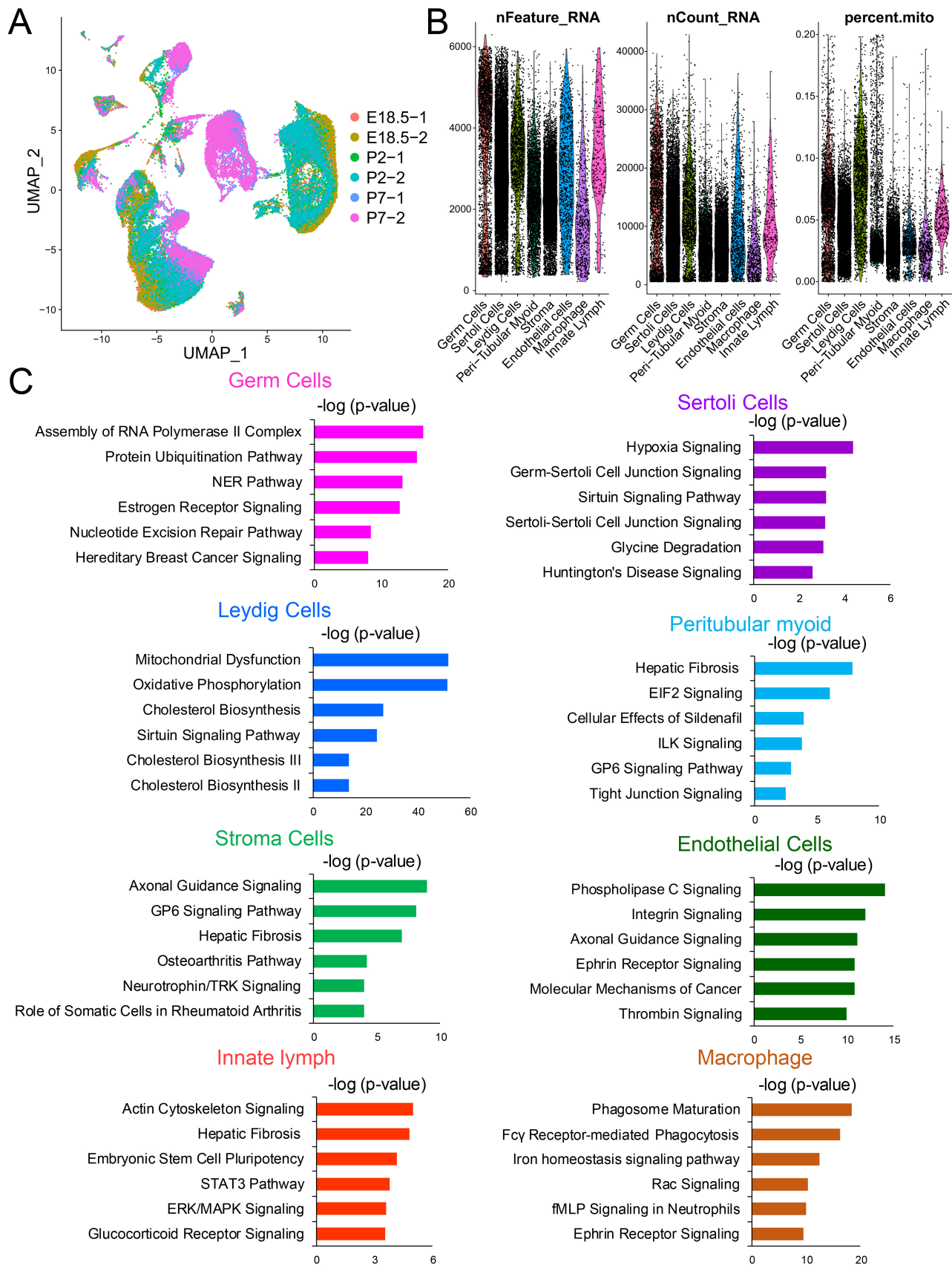# Figure S1

Figure S1. Identification and characterization of the major cell types from perinatal testes.
(A) UMAP plot of cells from E18.5, P2, and P7 mouse testes.
(B) Violin plots of cell types from E18.5, P2, and P7 mouse testes, showing nFeature_RNA, nCount_RNA, and mitochondrial gene expression profiles—post filtering—using the parameters indicated in the Materials and Methods.
(C) The most statistically significant ingenuity canonical pathways for each cell type.

Figure S2

A — Cell proliferation genes

Clusters: T1-ProSG, I-ProSG, T2-ProSG, SSC-1, SSC-2, Diff-SG

Genes (top to bottom): Mcm5, Pcna, Mcm2, Mcm4, Rrm1, Ung, Gins2, Mcm6, Cdca7, Dtl, Prim1, Uhrf1, Hells, Rfc2, Nasp, Rad51ap1, Gmnn, Slbp, Msh2, Rad51, Rrm2, Cdc45, Exo1, Tipin, Clspn, Brip1, Hmgb2, Cdk1, Ube2c, Birc5, Tpx2, Top2a, Cks1b, Mki67, Tacc3, Ttk, Kif2c, Cdca8, Cenpa

B — T1-ProSG gene markers: Dnmt3l, Dnmt3a, Kdm1b, Piwil4, Rac3, Utf1

C — Gene markers for different SG subsets:
SSC-1: Gfra1, Id4, Bcl6b
SSC-2: Rarg, Neurog3, Nanos3
Diff-SG: Kit, Stra8, Dmrtb1

D
CDKN2A+ 3.79%
CDKN2A- 53.2%
a. Unfractionated
b. CDKN2A+
c. CDKN2A-
Genes: Elmo1, Etv4, Gfrα1, Amh, Vim

E
Legend: T1-ProSG, I-ProSG, T2-ProSG, SSC-1, SSC-2, Diff-SG
Axes: Component 1, Component 2

F
Cytoskeleton organization: Pdlim4, Cln3, Elmo1, Palld, Ssh1, Ssh2
Cell migration: Jak1, Tiam1, Nf2, Lamb1, Mfge8, Prkci
Cell proliferation: Mki67, Mybl2, Cdk1, Bub1, Ccnb1, Plk1
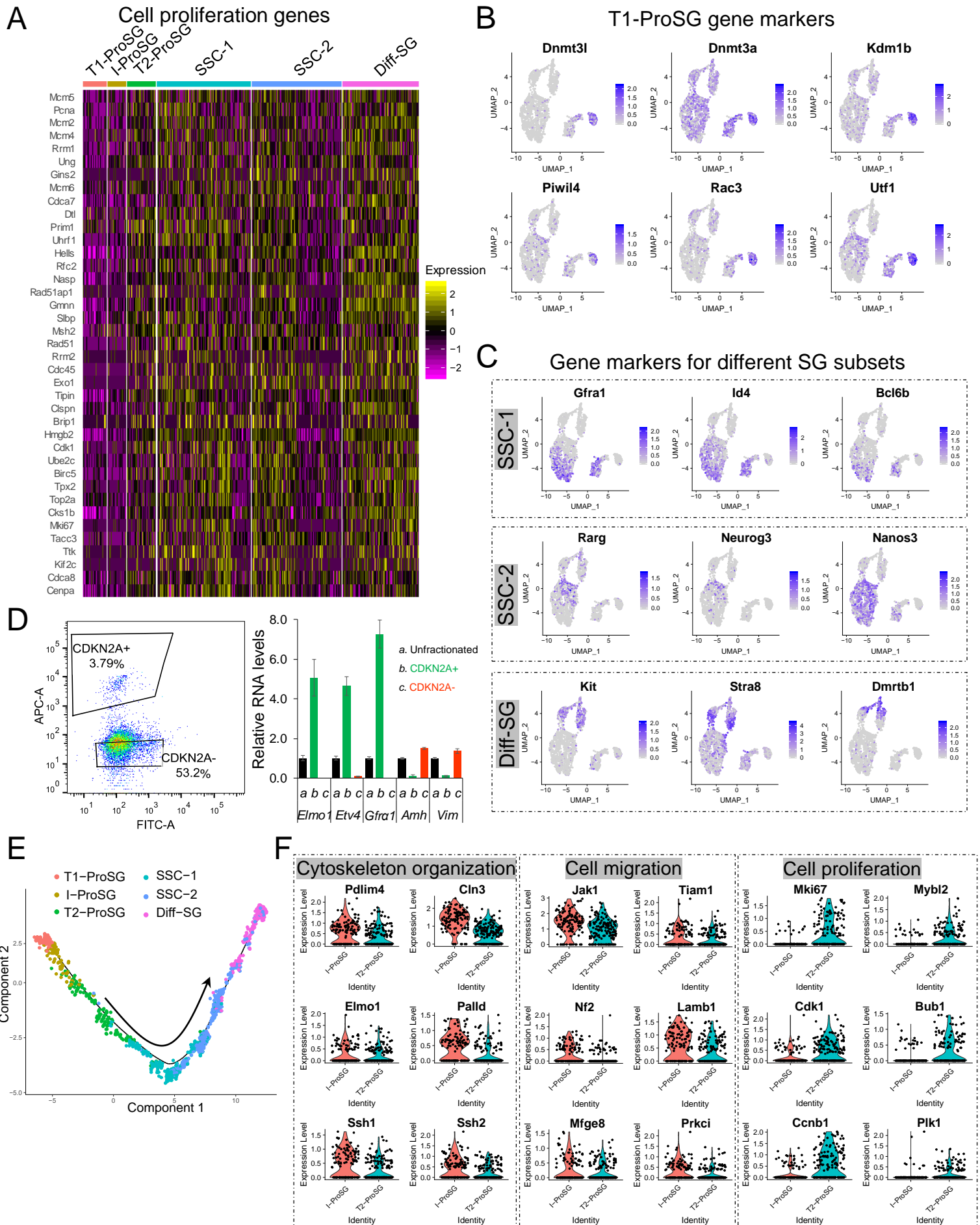(Identity: I-ProSG, T2-ProSG)

Figure S2 The expression of gene markers for perinatal germ cell subsets.

(A) Heatmap of cell proliferation-related genes.

(B) UMAP plots (from Figure 2A) of gene markers specifically expressed in the T1-ProSG.

(C) UMAP plots (annotated from Figure 2A) of genes differentially expressed in different germ cell subsets.

(D) FACS plot of mouse P2 testicular cells stained with CDKN2A antibody. The percentage of positive and negative cells are indicated in upper and lower regions; CDKN2A-positive cells were gated by cells stained with secondary antibody only ($< 0.01\%$ positive). qPCR analysis was performed on sorted positive and negative cells, as well as unfractionated P2 testicular cells. The values shown are from FACS-purified cells relative to unfractionated cells (mean $\pm$ SD from two biological replicates).

(E) Monocle trajectory analysis of germ cell subsets from E18.5, P2, and P7 mouse testes.

(F) Violin plots showing expression of genes associated with actin cytoskeleton organization, cell migration, and cell proliferation in both I- and T2-ProSG.
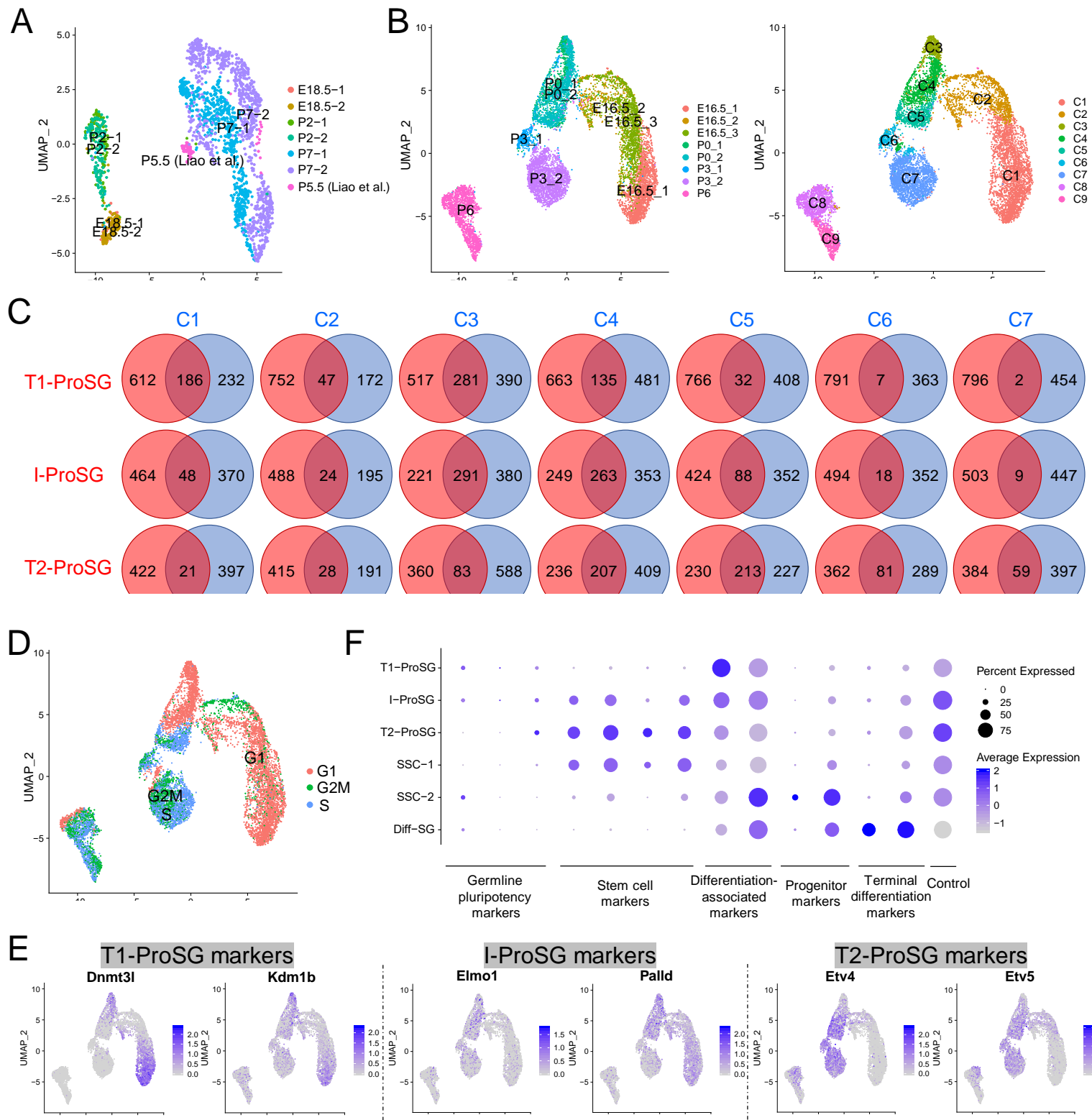
Fig. S3

Figure S3. Comparison of our findings of ProSG-to-SSC development with published datasets.

(A) UMAP plot of germ cells from E18.5, P2, P7 mouse testes (from our study) and P5.5 testes from Liao et al.

(B) UMAP plot of germ cells from Law et al. Left, sample information; Right, cell clusters.

(C) Venn diagrams of DEGs from indicated ProSG subsets (from our study) and germ cell clusters (from Law et al.).

(D) UMAP plot inferring the cell-cycle phase based on expression of a large set of G2M- and S-phase genes (Kowalczyk et al., 2015).

(E) UMAP plots of ProSG markers we identified in our study using Law et al. dataset.

(F) Dot plots of markers that Law et al. used in their study using our dataset.
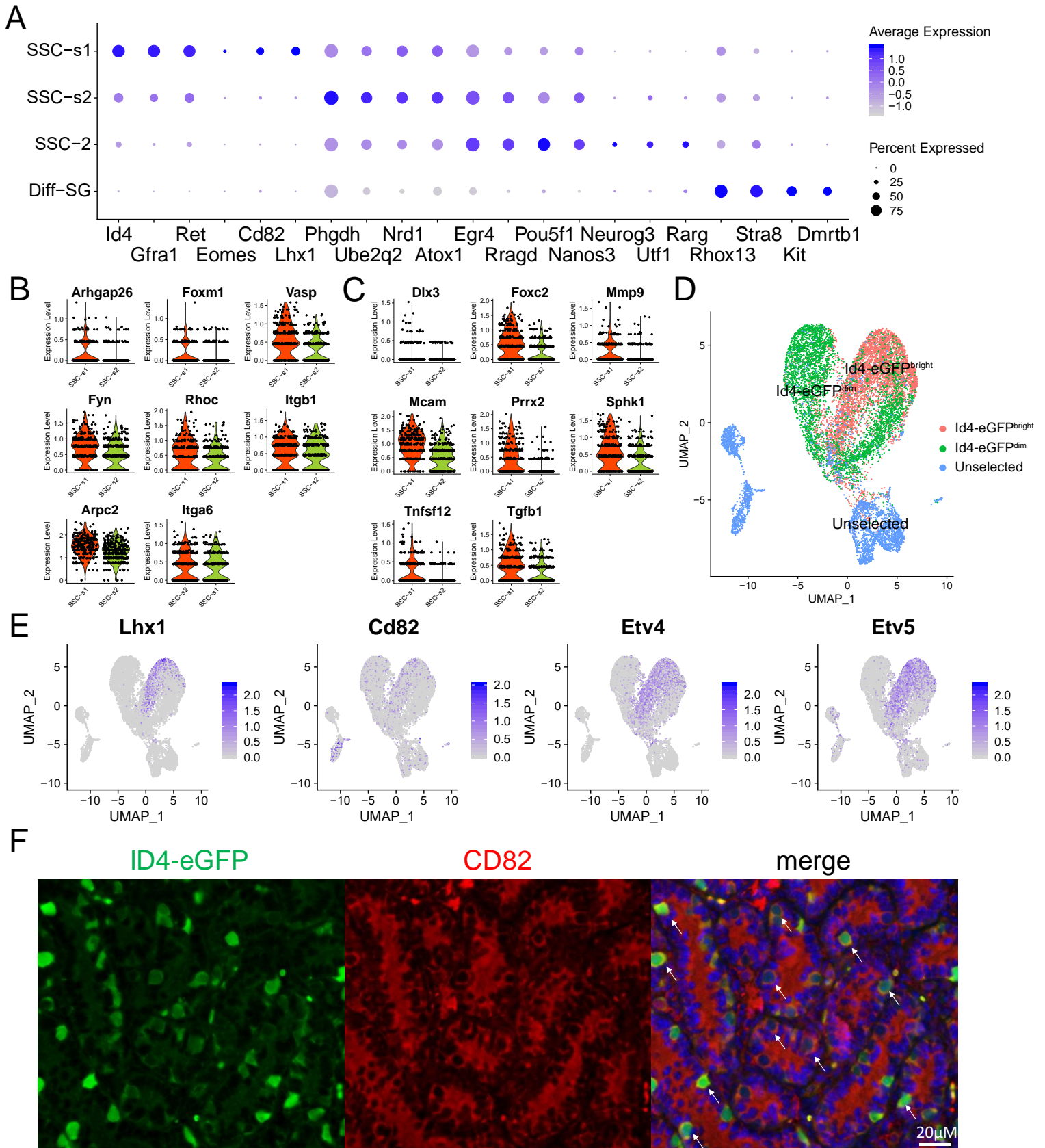
Figure S4

Figure S4. Undifferentiated SG states during the SSC establishment period.

(A) Dotplots of genes differentially expressed in different SG subsets/states.

(B) Violin plots of integrin signaling-related genes in different SSC-1 states.

(C) Violin plots of vessel development-related genes in different SSC-1 states.

(D) UMAP plot of germ cells purified by FACS against Id4-eGFP from Hermann BP et al. 2018.

(E) UMAP plots of SSC-s1 markers we identified in dataset shown in (D).

(F) IF analysis of P7 testes sections from Id4-eGFP mice stained with CD82 antibody. Scale bar, 20 µm. Write arrow showing the labeled Id4-eGFP+ germ cells.
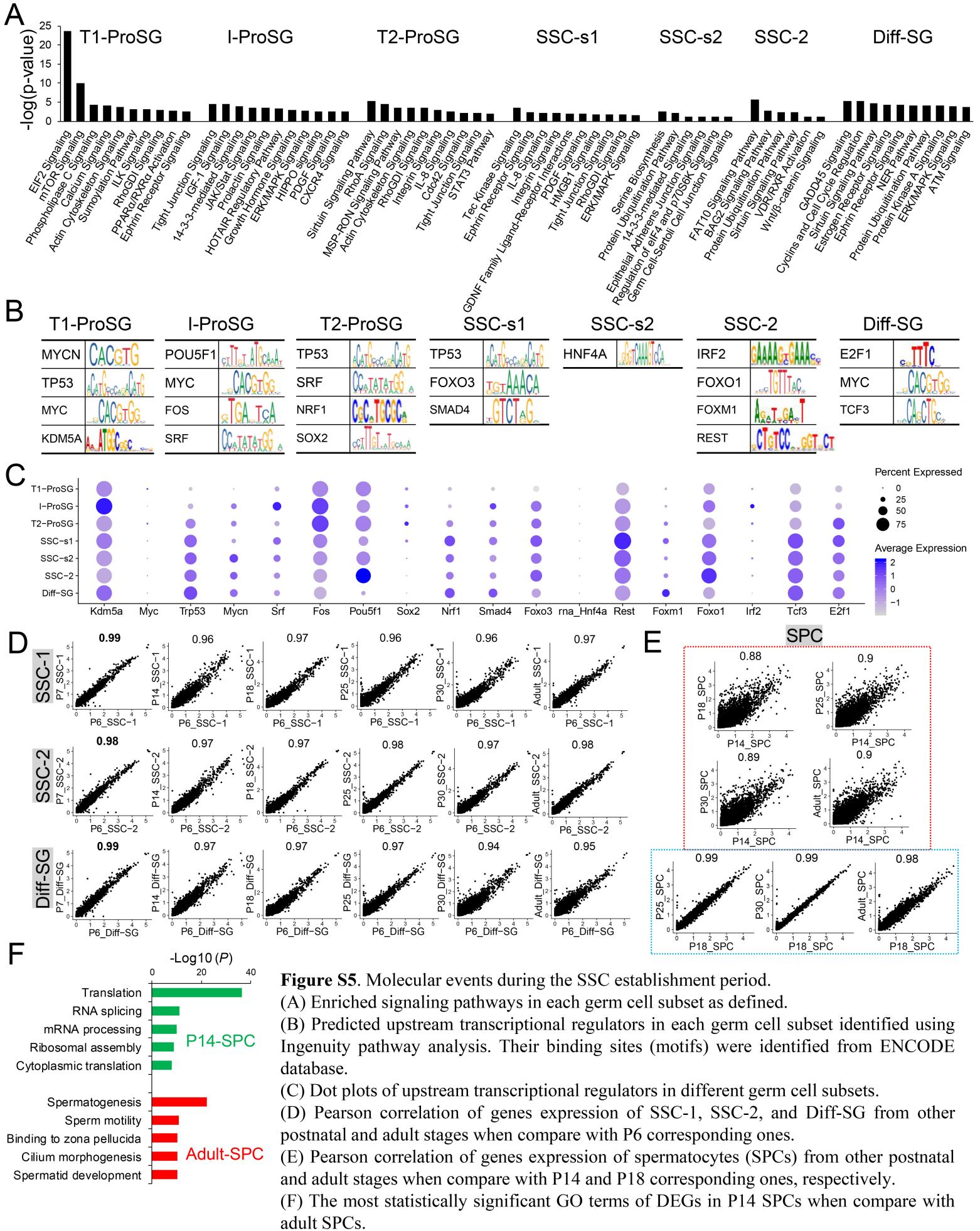
Figure S5

Figure S5. Molecular events during the SSC establishment period.

(A) Enriched signaling pathways in each germ cell subset as defined.

(B) Predicted upstream transcriptional regulators in each germ cell subset identified using Ingenuity pathway analysis. Their binding sites (motifs) were identified from ENCODE database.

(C) Dot plots of upstream transcriptional regulators in different germ cell subsets.

(D) Pearson correlation of genes expression of SSC-1, SSC-2, and Diff-SG from other postnatal and adult stages when compare with P6 corresponding ones.

(E) Pearson correlation of genes expression of spermatocytes (SPCs) from other postnatal and adult stages when compare with P14 and P18 corresponding ones, respectively.

(F) The most statistically significant GO terms of DEGs in P14 SPCs when compare with adult SPCs.
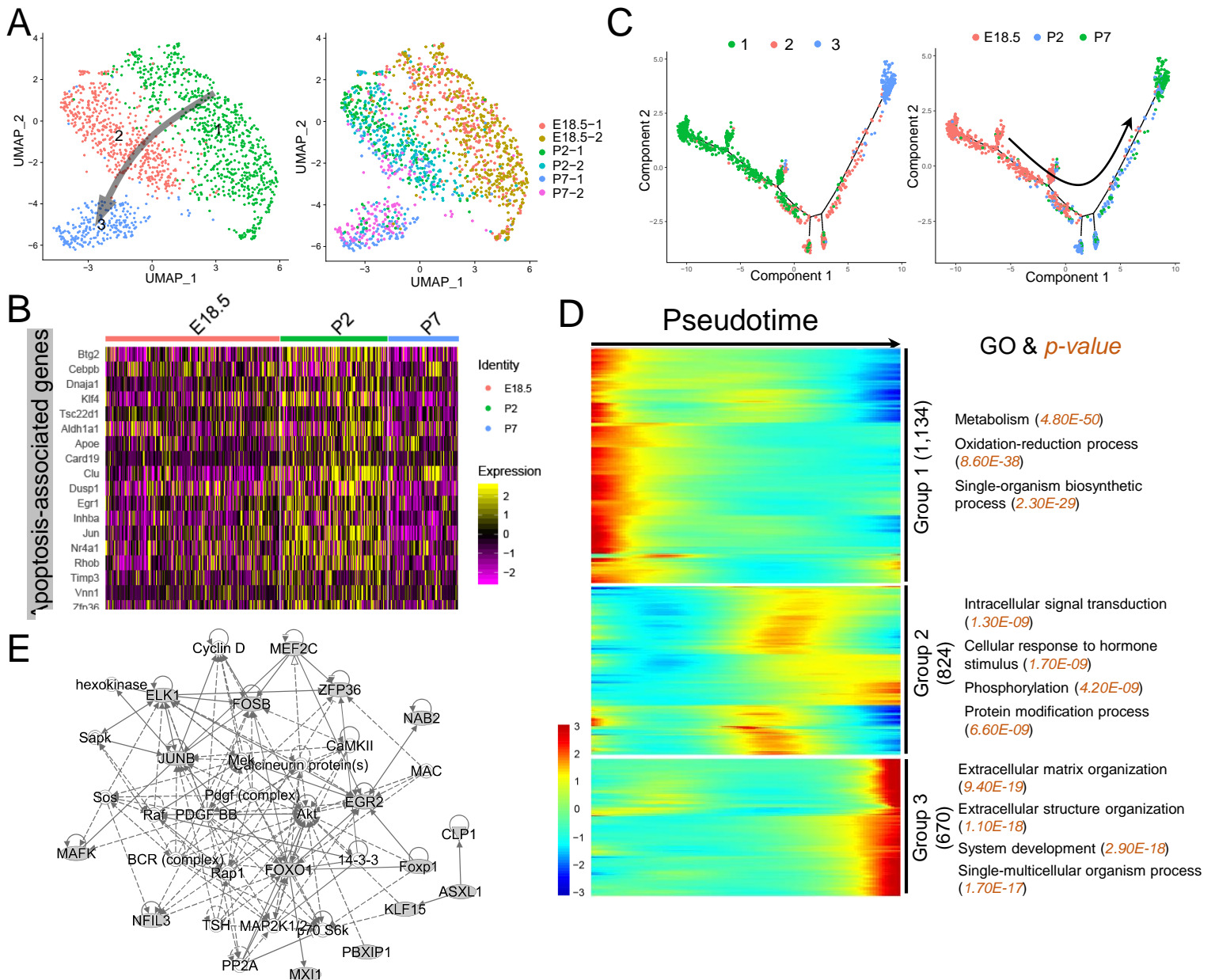
Figure S6

Figure S6. Identification and characterization of Leydig cells during the perinatal stage.

(A) UMAP plot of Leydig cells (annotated from Fig. 1A) from E18.5, P2, and P7 mouse testes. Left, Leydig cell clusters; Right, sample source. Arrow shows the predicted developmental direction.

(B) Heatmap of apoptosis-associated genes from different stages of Leydig cells.

(C) Monocle pseudotime trajectory analysis of the Leydig cell clusters defined in (A). Left, Leydig cell clusters; Right, developmental stage information.

(D) Heatmap of differentially expressed genes (DEGs) from different Leydig cell clusters following the trajectory timeline shown in (C). Top, pseudotime direction; Right, the number of DEGs and the representative biological processes and P-values in each group.

(E) Gene network of enriched transcription factors (TFs) identified from gene group 2 in (D).
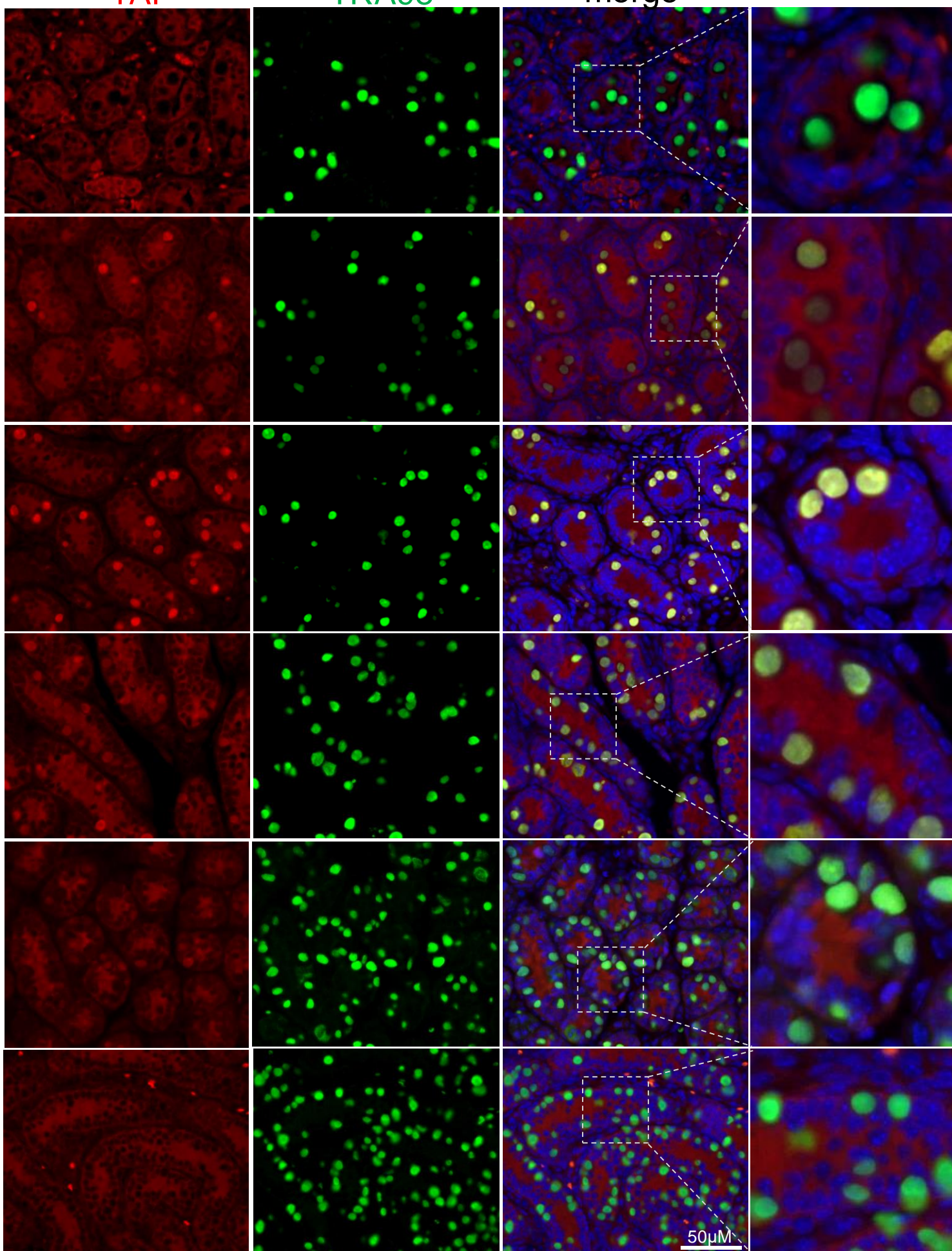
Fig. S7

Figure S7. Immunofluorescence analysis of P0-P7 testes sections co-stained with antibodies against YAP and TRA98. Scale bar, 50 μm.
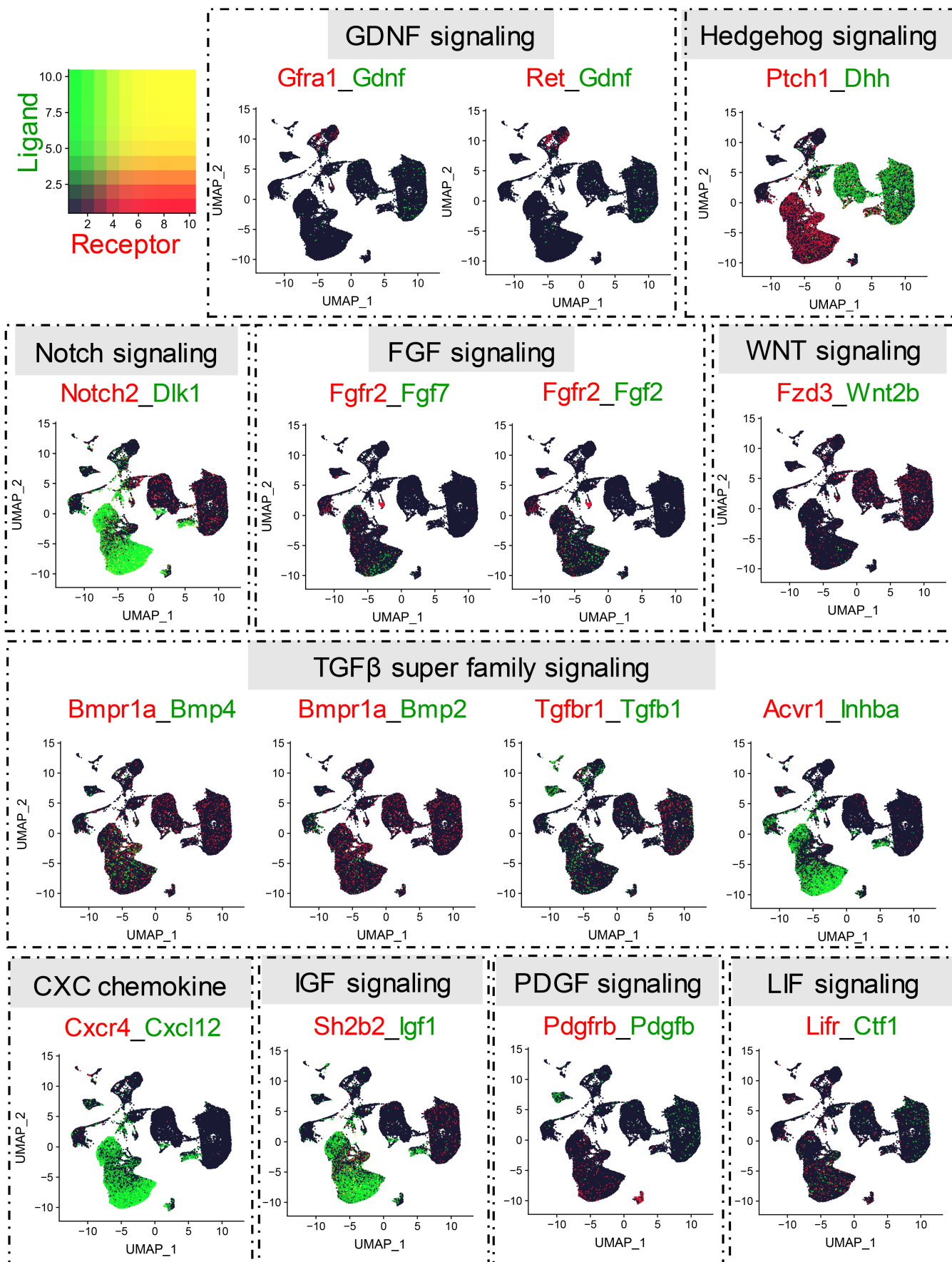
Figure S8

Figure S8. UMAP plots of candidate Receptor-ligand signaling (annotated from Fig. 1A)

Table S1.

Click here to Download Table S1

Table S2.

Click here to Download Table S2

Table S3. Mapping results of testes samples analyzed by scRNAseq in this study

Samples	Total reads	Mean reads / cell	Median gene / cell	# genes detected	# cell captured
E18.5-1	216,126,647	28,411	2,633	21,620	7,607
E18.5-2	269,007,368	24,788	2,407	27,152	10,852
P2-1	247,447,539	26,963	2,448	21,923	9,177
P2-2	245,639,102	25,037	2,650	22,465	9,811
P7-1	205,691,816	26,279	2,540	21,759	7,827
P7-2	309,204,385	35,733	3,179	21,832	8,653

Table S4. The number of the cells in each cell type from each time point in this study

Cell types	Samples					
	E18.5-1	E18.5-2	P2-1	P2-2	P7-1	P7-2
Germ Cells	57	200	384	266	728	1263
Sertoli Cells	2286	4458	4411	4771	3292	4609
Leydig Cells	412	529	295	303	143	228
Peri-Tubular Myoid	384	592	381	408	1153	681
Stroma	3439	3847	2906	3413	1806	1293
Endothelial cells	210	422	95	136	58	80
Macrophage	135	194	63	98	86	65
Innate Lymph	63	83	24	63	21	25

Table S5. Antibodies used in this study

Rabbit polyclonal anti-CDKN2A antibody against cyclin dependent kinase inhibitor 2A	Novus Biologicals	Cat # NB200-111SS
Rabbit monoclonal anti-DNMT3L	Abcam	Cat # ab194094
Rat polyclonal anti-KI-67	Antibodies-Online	Cat # ABIN1032382
Polyclonal Sheep IgG against KI-67	R&D Systems	Cat # AF7649-SP
Rabbit polyclonal anti-ELMO1 antibody [C3], C-term	GeneTex	Cat # GTX106301
Monoclonal mouse antibody against PALLD	Novus Biologicals	Cat # NBP1-25959SS
Rat monoclonal anti-TRA98	B-Bridge International	Cat # 73-003
Monoclonal Mouse anti-LHX1	Developmental Studies Hybridoma Bank	Cat # 4F2
Monoclonal Mouse anti-CD82	Proteintech	Cat # 66803-1-Ig
Polyclonal Rabbit anti-CD82	LifeSpan Biosciences	Cat # LS-C100497-400
Rabbit polyclonal anti-ETV4	Proteintech	Cat # 10684-1-AP
Monoclonal Mouse anti-YAP	Santa Cruz Biotechnology	Cat # sc-101199
Goat polyclonal anti-GFP (FITC)	GeneTex	Cat # GTX26662
Goat anti-Rat IgG (H+L) 2nd antibody, 488	Thermo Fisher Scientific	Cat # A-11006
Goat anti-Rat IgG (H+L) 2nd antibody, 555	Thermo Fisher Scientific	Cat # A-21434
Goat anti-Rabbit IgG (H+L) 2nd antibody, 555	Thermo Fisher Scientific	Cat # A27039
Goat anti-Rabbit IgG (H+L) 2nd antibody, 488	Thermo Fisher Scientific	Cat # A-11008
Goat anti-Mouse IgG (H+L) 2nd antibody, 555	Thermo Fisher Scientific	Cat # A28180