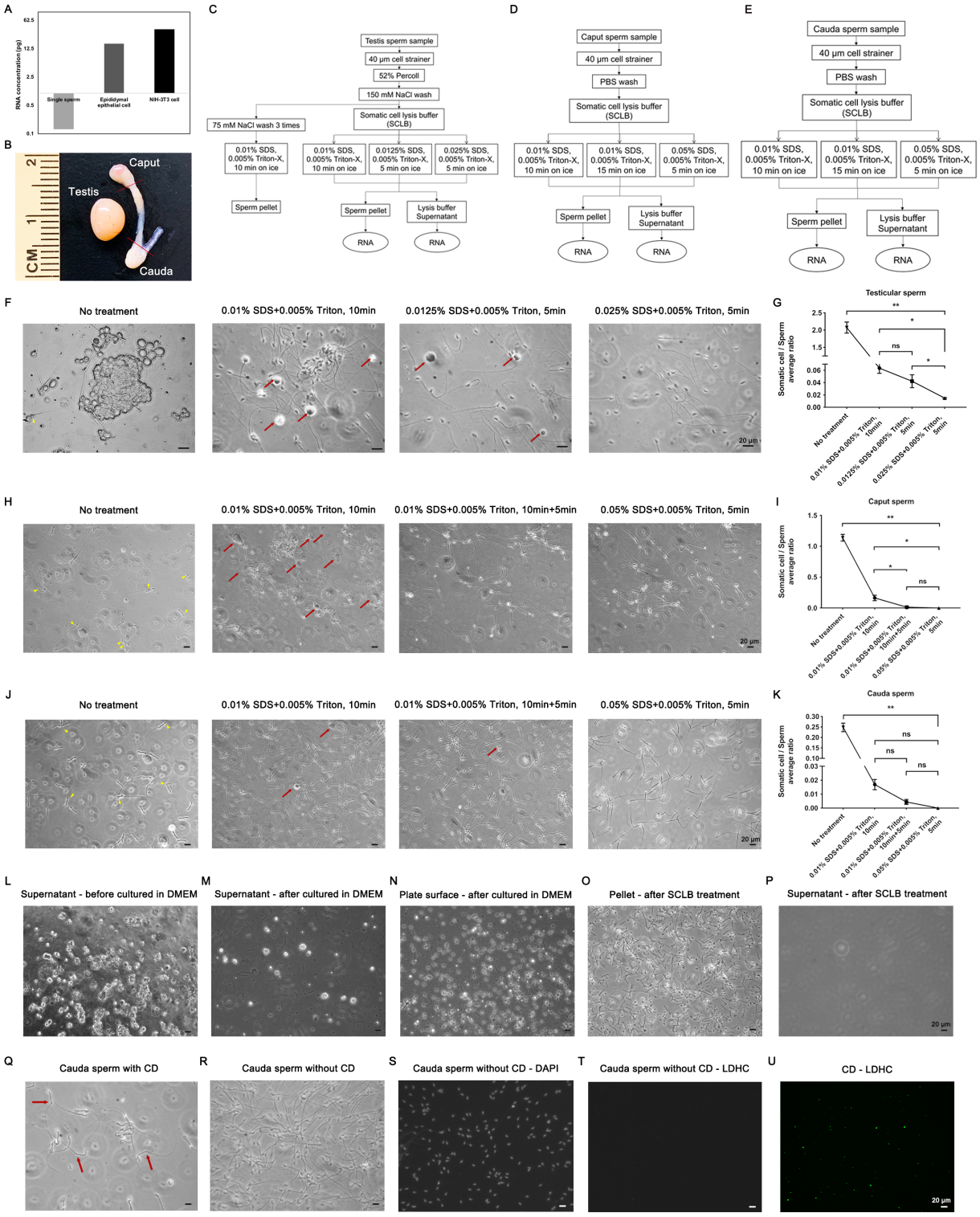

Figure S1. RNA quantity in different cells, workflows of sperm purification, and representative images of sperm, related to STAR Methods and Figure 1. (A) Quantitative analyses of RNA in single sperm, single epididymal epithelial cells, and NIH-3T3 cells. (B) The manner of dissecting the murine caput and cauda epididymis for sperm purification. (C-E)

Workflow for optimized purification protocol of testicular (C), caput (D) and cauda epididymal sperm (E). (F) Preparation of ultrapure testicular sperm. Images showing testicular sperm suspensions before and after treatment with SCLB at different concentrations for different durations. (G) Quantitative analyses of purity of the testicular sperm after various SCLB treatments. (H) Preparation of ultrapure caput epididymal sperm. Images showing caput epididymal sperm suspensions before and after treatment with SCLB at different concentrations for different durations. (I) Quantitative analyses of purity of the caput epididymal sperm after various SCLB treatments. (J) Preparation of ultrapure cauda epididymal sperm. Images showing cauda epididymal sperm suspensions before and after treatment with SCLB at different concentrations for different durations. (K) Quantitative analyses of purity of the cauda epididymal sperm after various SCLB treatments. Data are presented as mean ± SEM, n=4. ns, not statistically significant. **, $p<0.01$; *, $p<0.05$. Yellow and red arrows point to CDs and contaminating somatic cells, respectively. (L-P) Preparation of highly enriched caput epididymal epithelial cells. (L) Epididymal epithelial cells remained suspended in the culture medium before the 4-hour culture. (M) Epididymal epithelial cells remained suspended in the culture medium after the 4-hour culture. (N) Non-epithelial cells attached to the bottom of the culture plate after the 4-hour culture. (O) Sperm separated from the epididymal epithelial cells by SCLB treatment. (P) Epithelial cells completely lysed by the SCLB. (Q-U) Purification of cytoplasmic droplets (CDs) from cauda epididymal sperm. (Q) Phase-contrast micrographs showing cauda epididymal spermatozoa with CDs (red arrows). (R) Phase-contrast micrographs showing cauda epididymal spermatozoa after sucrose gradient centrifugation-based CD removal. (S) The nucleus was stained blue with DAPI. (T) Immunofluorescent staining of LDHC, a CD marker protein, on cauda epididymal spermatozoa after CD removal. (U) Immunofluorescent staining of LDHC in purified CDs (>98%). Scale bar = 20μm.

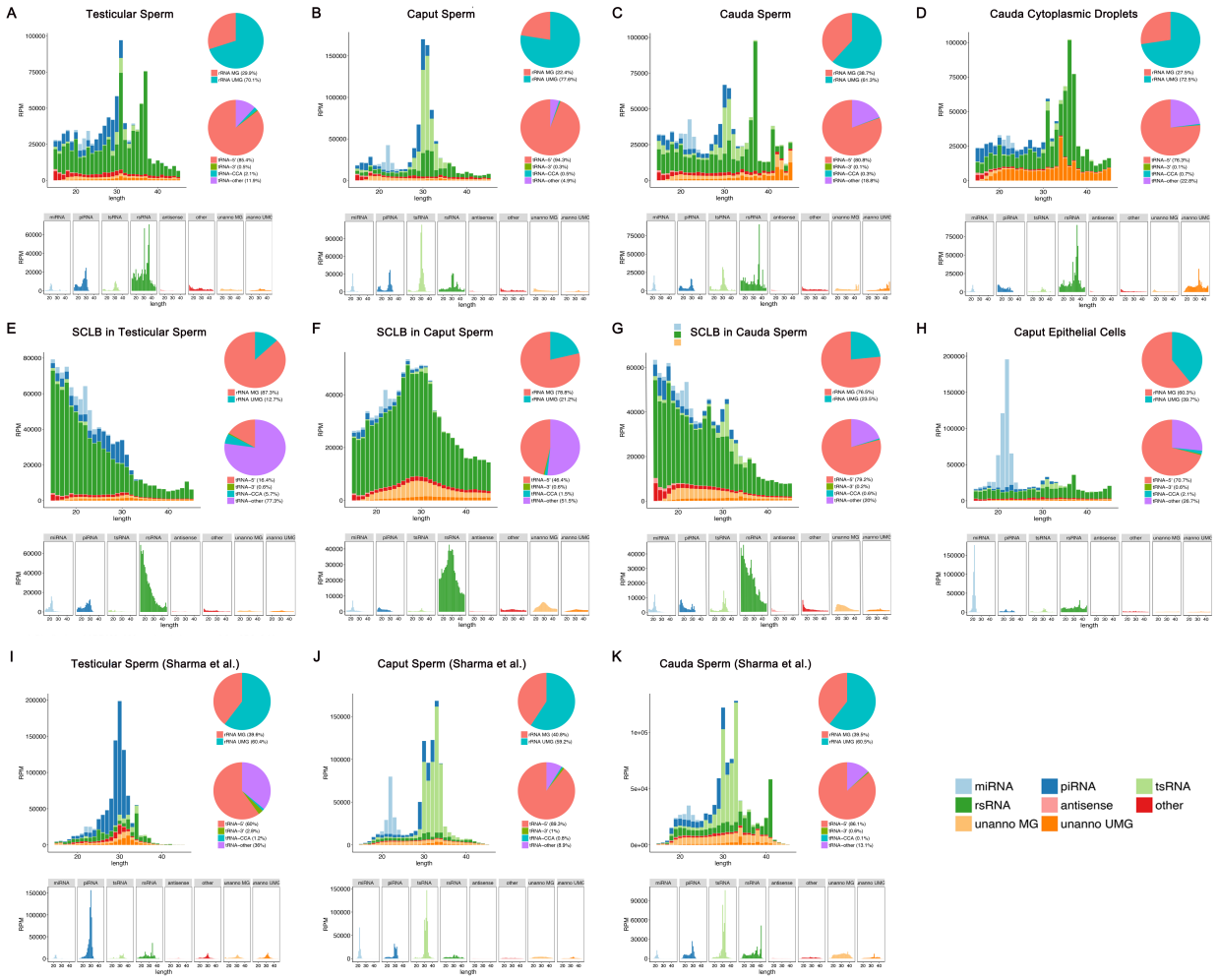

Figure S2. Size distribution of small RNA population, related to Figure 1. (A-H) Profiling and size distribution of sRNAs in spermatozoa (A-C), cauda CDs (D), SCLB (E-G), and caput epithelial (H) in this study. **(**I-K) Profiling and size distribution of small RNAs in spermatozoa in a published report [S1].

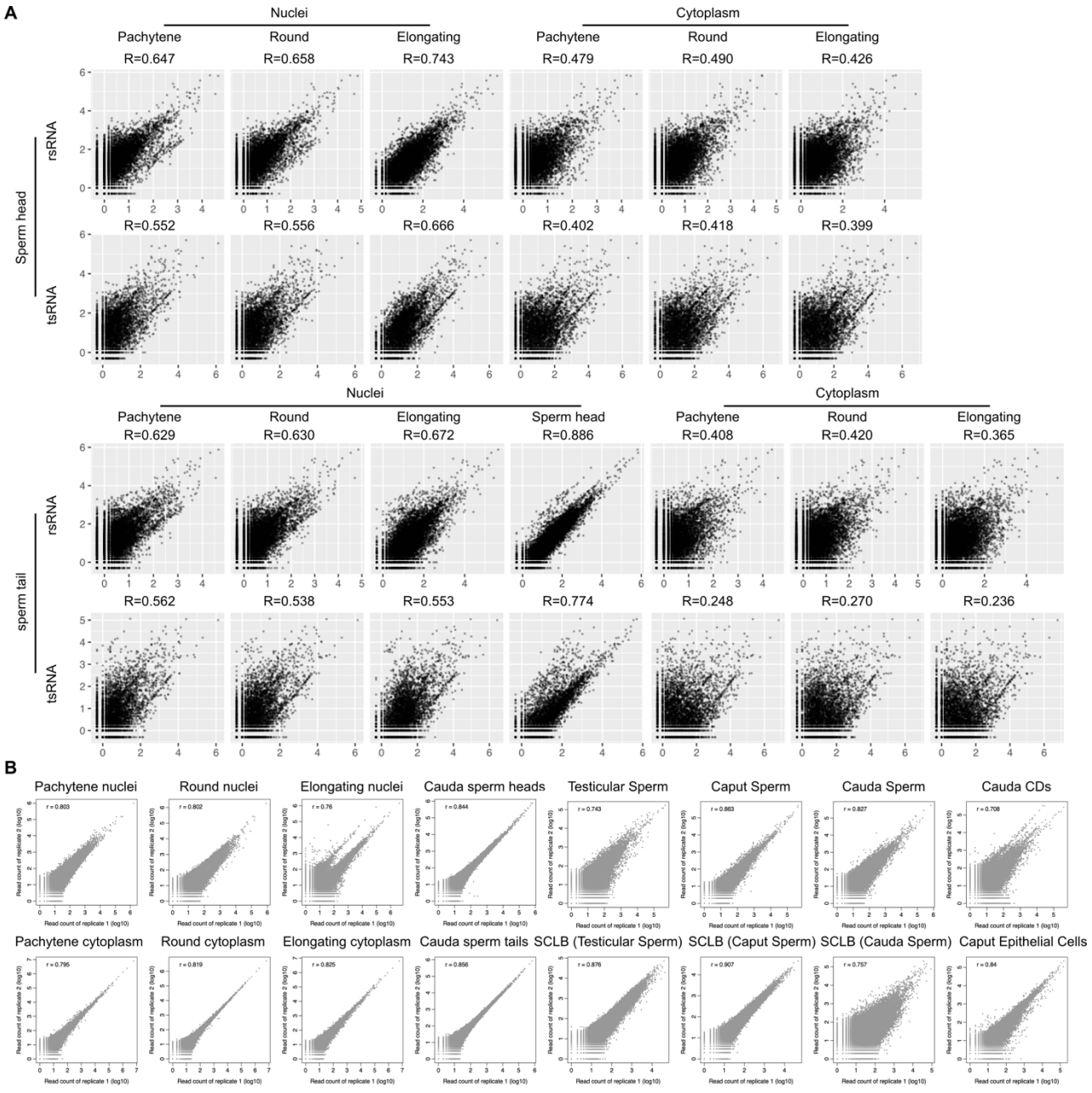

Figure S3. Correlation plots of expression levels (in log10 read counts) between cauda epididymal sperm heads/tails and three spermatogenic cell types (A) and between two replicates (B), related to Figure 1. (A) Correlation plots of tsRNA/rsRNA expression levels (in log10 read counts) between cauda epididymal sperm heads (upper two panels)/tails (lower two panels) and nuclei/cytoplasm of pachytene spermatocytes, round, and elongating spermatids. (B) Correlation plots of total small RNA levels (in log10 read counts) between the two replicates analyzed, with each containing two samples pooled from four mice each.

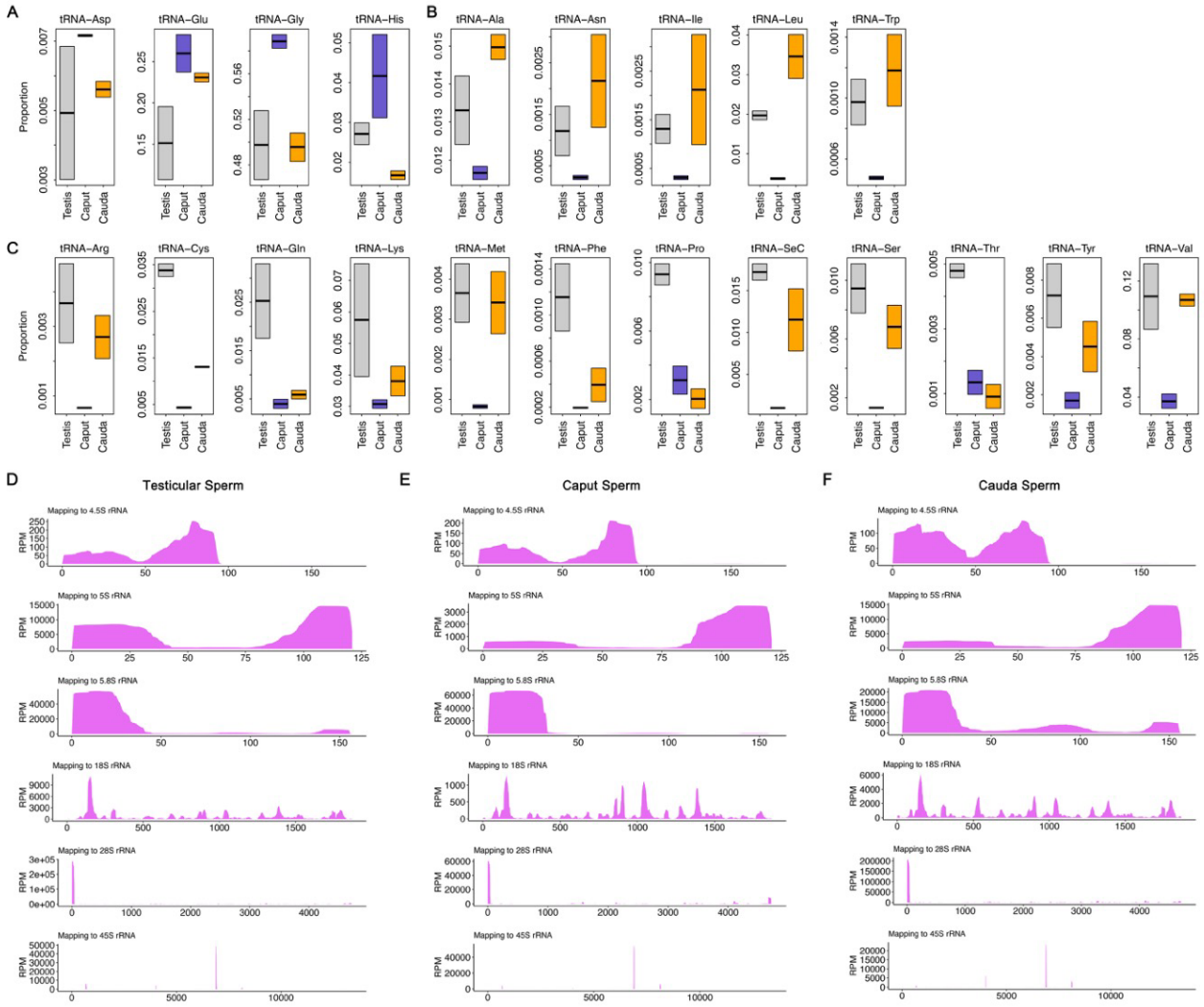

Figure S4. tsRNAs and rsRNAs profile in testicular, caput and cauda epididymal sperm, related to Figure 2. (A-C) Dominant tsRNAs detected by sRNA-seq in caput (A) and cauda (B) epididymal sperm, as well as testicular sperm (C). (D-F) Cell-specific rsRNA subtype profiles in mouse testicular (D), caput (E), and cauda (F) epididymal sperm. RPM, reads per million clean reads.

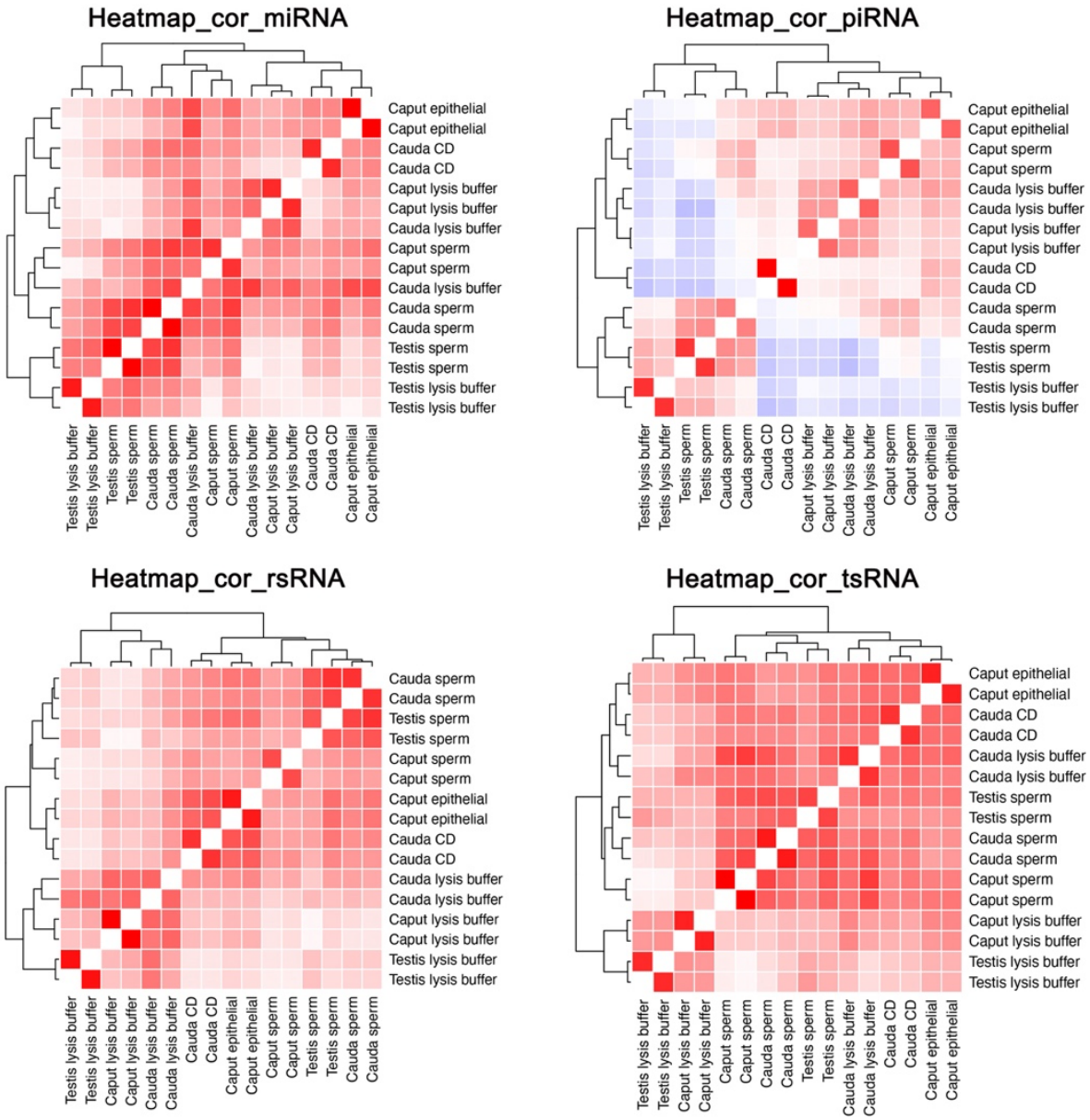

Figure S5. Hierarchical clustering analyses of the transcriptomes of four major small RNA species (miRNA, piRNAs, tsRNAs and rsRNAs) in the eight samples, related to Figure 4.

Supplemental references:

[S1] Sharma, U., Sun, F., Conine, C.C., Reichholf, B., Kukreja, S., Herzog, V.A., Ameres, S.L., and Rando, O.J. (2018). Small RNAs Are Trafficked from the Epididymis to Developing Mammalian Sperm. Dev. Cell *46*, 481-494.e486. 10.1016/j.devcel.2018.06.023.