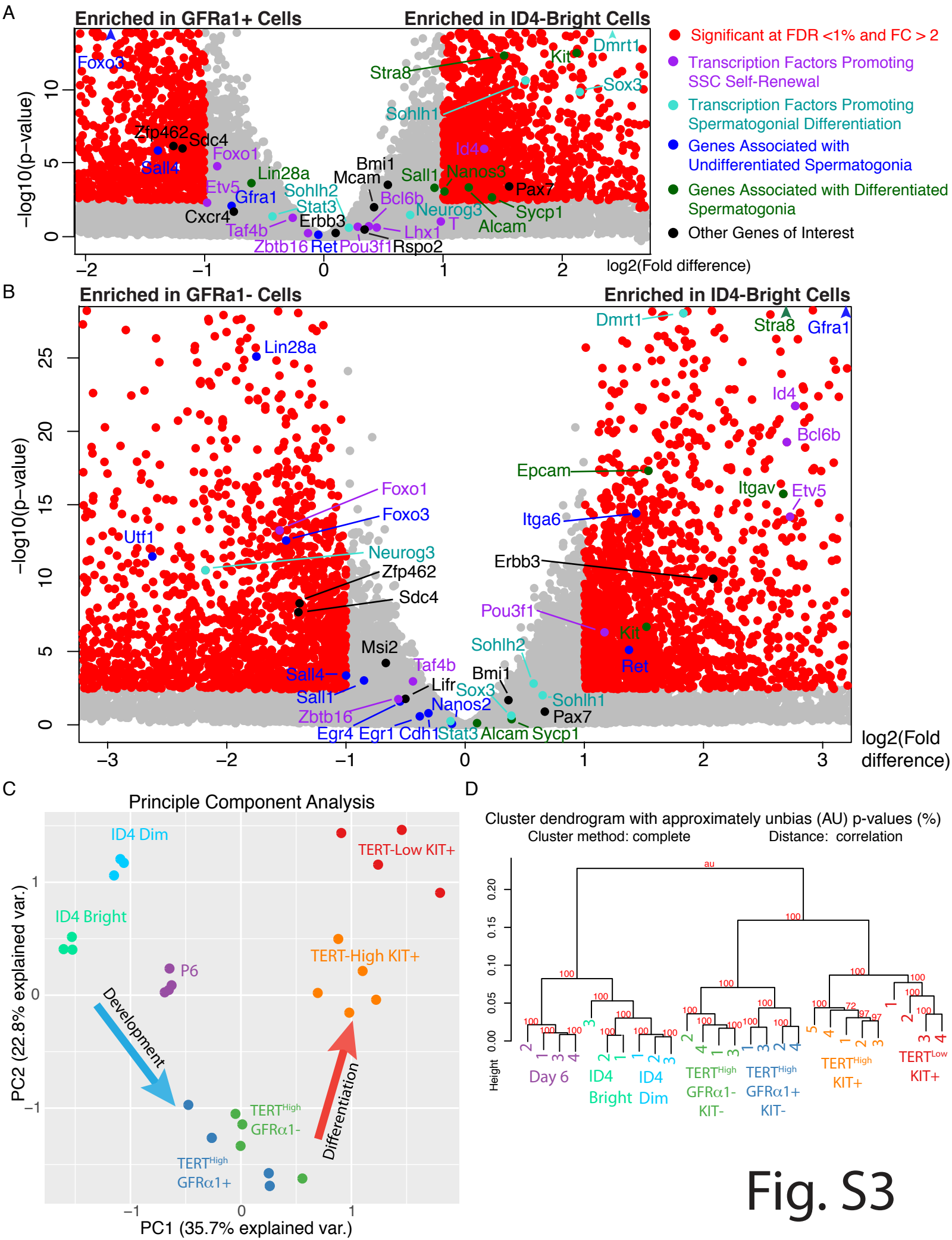
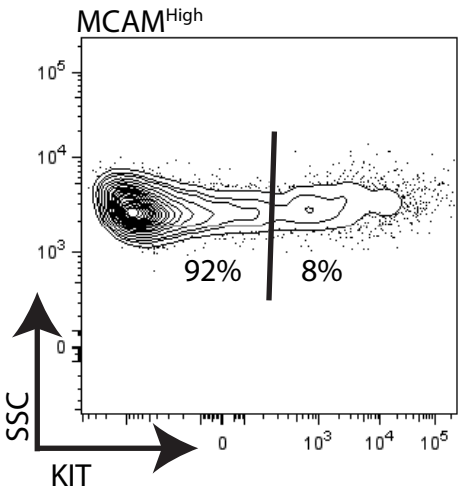
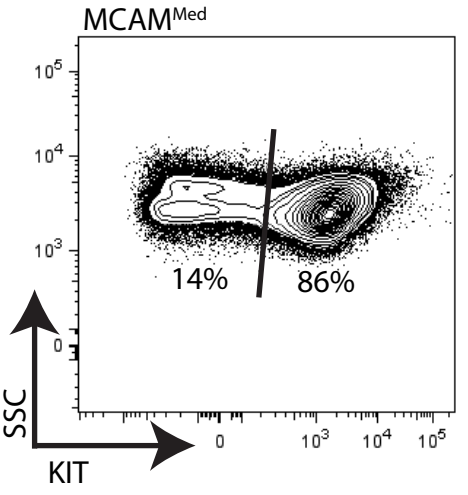

A
Enriched in GFRa1+ Cells
Enriched in ID4-Bright Cells
−log10(p−value)
log2(Fold difference)
Foxo3
Zfp462
Sdc4
Sall4
Foxo1
Etv5
Gfra1
Lin28a
Cxcr4
Sohlh2
Stat3
Taf4b
Erbb3
Zbtb16
Ret
Bmi1
Mcam
Pou3f1
Rspo2
Bcl6b
Lhx1
Sall1
Id4
Nanos3
Neurog3
T
Alcam
Pax7
Sycp1
Stra8
Kit
Dmrt1
Sox3
Sohlh1
Significant at FDR <1% and FC > 2
Transcription Factors Promoting SSC Self-Renewal
Transcription Factors Promoting Spermatogonial Differentiation
Genes Associated with Undifferentiated Spermatogonia
Genes Associated with Differentiated Spermatogonia
Other Genes of Interest
B
Enriched in GFRa1- Cells
Enriched in ID4-Bright Cells
−log10(p−value)
log2(Fold difference)
Lin28a
Foxo1
Foxo3
Utf1
Neurog3
Zfp462
Sdc4
Msi2
Sall4
Taf4b
Lifr
Sall1
Zbtb16
Egr4
Egr1
Cdh1
Nanos2
Stat3
Bmi1
Sox3
Alcam
Pax7
Sohlh1
Sycp1
Pou3f1
Sohlh2
Kit
Ret
Erbb3
Epcam
Itga6
Itgav
Etv5
Id4
Bcl6b
Dmrt1
Stra8
Gfra1
C
Principle Component Analysis
PC2 (22.8% explained var.)
PC1 (35.7% explained var.)
ID4 Dim
ID4 Bright
P6
TERT-Low KIT+
TERT-High KIT+
TERTHigh GFRα1-
TERTHigh GFRα1+
Development
Differentiation
D
Cluster dendrogram with approximately unbias (AU) p-values (%)
Cluster method: complete
Distance: correlation
Height
Day 6
ID4 Bright
ID4 Dim
TERTHigh GFRα1- KIT-
TERTHigh GFRα1+ KIT-
TERTHigh KIT+
TERTLow KIT+

Fig. S3

A


MCAM^High
92%
8%
SSC
KIT

B


MCAM^Med
14%
86%
SSC
KIT

C

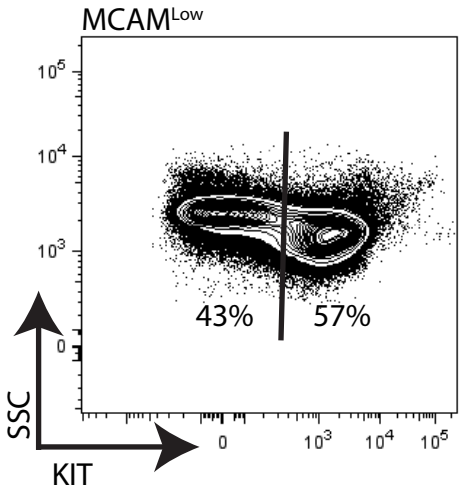
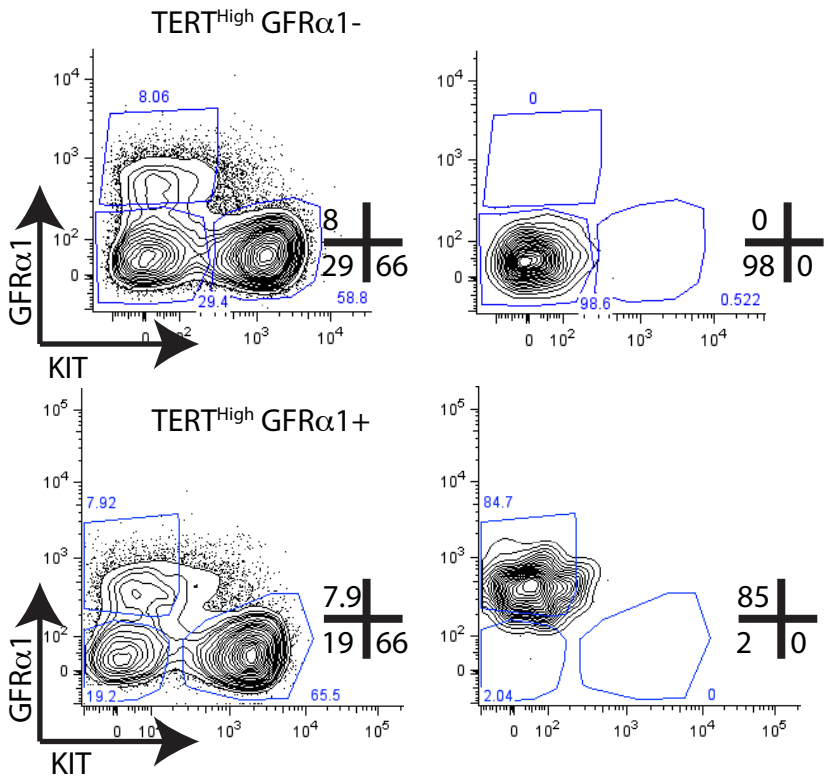

MCAM^Low
43%
57%
SSC
KIT

Fig. S4

A


TERT^High GFRα1-
8.06
8
29
66
29.4
58.8
0
0
98
0
98.6
0.522
GFRα1
KIT
TERT^High GFRα1+
7.92
7.9
19
66
19.2
65.5
84.7
85
2
0
2.04
0
GFRα1
KIT

B

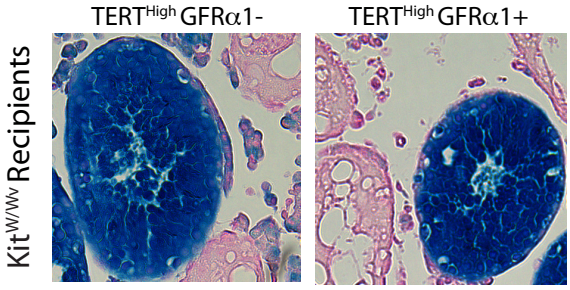

TERT^High GFRα1-
TERT^High GFRα1+
Kit^W/Wv Recipients

Fig. S5

Supplemental Figure Legends

Figure S1. Additional immunophenotyping of adult Tert$^{Tomato/+}$ testis cells, related to Figure 1.

A. Live singlet Tert-Tomato testes cells stained with GFRα1-biotin, streptavidin-APC and cKit antibodies. Control cells are stained with streptavidin-APC only. Background staining of GFRα1 and cKit in meiotic cells is also shown.

B. Live singlet Tert-Tomato testes cells stained with α6-integrin (ITGα6) antibodies. Control cells are unstained.

C. ITGα6 expression in Tert$^{Neg1\text{-}2+Low\text{-}meiotic}$, Tert^{Low} and Tert^{High} cells, graphed against forward-scatter.

D. Live singlet Tert-Tomato testes cells stained with EPCAM antibodies. Control cells are unstained.

E. Histogram of EPCAM expression in the indicated cell populations

F. EPCAM expression in Tert^{Neg1}, Tert$^{Low\text{-}Meioic}$, Tert^{Low} and Tert^{High} cells, graphed against forward-scatter.

G. Summary of immunophenotyping the adult testis. "-" indicates absence of staining. "+" indicates relative degree of staining intensity. "+ or -" indicates bimodal staining pattern.

Figure S2. Clustering of RNA-Seq libraries from biological replicates, related to Figure 2

A. Indicated purified populations were sequenced and the resulting expression data was analyzed using the pvClust package.

B. Shown tree is a result of unsupervised hierarchical clustering using pvClust.

C. Morphogenesis/epithelial development cluster generated by Cytoscape Enrichment Map of GSEA results for Tert^{High} GFRα1+.

Figure S3. Comparison of ID4 RNA-Seq to our sequencing of GFRα1+ and GFRα1- spermatogonia, related to Figure 2.

A. Volcano plot showing differentially expressed genes between GFRα1+ and ID4-Bright cells.

B. Volcano plot showing differentially expressed genes between GFRα1- and ID4-Bright cells.

C. PCA analysis showing relatedness between ID-Bright and ID-Dim cells and our five populations.

D. Unsupervised hierarchical clustering using pvClust of ID-Bright and ID-Dim and our five indicated populations.

Figure S4. cKit Expression in MCAM fractions, related to Figure 4

A. Percent of cKit+ and cKit- cells in the MCAM-High population by flow cytometry.

B. Percent of cKit+ and cKit- cells in the MCAM-Med population by flow cytometry.

C. Percent of cKit+ and cKit- cells in the MCAM-Low population by flow cytometry.

Figure S5. Purity and MCAM status of transplanted GFRα1- cells, related to Figure 5.

A. Representative purity of FACS-sorted cells used for transplantation experiments. Initial gating strategy is shown in the left panel; in the right panel, an aliquot of 500-1000 sorted cells was re-analyzed to determine purity levels

B. Representative histological cross-sections from transplant recipients. R26-lacZ^{+} donor cells of the indicated immunophenotype were labeled by X-gal staining.

Supplemental Experimental Procedures

Wholemount analysis of seminiferous tubules

Wholemount analysis was performed as previously described (Pech et al., 2015). For MCAM wholemounts, the NP40 and methanol treatments were replaced by treatment with -20C acetone for 10min. For wholemounts of GFRα1 expression in the post-transplant testis, no permeabilization steps were used.

Immunofluorescence of FACS-sorted cells

Cytospins and microscopy for Edu, PLZF, and GFRα1 were performed as previously described (Pech et al., 2015). Statistical analysis of Edu incorporation was done using the paired two-tailed Student's t-test. N=5 mice; N=900-10,000 cells per condition. For PLZF and GFRα1 staining, N=3 mice pooled; N=1524 cells were used.

In situ hybridization for Ngn3

In situ hybridization was performed using the RNAScope 2.0 HD reagent Red Kit (Advanced Cell Diagnostics) with a Ngn3 probe (ACD; cat no. 422401) according to the manufacturer's protocol on sorted cells cytospin onto coverslips. Statistical analysis of Ngn3 expression was done using the paired two-tailed Student's t-test. N=5-6 mice; at least N=2000 cells counted per condition.

In vitro germ cell culture

Germ cells were cultured as previously described (Kanatsu-Shinohara et al., 2003a), with minor modifications. Germ cells were sorted using a flow cytometer directly into "basal GS media" (StemPro34 Serum Free Media (Invitrogen), and appropriate supplements. Sorted cells were then transferred to 24-well plates containing irradiated MEF-coated coverslips. Cells were cultured in 2mls of the basal GS media, or basal GS media supplemented with 10ng/ml FGF2 and 50ng/ml GDNF (Peprotech). Immunofluorescence was performed as described above. Statistical analysis of MCAM expression was performed using the unpaired two-tailed Student's t-test. For the experiment, N=4 mice; N=50 cells per condition were used.

RNA-Seq library preparation

Dissociated testes cells were prepared and sorted from both testes of adult mice, as described previously (Pech et al., 2015). Four to five biological replicates were sorted. Cells were sorted directly into Trizol LS (Invitrogen), and RNA extracted following the manufacturer's recommendation. RNA was further purified on Rneasy Micro columns (Qiagen). cDNA was prepared and amplified using the Nugen Ovation V2 kit, starting from 5 to 10ng of total RNA. The cDNA was sonicated to 200bp size using a Covaris S2 machine, and 25ng of cDNA was used to make the libraries, following standard Illumina Truseq V2 protocols. Samples were sequenced on an Illumina Hiseq2500 machine, with paired-end 101bp reads.

Germ Cell Transplantation

Four independent transplantations were performed. For each transplantation experiment, testes cell suspensions were prepared from two pooled adult mice and sorted as described previously. A total of 16-18 recipient testes were analyzed per cell type. Donor cells were genetically marked using either a R26-lacZ allele (Jackson Labs), or a ubiquitously expressed CAG-EGFP allele (Lin and Artandi, unpublished). Cells were sorted in HBSS+5%FBS. Sort purity was confirmed by re-analysis post-sort. Cell yields were confirmed post-sort by hemocytometer. Donor cells were introduced into infertile Kit^{W/W^v} recipients (Jackson Labs) via efferent duct injection (Ogawa et al., 1997). Colonization was determined eight weeks after injection, either by X-gal staining or by EGFP epifluorescence. Colony numbers were normalized to 100,000 cells transplanted. Statistics were calculated using Prism (GraphPad Software, La Jolla, CA) using the Mann-Whitney non-parametric U test.

RNA-Seq data analysis

Differential expression analysis was performed as previously described (Pech et al., 2015). Expression datasets were ordered from the least to most differentiated and submitted with four replicates each to STEM software analysis (Short Time Series Expression Mining, Version 1.0) (Ernst and Bar-Joseph, 2006) using the default parameter setting. Gene Set Enrichment Analysis (GSEA) (Subramanian et al., 2005) was performed using version 2.0.13. Genes were rank-ordered by their T-test value. Genes that could not be converted to a HUGO gene name were excluded. GSEA was run on this pre-ranked list of genes, using default parameters. Results from GSEA were submitted to Cytoscape 3.4 using the Enrichment Map plugin (Merico et al., 2010) using a p-value cutoff of 0.001, a q-value cutoff of 0.05, and a similarity cutoff of 0.5.