A spatiotemporal atlas of human spermatogenesis based on single-cell transcriptomics and multiplex antibody imaging

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Summary

Human health depends on complex processes of intercellular interactions and single-cell type-specific functions. Dramatic improvements in single-cell RNA sequencing (scRNA-seq) have opened up the possibility to identify functional states of single cells. Still, spatial methods are crucial for understanding transcript location in relation to tissue architecture. Here, we constructed a large-scale mapping strategy for proteins based on scRNA-seq and fluorescent-based multiplex immunohistochemistry (mIHC). We focused on the spatiotemporal landscape of 12 distinct germ cell states in human testis and performed an in-depth characterization of ~500 germ cell proteins. Quantitative spatial localization data based on a custom-built image analysis pipeline allowed us to cluster proteins according to expression, forming the basis for functional analysis. mRNA and protein expression dynamics showed multiple cases with low levels of co-expression, highlighting the necessity of studying protein levels in single-cell mapping projects. The presented workflow holds promise for proteome-wide tissue studies in health and disease.

Keywords: testis, multiplex immunohistochemistry, spermatogonial stem cells, male germ cell differentiation

Introduction

The dramatic improvements in single-cell RNA sequencing (scRNA-seq) have resulted in many worldwide single-cell mapping efforts, including the Human Cell Atlas (HCA) (1), the Chan Zuckerberg Initiative (CZI) (2), the Human BioMolecular Atlas Program (HuBMAP) (3) (funded by the National Institute of Health (NIH)), and the Human Cell Landscape (4). In most single-cell mapping efforts, reproductive organs are underrepresented. With almost one-third of the world's population being affected by reproductive disorders, the increased incidence of infertility, and the close relationship between reproductive health and overall health, there is an urgent need to map the reproductive processes at a molecular level. Examining the molecular constituents at a single-cell resolution is necessary to facilitate a deeper understanding of these processes. Most ongoing initiatives focus on scRNA-seq analysis, and data integration across platforms is rare, limiting the translation of findings into biologically relevant knowledge and applications due to the loss of transcript location about the tissue geography. While transcriptomic analysis is superior to proteomic methods in terms of gene coverage of low-abundant genes, proteomic methods exceed in dynamic range (5). As transcriptomic methods also suffer from drop-out (6), proteomic methods can be used to better describe the heterogeneous temporal expression patterns and the extent to which proteins are activated or repressed (7, 8). The most significant initiative for mapping proteins is the Human Protein Atlas (HPA) project, which generates an open-access database (www.proteinatlas.org) using an integration of spatial antibody-based proteomics and transcriptomics technologies. The large-scale database covers the major human tissues and cell types using bulk RNA, scRNA (9), and antibody-based data (10), with the classification of genes according to tissue and cell type specificity from a body-wide perspective. The HPA has utilized immunohistochemistry (IHC) to visualize the expression of human proteins in all male and female reproductive tissues. Antibody-based proteomics provides a pixel resolution not currently attainable by other spatial methods; however, classical IHC is primarily used to study one protein at a time, and the resulting data is only semi-quantitative (11). In addition, the visualization of different cell types is limited to what the human observer can distinguish, and the transition between cell states or stages of differentiation is not always possible to determine based on histology only (12). Finally, manual inspection of images is highly subjective, which may lead to wrong interpretation or bias, further highlighting the need for more advanced imaging methods (13).

Recently, an increasing number of methods for multiplex analysis of proteins have been introduced, enabling novel insights into co-expression and tissue

heterogeneity. Some examples of highly multiplexed methods include the CODEX (CO-Detection by indEXing), Immuno-SABER, InSituPlex, and TOF-based methods such as imaging mass cytometry and Mass Ion Beam Imaging. While several of these methods allow for high-plex analyses with dozens of markers, they are mainly used together with ready-made antibody panels, in particular in the field of immuno-oncology. Setting up custom-made panels requires tedious validation and conjugation of antibodies, e.g., with DNA barcodes. Thus, these methods are not optimal for mapping subsets of cells and single-cell states in any kind of tissue, and are not applicable for proteome-wide efforts (11).

Here, we sought to set up a high-throughput multiplex immunohistochemistry (mIHC) workflow for proteome-wide analysis of human tissues. Build-ing upon existing pipelines for regular IHC that are available in most histological laboratories, we used a combination of scRNA-seq data analysis, fluorescent-based antibody-based imaging, and automated image analysis. We have focused on the testis and the development of sperm cells by spermatogenesis, in which the undifferentiated germ cells are subjected to several germ-specific events such as the reduc-tion of the number of chromosomes, condensation of the nucleus, and removal of excess cytoplasm within the sperm. The sperm cells acquire unique morphological features and cell structures, such as the flagellum and the acrosome, which grants them the ability to motility necessary for successful fertilization (14). The complex nature of spermatogenesis involves the expression of thousands of genes and proteins (15), and the testis has previously been shown to be the human tissue with the highest number of tissue-specific genes (10, 13, 16), out of which many have an unknown function (17). We selected a set of ~500 proteins that, based on regular IHC, showed specific locations to germ cells within the testis. With the help of scRNA-seq data, we built antibody panels outlining 12 discrete testicular cell states or stages of differentiation, a resolution not attainable by regular IHC. The fixed panels were used to study one candidate protein at a time and to quantify the expression based on pixel overlap using an automated image analysis pipeline. The integrated analysis and detailed spatial proteomics data allowed us to determine the single-cell state-specific localization of a large set of previously uncharacterized proteins, thereby being able to assign presumed functions in the human testis. We were also able to study mRNA and protein expression dynamics and identified many mRNA-to-protein pairs that varied from a spatio-temporal aspect, further emphasizing the importance of including protein analyses in single-cell mapping efforts.

In summary, we here introduced a validated and robust multiplex pipeline for high-throughput proteome-wide analyses of single-cell states in human tissues, constituting a foundation for hypothesis-driven protein research in

health and disease. The data is presented as an open-access spatiotemporal single-cell type reference map of the adult human testis that decodes the complexity of human germ cells, tissue heterogeneity, and homeostasis and links quantitative data with tissue morphology, thereby accelerating research on infertility and other disorders of the reproductive system.

Results

A methodological approach for comprehensive tissue studies at the single-cell level

The testis constitutes a challenging tissue type of study due to its dynamic and complex cellular composition during spermatogenesis when spermatogonial stem cells differentiate into mature sperm, supported by the testicular niche cells (Figure 1A). A high-resolution image-based atlas of the testis proteome was created by integrating IHC and scRNA-seq datasets to decipher the tissue-based spatial protein expression profiles of male germ cells (Figure 1B). With this multimodal data, we set out to build multiplex IHC (mIHC) antibody panels. The mIHC panels were used for determining the spatial profiles of germ-cell-associated proteins to specific testicular cell states (Figure 1C). Next, we applied a custom image analysis pipeline to achieve a single-cell quantitative protein expression read-out (Figure 1D) and performed downstream analyses for deep molecular interrogation of histological features (Figure 1E). An overview of the workflow is presented in Figure 1.

The tissue-based testis proteome as defined by antibody profiling and single-cell transcriptomics

To generate a high-resolution image-based atlas of the testis proteome, we utilized an IHC-based dataset comprising 4,431 proteins that have been analyzed with single-targeting validated antibodies. From here, 3,752 unique proteins showed at least weak IHC staining in at least one of the germ cell types. For 1,124 proteins, more than one antibody toward the same protein was analyzed, and the antibody that had the highest signal-to-noise ratio was selected to represent the final results (Figure 2A). This set of 3,752 proteins expressed in testicular germ cells constitutes the basis for the selection of both panel markers and candidate proteins to be investigated more in-depth in the current study. The preleptotene and pachytene spermatocytes have been merged based on the highest staining intensity value. The IHC dataset included numerous proteins expressed in one of the germ cell types only, e.g., proteins specific to spermatogonia, spermatocytes, early spermatids, or late spermatids, but also proteins that appear to be expressed

in more than one cell type. When considering also the staining intensity (Figure 2B), it is evident that there are specific clusters of proteins that appear to be more specific, with high staining intensity in particular cell types. It can also be noted that some proteins seem to have a gradient in expression, with higher staining intensity in some cell types and weaker in others, mainly observed in the “Mixed” and “All germ cells” groups (Figure 2B). Overall, there was a high global correlation between spermatogonia, spermatocytes, and early spermatids, with an even higher association between spermatocytes and early spermatids (Figure 1C).

Next, we identified the different cell states and stages of differentiation throughout spermatogenesis that we aimed to target using high-resolution spatial proteomics and mIHC. We integrated scRNA-seq from two sources (Supplementary Figure 1A) (18, 19). In total, 26 clusters were identified and annotated with relevant hallmark gene expression profiles, with each cluster represented from both data sources (Supplementary Figure 1B-D). A total of 37,022 cells were retained for downstream analysis, and clusters were grouped on the cell type basis, with clusters of spermatogonia (SPG), spermatocytes (SPC), and early and late spermatids (SPT), as well as somatic cell niches (Sertoli cells, Leydig cells and supporting cells). Each dataset was represented after grouping the clusters into their main cell type, with a higher percentage distribution from one of the datasets (Sup-plementary Figure 1E-F). Pseudo-time trajectory analysis only on the extracted germ cell populations showed clear transitions between early and late stages, visualizing the stepwise differentiation and developmental progression (Supplementary Figure 1G).

Next, we extracted clusters corresponding to the three primary germ cell groups (SPG, SPC, and SPT). We performed sub-clustering, which revealed 12 sub-clusters of SPGs, 14 sub-clusters of SPCs, and 16 sub-clusters of SPTs (Supplementary Figure 2). In almost all sub-clusters, both datasets were well-represented, but some sub-clusters were mainly retained from one of the datasets (data not shown). Based on genes representing specific cell types and stages during spermatogenesis, we defined each sub-cluster to specific primary states or stages of differentiation (Supplementary Figure 2). For SPGs, the sub-clusters capture the spermatogonial cell states, defined as SPG.0, SPG.1, SPG.2-3, and SPG.4, as well as a cluster that expressed spermatocyte markers, therefore named SPC.1. (Supplemen-tary Figure 2). For SPCs, we were able to identify three main cell states related to the meiotic events in SPCs, termed SPC.1, SPC.2, and SPC.3. Here, a smaller subcluster expressed spermatid genes and was different from the SPC.3 subcluster, therefore named SPT (Supplementary Figure 2). Finally, for SPTs, we could observe two sub-clusters of cells characterized as early-state

spermatids (named SPT.early.1 and 2) and three transformed states where sperm cells begin to develop their characteristic shape and appearance. The later states were named SPT.late.1, 2, and 3 and generally expressed proteins related to sperm cell flagella and motility (Supplementary Figure 2). The final UMAP projec-tions for each sub-clustering can be viewed in Figure 2D and Supplementary Figure 2. Cell counts for unannotated and annotated clusters can be viewed in Supplementary Tables 1 and 2.

As a basis for further in-depth spatial proteomics analysis using mIHC, we aimed to visualize the cell states corresponding to each sub-cluster for SPGs, SPCs, and SPTs with specific antibodies. Based on differential expression analysis and fold difference filter using the set of 3,752 proteins for which validated antibodies were already available as part of the HPA project, we retrieved lists of genes enriched in each sub-clusters. We identified 969 DEGs for SPG states (SPG.0 = 382, SPG.1 = 125, SPG.2.3 = 69, SPG.4 = 383, and SPC.1 = 410), 11,543 DEGs for SPC states (SPC.1 = 883, SPC.2 = 810, SPG.3 = 400, and SPT = 168), and 861 DEGs for SPT states (SPT.early.1 = 367, SPT.early.2 = 513, SPT.late.1 = 116, SPT.late.2 = 133, and SPT.late.3 = 203). Among those, well-established markers known to be specific for a particular cell state were prioritized, such as UTF1 for SPG.0 and DMRT1 for SPG.2-3. For cell states where no well-established marker was present, candidate markers for each cell state were selected based on manual inspection of the IHC data, considering signal-to-noise ratio and staining pattern (e.g., avoiding subcellular patterns that are challenging for downstream automated image analysis). Next, we utilized multiplex IHC (mIHC) to develop three antibody panels for spatial mapping of each of the cell state markers: An SPG panel, an SPC panel, and an SPT panel (dot plots for the corresponding genes are shown in Figure 2E).

High-throughput mIHC for determining cell state-specific protein expression

Antibodies towards proteins representing each specific cell state were optimized, and the final sets of antibody panels were chosen based on robust staining, marking out the expected germ cells with as little overlap as possible to other markers within the same panel. This allowed us to confidently distinguish each cell state corresponding to the different sub-clusters using mIHC. The final mIHC antibody panels for SPG, SPC, and SPT are shown in Figure 3A, and IHC stainings for each panel marker are presented in Supplementary Figure 4. In the SPC panel, we added well-established markers for both SPT early and SPT late despite these being represented by only one sub-cluster. The panels are visualized schematically in Figure 3A.

The three SPG, SPC, and SPT antibody panels, each containing five different markers, were next used to stain with mIHC and novel proteins, here named “candidate proteins.” By leaving a specific spot corresponding to a particular fluorophore in a cyclic 6-plex workflow, one candidate protein at a time was stained with either the SPC, SPC, or SPT panel, allowing us to determine the main cell state-specific location of each candidate protein based on expression overlap. A set of 499 candidate proteins was selected for mIHC analysis (Figure 3C), representing each of the main expression clusters previously identified by IHC (Figure 1B-C). For a complete list of candidate proteins and corresponding antibodies, view Supplementary Table 3. Mainly, proteins stained in, e.g., SPG panel were mainly expressed in spermatogonia according to IHC annotation (Supplementary Figure 5), as well proteins stained in the SPC panel and SPT panel, primarily showing an IHC annotation in spermatocytes and spermatids, respectively (Supplementary Figure 5). Candidate proteins that appeared to be expressed in the transition between two panels, e.g., SPC.1, that are present in both SPG and SPC panels, were stained with both panels to be able to conclude at which cell state the expression of these 69 candidates proteins starts and stops. In total, 228 candidate proteins were stained with the SPG panel, 264 with the SPC panel, and 78 with the SPT panel.

All the generated fluorescent images based on 6-plex mIHC for 499 different candidate proteins were analyzed employing an image analysis pipeline. (Supplementary Figure 5). Due to the flexible setup of the analysis pipeline, we could leverage the same image analysis strategy for stainings generated by all three antibody panels (Figure 3A). Images were extracted, and cell (objects) segmentation was performed first to identify all cells in each stained slide. Cells were then phenotyped based on their panel marker staining in each cell. The total count of each cell state for the three different antibody panels is visualized as percentages in Figure 3B. The expression of the candidate protein for each phenotyped cell was quantified using a pixel-based model available in the Ilastik software. Ilastik uses machine learning algorithms to segment and classify cell- and tissue-based stainings. By drawing and teaching the algorithm what was regarded as a positive signal vs background in approximately 10% of the generated images, the algorithm could be used to create image masks for all candidate protein images and was effectively used to filter out non-specific staining and uncertain staining intensities (Supplementary Figure 6). The number of cells and the mean intensity expression from image analysis per protein and panel can be viewed in Supplementary Tables 8 and 9.

Functional analysis of spermatogenesis proteins in single-cell states

To describe the image analysis protein quantification, we applied hierarchical clustering using the mean intensity for each candidate protein stained with each mIHC panel based on the cell state-specific expression. The proteins retained in each of the generated clusters were functionally assessed by Gene Ontology (GO) enrichment.

Five main clusters were identified and named for the 228 candidate proteins stained with the SPG panel according to their main expression attributes (Figure 5A and Supplementary Table 4). Most candidate proteins stained with the SPG panel were predominantly expressed in SPG.0 and SPG.1 cells (Cluster 2). GO enrichment analysis showed that the 81 proteins within this cluster were mainly associated with transcriptional activity (Figure 5A). None of these proteins showed an apparent staining in the spermatocyte stage. Cluster 1 was dominated by proteins expressed in SPG.2-3. Among these 52 proteins, lamellipodium was the only significant function retained. Cluster 4 mainly contained proteins expressed in SPG.4 (53 proteins), associated with cell cycle checkpoint and transition, which is expected as this is the final cell stage before mitosis and transitioning into spermatocytes. Cluster 5, which contained 23 proteins, included a group of proteins expressed in both SPG.0 and SPG.1, with functions mainly related to transcription. Finally, Cluster 3, with 19 proteins, was mixed.

Next, the SPC panel proteins were subjected to hierarchical clustering (Figure 5B and Supplementary Table 4). We obtained clusters associated with specific states and clusters with a broader expression pattern. Most proteins were expressed in SPC.3 cells, and expression in this cell dominated most clusters (Figure 5B). However, the largest cluster, Cluster 7, consisted of 73 proteins and also included expression in SPC.2. Clusters 3, with 37 proteins, and Cluster 1, with 48 proteins, were also associated with SPC.3 cells but with lower expression in SPC.2 and SPT.early cells, respectively. Interestingly, the 14 proteins in Cluster 5 were predominantly expressed in SPC.1 cells, while the 13 proteins in Cluster 6 were mainly SPC.2-proteins. Cluster 4, with 60 proteins, and Cluster 2, with 19 proteins, were mainly of a mixed nature, with the primarily former including proteins in any of the spermatocyte states and the latter predominantly being expressed in SPC.3 and SPT.early germ cells. The functional assessment of these groups of proteins by GO analysis showed an overall association with RNA processing, nuclear condensation, and structural chromosomal changes (Figure 6B).

For the SPT candidate proteins, six main expression clusters were identified (Figure 5C and Supplementary Table 4), including proteins specific to the early phases of spermiogenesis, proteins representing the transformation of

haploid round cells, and finally, proteins present during the maturation of elongated sperm cells. Most proteins stained with the SPT panel were found in the later stages of sperm development, such as Cluster 5 and 6, with 21 and 14 proteins, respectively. The closely related Cluster 4 only included 2 proteins and showed a similar expression distribution to those in Cluster 5 and 6 but with more pronounced expression in SPT.late.1 cells. The 20 proteins in Cluster 3 were mainly specific to SPT.late.2 cells, while Cluster 2 (18 proteins) and Cluster 1 (3 proteins) were associated with the earlier phases of spermatids, SPT.early.1 and 2 cells. (Figure 5C). In general, the GO analysis indicated that these proteins are related to sperm flagellum assembly, development, and motility, as well as functions associated with fertilization and acrosomal vesicle generation.

To exemplify expression patterns for each of the different SPG, SPC, and SPT clusters, representative proteins from each cluster are shown in Figure 6. For proteins stained with SPG panel, PABPC4, which may be involved in cytoplasmic regulatory processes of mRNA metabolism (20), in Cluster 1 was predominantly expressed in SPG-2.3 cells. The melanoma-associated antigen B2 (MAGEB2) in Cluster 2 was mainly present in SPG.0 cells. This protein is a cancer-testis antigen member of the MAGEB family, suggested to be involved in transcriptional regulation (21). CES2 protein is believed to participate in cholesterol metabolism (22) and was one of the proteins in Cluster 3. CES2 was shown to be expressed in SPG.0 and 1. In Cluster 4, the unknown protein HHIPL2 was mainly expressed in SPG.4 cells. FBL in Cluster 5 showed a general speckle-like expression in all SPG states and SPC.1 cells. FBL (also known as fibrillarin) has a multi-faceted function and is a component of a nucleolar small nuclear ribonucleoprotein particle. FBL is thought to participate in processing pre-ribosomal RNAs (23) (Figure 6A). For proteins stained with SPC panel, representative stainings are presented as follows: the cluster 1 protein RFX3 was mainly positive in the nuclei of SPC.3 and SPT.early cells. RFX3 is involved in ciliogenesis (24). The Cluster 2 protein, UBAC2, known to be involved in WNT-signaling (25) showed mainly a broad SPC and SPT staining pattern. HMMR, which is also known as CD168 or RHAMM, was included in Cluster 3, and was mainly found in SPC.3 cells. HMMR is involved in cell motility (26). The DEAD box protein 11, or DDX11 was among the proteins in Cluster 4 and mainly expressed in the earlier states of spermatocyte cells but with distinct staining in the other SPC cell states. DDX11 is essential for the cohesion of the chromosome arms and centromeres. If depleted, replicated chromosomes fail to segregate after the prometaphase arrest and ultimately lead to mitotic failure (27). The Cluster 5 protein Cyclin-dependent kinase 1 (CDK1) is highly conserved and known to promote the G2-M phase during the cell cycle (28), and was confined to SPC.1 cells. The class I MHC molecule HLA-B was mainly found

in Cluster 6, dominated by expression in SPC.2 cells. Class I molecules play a role in antigen presentation (29). Lastly, among Cluster 7 proteins, Spondin-2 or SPON2 is known to play a role in cell adhesion (30). SPON2 was observed in almost all cell states present in the SPC panel, however slightly more pronounced in SPC.2 and 3.

Among proteins stained with SPT panel, the cluster 1 protein, DNAAF6, which is needed for sperm flagella motility (31), was mainly found in earlier SPT states, namely SPT.early.2 cells. HMGB2 in Cluster 2 was expressed in SPT.early.1 state and is known to be able to bend DNA and involved in double-strand breaks repair and recombination (32). SPACA9 is part of the dynein. associated microtu-bule proteins (33). SPACA9 was observed in Cluster 3 and was seen in SPT.late.2 cells. From Cluster 4, CLEC11A was shown to be in most SPT states, however mainly in SPT.late.1, 2 and 3 cells. CLEC11A is known to promote differentiation in mesenchymal cells (34). Of Cluster 5 and 6 in SPT-stained proteins, IZUMO1 and ZPBP were proteins detected distinctly in SPT.late.2 and 3 cells. Both proteins are important for sperm-egg binding and IZUMO1 is essential for sperm-egg binding and fusion (35), and ZPBP is associated with the acrosome in sperm (36) (Figure 6C).

Cell state-specific expression dynamics between mRNA and protein levels

During spermatogenesis, two phases of inactive transcription have been identified and are known to be associated with homologous recombination of early meiosis and the cessation of mRNA production during the cellular transformation of haploid cells into elongating spermatids (37). Therefore, two major waves of transcription occur before and after the meiotic events (38).

We theorized that our in-depth cell state-specific characterization at the RNA and protein level could identify cases where mRNA expression is detected at earlier stages of the spermatogenesis process compared to the protein expression. By analyzing correlation coefficients between mRNA and protein levels and singling out cases of negative correlation, we identified pairs of mRNA and protein in all three panels with discordances in their co-expression dynamics (Figure 7A). This allowed for the identification of genes with both high expression and strong RNA-protein correlations, aiding in the exploration of expression dynamics and molecular regulation. Positive correlations were found for most of the candidates, indicating that in many cases, mRNA is translated into protein within the same cell state. Among these concordant cases, the early spermatogonia marker, RHOXF1, shows a posi-

tive correlation between mRNA and protein expression (Figure 7B), with highest expression in the SPG.0 state as displayed in the line plots. Similarly, the transcription factor RFX2, which is specific to the end of meiosis, is expressed in SPC.3 and colocaliz-es with the magenta signal on the mIHC staining (Figure 7B). ACSBG2 increases linearly from SPT.early.1 to SPT.early.3 for both mRNA and protein (Figure 7B). We also identified inverse correlation for SPG, SPC, and SPT (Figure 7A). Among these discordant RNA-protein pairs, the mRNA expression peaks in an earlier cell state than the protein. One such example is PIWIL4, already described as a SPG.0 marker (18), which is highly specific for SPG.0 at the mRNA level, but interestingly its corresponding protein is mainly expressed in SPG.2-3 and SPG.4 (Figure 7A). As another example a protein with a well-studied role in spermatogenesis with a discordant expression profile is WNK1. The WNK1 transcript is primarily detected in SPC.1 with a steady decrease to SPT stage, but its corresponding protein expressed peaks SPC.3 state (Figure 7B). Similarly, ACTRT3 is expressed in SPT.early.1 and 2 at the RNA level, but the protein is present in later stages, predominantly in SPT.late.2 but also in SPT.late.3 (Figure 7B). Furthermore, two candidates, VSIG4 in the SPG panel and R3HDML in the SPC panel, returned N/A values for the correlation analysis due to a lack of variability in the scRNAseq dataset (Supplementary Table 9).

Discussion

With the introduction of scRNA-seq, multiple efforts worldwide have generated high-quality datasets of human tissues (1, 9), which gives us the possibility to distinguish novel subsets of cells based on specific gene signatures. One such example is the identification of the spermatogonial SPG.0 state, with a gene expression profile closely related to infant germ cells, which suggests that these cells represent naïve stem cell-like cells (19). No large-scale study has however looked at the corresponding protein expression levels at a single-cell resolution comparable with the transcriptomics data, which is necessary for understanding how mRNA levels are translated into physiological and functional phenotypes. While it is known that transcription in testis occurs during two extensive waves before and after meiosis, and that the second transcription leads to the storage of mRNAs that will be needed at a later time-point during spermatogenesis, it has not been concluded which mRNAs that are directly translated into proteins, and which mRNA and protein expression patterns that vary from a temporal aspect. Mechanisms for post-transcriptional control have been shown to play a necessary role for normal spermatogenesis, and it can be anticipated that detailed analysis of the testis proteome ultimately leads to increased understanding of testicular function in health and disease.

Spatial analysis has the advantage of studying the expression in intact tissue samples in the context of neighboring cells, not biased by limitations in tissue dissociation that may affect proportions of cells. The testis constitutes a challenging tissue type to study due to its dynamic and complex biological processes during spermatogenesis when adult spermatogonial stem cells differentiate into mature sperm. Spermatogenesis can classically be divided into three main phases: (i) the proliferative mitotic phase in which spermatogonia undergo a series of mitotic divisions; (ii) the meiotic phase when genetic recombination occurs in primary diploid spermatocytes, which divide twice to produce haploid sper-matids; (iii) and finally spermiogenesis when the spermatids morphologically transform into spermatozoa. During spermatogenesis, which lasts approximately 74 days in humans, thousands of genes and proteins are activated or repressed (39). To which extent and at which time-point during this process all gene products are expressed and what functions they have to maintain a healthy reproduction however remains to be explored. Since spermatogenesis is a continuous process, the definition of what can be called a cell type adds another level of complexity. Traditionally, the cell types have been distinguished on the basis of their appearance, e.g. “dark” and “pale” spermatogonia according to the condensation of the nuclei, and round or elongated spermatids by looking at the shape. Already this distinction is however subjective and has challenges in manual interpretation. During the last phase of spermatogenesis, spermiogenesis, histones are replaced which causes a compaction of the spermatid chromatin. Genes such as Protamine 1 and 2 (PRM1 and 2) are transcribed at the early spermatids, before the cells transform and adopt their unique and characteristic appearance. For example, the 3′ UTRs of PRM1 mRNA have specific sequences that cause a “delayed” translation as these mRNAs are kept in a translationally repressed state bound to inactive ribonucleoprotein particles (40). Premature expression of PRM1 causes spermatid differentiation failure (41). In fact, the spermatogenesis process experiences periods of large translational output in an environment of inactive transcription (37). Our approach to couple scRNA-seq data with spatial antibody-based protein data with image analysis to pinpoint the exact cell state of proteins could offer a prerequisite to identify novel proteins with similar expression dynamics as the Protamine proteins.

We have previously done an extensive effort to classify IHC images stained as part of the HPA project in 5 different germ cell types and 3 somatic cell types (13, 16), giving a first clue towards the involvement of these proteins during spermatogenesis. Despite involving an expert for the interpretation, we could however show that when building an image analysis algorithm for automatic detection of the cell type-specific localization, multiple examples

were identified where the human observer had made errors (13), highlighting the need for more quantitative measurements. Here, we sought to develop a stringent workflow for proteome-wide antibody-based analysis with a resolution not attainable with traditional IHC. By utilizing scRNA-seq data for identification of specific cell states and validating these with mIHC, we established multiplex panels that could be used for determining the exact single-cell state localization of ~500 testicular germ cell-specific proteins based on expression overlap with the fixed antibody panels. To make the spatial proteomics data comparable with scRNA-seq, an automated image analysis pipeline was developed, allowing us to generate quantitative measurements of protein expression levels in each of the cell states. We were then able to cluster the proteins based on expression pattern, highlighting specific groups of proteins that form the basis for further functional analysis. Most of the proteins studied have not previously been described at this detailed level in the context of spatial localization within testis, thereby adding important knowledge into their presumed role within spermatogenesis. Interestingly, we also identified several proteins that showed a mismatch in correlation between mRNA and protein levels within the cell types studied in each of the panels, indicating that mRNA is stored and the protein is translated at a later time-point during spermatogenesis. Such conclusions would not be possible to draw from transcriptomics data alone, and these proteins constitute important targets for further functional studies on testis physiology. In addition to correlations of mRNA versus protein levels analyzed within each panel, it would be interesting to analyze the similar set of proteins across multiple panels and gross cell states.

Interestingly, our analysis revealed that many of the 499 investigated candidate proteins showed a variable correlation coefficient between RNA and protein levels in terms of cell state-specific expression pattern. It is in particular important to highlight that among these proteins, some have been described in literature previously as important markers for a certain cell state, but we here show that the actual protein is expressed at a later differentiation stage. One such example is the SPG protein PIWIL4 which at the mRNA level was shown to be expressed in SPG.0, while the maximum protein expression was detected later in SPG.2-3 and SPG.4 states. PIWIL4 was among the Cluster 1 proteins in SPG analysis and related to lamellipodium, a type of cell membrane protrusion that drives migration in not only normal processes, but also pathological event (42). In fact, the highly conserved PIWI proteins are essential for self-renewal and as highlighted in a previous report, knock-down of PIWIL4 expression in vitro in a breast cancer cell line significantly decreased the migration ability of cancer cells (43). As a concordant case in SPG, RHOXF1 was identified as mainly expressed in SPG.0 with decreasing expression towards SPC phenotype. RHOXF1 is commonly

used as an SPG hallmark, especially in some scRNA-seq studies (44). RHOXF1 belongs to the X-linked reproductive homeobox gene cluster and encodes a transcription factor with a crucial role in controlling spermatogonial proliferation and differentiation Expectedly, deleterious RHOXF1-variants cause severe oligo and azoospermia (45). In SPC Cluster 1, the transcription factor RFX2 showed both mRNA and protein mainly in SPC.3 cells. Among the enriched functions in Cluster 1, acrosome assembly was highly significant for the cluster and in RFX2-null mice, acrosomal defects have been detected (46). Further, RFX2 is previously reported as mainly being expressed in late pachytene stage which further cements its presence as a pachytene-elevated protein. In fact, the SPC.3 panel marker TCFL5 is also regarded as a pachytene protein, which increases the reliability in our findings (47). Relating to that, WNK1 is known to be expressed at pachytene stage (48), confirming our staining results. Howev-er, the mRNA expression peaks were mainly detected in SPC.1 state. WNK1 is essential for cell division (49) and its has also been shown that disruption of WNK1 might lead to premature protein synthesis of other mRNAs due to upregulation of MTOR (48). ACTRT3 is suggested to be involved in cytoskeletal organization and is highly similar to conventional actin protein (50). Confirming our results, ACTRT3 is known to localize to the nucleus mainly in elongating and elongated spermatids (51).

Our analysis focused on the testicular germ cells, and to cover all the main cell states, we built three different custommade panels. In addition to germ cells, it is known that the somatic niche cells (Sertoli cells, Leydig cells, and peritubular (myoid) cells) provide support for completing spermatogenesis, from spermatogonial stem cell stage to mature sperm. Future studies should therefore also include these cells, especially Sertoli cells, which are in direct contact with the sperm cells throughout spermatogenesis (52), to further elucidate protein-protein interactions between these cells. Nevertheless, we here show that by using a 6-plex mIHC workflow, we were able to perform a large-scale analysis of almost 500 proteins, and based on the quantitative spatial proteomics data identify link expression patterns with specific testicular functions. By utilizing entirely custom-made panels and a workflow that is highly compatible with regular IHC, our strategy can easily be set up in histological laboratories for increasing the resolution of antibody-based imaging to a level comparable with scRNA-seq data. While fixed panels of five markers at a time does not cover all various cell types and cell states, multiple panels per tissue type can be built depending on the questions asked, and for visualization, the human eye is anyway not able to interpret dozens of markers in the same field of view. Instead, our pipeline can be used in a proteome-wide manner, since the method does not require antibody conjugation and each candidate protein can be studied by just replacing one antibody at a specific step of the staining cycle.

In summary, we here show that the integrated workflow combining quantitative scRNA-seq analysis with qualitative information provided by mIHC we were able to leverage novel insights at different resolutions and study specific expression in cell states not previously described or distinguishable by one method alone. The stringent workflow with downstream automated image analysis is applicable for any type of human tissue and holds promise for defining the proteomes of each cell state and differentiation stage at a high molecular resolution, ultimately generating a detailed map of human cells. The data presented here is expected to be of major importance in research on male reproduction, and the validated antibody panels constitute an important resource for further spatial proteomics studies on human testis.

Limitation of the study

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Acknowledgments

The project was funded by the Knut and Alice Wallenberg Foundation (2015.0344) and the Swedish Research Council (2022-02742). This publication has been made possible in part by the BioImage Informatics Facility BIIF, a unit of the National Bioinformatics Infrastructure Sweden NBIS, with funding from SciLifeLab, National Microscopy Infrastructure NMI (VR-RFI 2019-00217), and Chan Zuckerberg Initiative DAF (DAF2021-225261, DOI 10.37921/644085ggkbos, an advised fund of Silicon Valley Community Foundation, DOI 10.13039/100014989). The computations for image analysis were in part enabled by resources provided by the National Academic Infrastructure for Super-computing in Sweden (NAISS), partially funded by the Swedish Research Council (2022-06725). Finally, the authors also want to thank all members of the HPA group for their efforts.

Author contributions

Conceptualization: CL and MU; Data curation: FH and LM; Formal Analysis: FH, LM, GM, JG, BK, and RS; Funding acquisition: CL and MU; Investigation: FH, LM, JG, BK, and RS; Methodology: CL, FH, LM, GM, and JG; Project administration: JG and FH; Resources: LM, KvF, GM, and MF; Software: LM, GM, FH; Supervision: CL, LM, CZ, and MU; Validation: FH, LM, and CL; Visualization: FH and LM; Writing – original draft: FH, LM, and CL; Writing – review & editing: FH, LM, CL, BK, RS, CZ, and MU. All authors have read and approved the final version of the manuscript.

Resource availability

Lead contact

Requests for further information and resources should be directed to and will be fulfilled by the lead contact, Dr. Cecilia Lindskog (cecilia.lindskog@igp.uu.se).

Material availability

This study did not generate new unique reagents.

Data and code availability

All data and images generated from this study are available in the Human Protein Atlas (https://www.proteinatlas.org/). All R codes and other source data are available upon request or at https://github.com/LindskogLab/A-spatiotemporal-atlas-of-human-spermatogenesis. The image analysis Python code is available on GitHub by accessing Jupyter notebooks and relevant src functions in this repository here: https://github.com/BIIFSweden/spermatogenesis-profiling (DOI:https://zenodo.org/doi/10.5281/zenodo.13788792).

Methods

Experimental model and study participant details

Tissue preparation and ethical declaration

Histologically normal formalin-fixed and paraffin-embedded (FFPE) tissue samples from adult human testis were used for mIHC. The tissue collection consisted of samples from in four total individuals (#4485 Male 41 years, #4506 Male 32 years, #3627 Male 25 years, #4845 Male 29 years), out of which three individuals were used in each of the analyzes. Testis tissues were obtained from the Clinical Pathology department, Uppsala University Hospital (Sweden), and collected within the Uppsala Biobank organization. Samples were anonymized as determined in the approval and advisory report from the Uppsala Ethical Review Board (ref nos. 2002-577, 2005-388, 2007-159). For generation of tissue microarrays (TMAs), testis tissue blocks were punched at 1 mm in diameter using a manual tissue arrayer (MTA-1, Beecher Instruments) and placed in a recipient block, generating a TMA block of six cores comprising of doublet testis cores from three individuals. To generate the public IHC stainings available in the HPA database, standard TMAs as part of the HPA workflow were used (REF), which included

single cores of three individuals of adult human testis. Different individuals were used throughout the HPA project, and all IHC stainings available on www.proteinatlas.org are thus not from the same testis samples. The FFPE TMA blocks were cut in 4 µm sections using a waterfall microtome (Microm H355S, ThermoFisher Scientific, Freemont, CA), placed on SuperFrost Plus slides (Thermo Fisher Scientific, Freemont, CA), to be dried overnight at room temperature (RT). Slides were baked at 50°C for 12-24 h and cooled and stored at -20°C before use.

Method details

Sample preparation and slide pretreatment

Slides were brought to RT, deparaffinized in xylene, and rehydrated in graded alcohols (99.9, 96, and 80%) down to deionized water. Endogenous peroxidases were blocked using 0.3% hydrogen peroxide in the 96% alcohol step. Heat-induced epitope retrieval (HIER) was performed in a decloaking chamber (Biocare Medical, Walnut Creek, California, USA) at 125°C for 4 min in pH 6.0 Target Retrieval Solution (Agilent Technologies Inc., Santa Clara, California, USA) and quickly cooled to 90°C and then left to cool to RT before staining. Both chromogenic single-plex IHC (HPA standard) and mIHC were performed by using Lab Vision Auto-stainer 480S (Thermo Fisher Scientific, Freemont, CA). For mIHC slides, slides were bleached after HIER in a bleaching solution consisting of 0.2 M glycine, and 1.5% hydrogen peroxide in TBS-Tween (Thermo Fisher Scientific, TA-999-TT). Slides and bleaching solution were inserted in Falcon tubes and placed on rotation under LED light for 1 h in RT to reduce the overall effect of tissue autofluorescence. Slides were kept in TBS-Tween before mIHC staining. Stainings with single-plex IHC and 3, 3'-diaminobenzidine (DAB) (hereinafter referred to as chromogenic IHC) have been performed as previously described (53).

Annotation of IHC images

Digital images of IHC-stained transverse sections of testis tissue available on the HPA version 23 (Ensembl version 109) were selected for extended testis annotation. In addition to antibody validation, an inclusion criterion was that the staining pattern in the testis should be of at least moderate intensity in at least one of the cell types in the testis. The manual annotation data of adult human testis seminiferous epithelium was generated as previously described (16) and validated by a multi-class, multi-label hybrid feature-based deep learning model (13). In summary, for each antibody, the staining intensity (strong, moderate, weak, negative), quantity of stained cells (>75%, 25–75%, 1–24%, <1%) and subcellular localization (nuclear, cytoplasmic, membranous) in eight different testicular cell types, including five germ cell

types (spermatogonia, preleptotene spermatocytes, pachytene spermatocytes, round/early spermatids, and elongated/late spermatids), and three somatic cell types (Sertoli cells, Leydig cells, and peritubular cells) was recorded. For this work, we only focused on the germ cell types, and therefore the somatic cells were excluded from the analysis. Please note that the annotation results of preleptotene spermatocytes and pachytene spermatocytes have been merged for all analyses in this work. A table with all annotations can be viewed in Supplementary Table 5.

Single-cells transcriptomics

To determine and characterize different populations of human testicular germ cells, publicly available UMI count tables were downloaded from the Gene Expression Omnibus (GEO) data repository. The inclusion criteria were adult and healthy tissue sam-ples, sequencing performed using the 10X Genomics system, and no prior cell sorting. Two studies were identified as suitable: Guo et al. (2018) ((18)/ GSE112013), which included testis cells from three young healthy donors aged 17 to 25 years old, and Nie et al. (2022) (19)/), which included testis cells from 12 donors aged 17 to 76 years old. In the latter study, data were divided into three groups: young adults ("Young," n=4, 17-22 years old), older adults ("Older group-1," n=5, 62-66 years old), and a third group ("Older group-2," n=3, 64-76 years), which was excluded due to spermatogenic impairment. Three UMI count tables (one from GSE112013 and two from GSE182786) were loaded into R and analyzed using the Seurat package v4 (54). For each dataset, Seurat objects were created with a minimum of 3 cells and 500 features per cell. After standard QC, cells were filtered to retain those with 500-5000 expressed genes and less than 20% of the read mapped to the mitochondrial genome. Each dataset was preprocessed separately following the Seurat package vignette (https://satijalab.org/seurat/archive/v4.3/integration_rpca). Datasets were integrated using the reciprocal PCA (RPCA) method, and UMAP and clustering analysis was performed on the integrated object, testing resolutions from 0.4 to 1.2, with a final selection of 0.8. Each cluster was assigned an identity based on well-known markers and named according to the main cell type in that cluster (Supplementary Figure 1D). The count data for cells labeled as main germ cell types—spermatogonia (SPG), spermatocytes (SPC), and spermatids (Early.SPT + Late.SPT)—were isolated and processed similarly to the previously described method (Supplementary Figure 2). This resulted in three independent Seurat objects for SPG, SPC, and SPT. Based on well-known markers (Supplementary Figure 2), cell states were assigned accordingly (Supplementary Figure 3). Only genes coding for proteins characterized by IHC with at least weak staining in any germ cells were kept for downstream analysis, and uncharacterized cells were removed.

To identify potential markers for constructing the three mIHC panels, the Seurat function FindAllMarkers was used with the Wilcoxon ranksum test, using the following settings: ‘min.pct = 0.20’, ‘logfc.threshold = 0.25’, and ‘only.pos = TRUE’; for the SPG, SPC, and SPT objects.. A fold difference variable was defined based on the mean expression per sub-cluster in the RNA assays, representing the fold change between the highest and the second-highest mean expression, and differentially expressed genes (DEG) with this a fold difference below 1.5 were removed. These resulting lists served as the starting point for comparison with IHC data to identify the best antibody combinations for each main germ cell type and their respective panels. Some DEGs could be shared by several cell states, but were still included. To visualize the expression level across the different testicular cell states, Seurat’s DotPlot function was used. Mean expression for the protein candidate has been recovered for each of the main germ cell Seurat objects for visualization.

To further validate the lineage progression and developmental trajectory of the identified germ cell populations, we performed pseudotime analysis using the Monocle 3 package on R (Supplementary Figure 1G and 2).

Multiplex panel generation and staining

To select suitable antibodies as panel markers, we placed a large emphasis on staining specificity, primarily selecting proteins with germ cell-specific expression patterns over those with additional staining in somatic cells and stromal cells outside of the seminiferous tubules in testis, from the DEG lists. If there was significant overlapping in panel marker stains (i.e., if a too large proportion of germ cells were double-positive), alternative markers were tested from the lists, or the order of the panel markers was re-ordered to ensure distinct and non-overlapping staining patterns.

Antibodies that were finally included in the antibody panels were first tested in single-plex mode to ensure compatibility with the OPAL detection system, similar specificity as in chromogenic IHC and to ensure an appropriately high signal-to-noise ratio, otherwise, antibodies were exchanged for another antibody/clone against the same antigen or ex-changed for a protein with similar expression pattern and cell type specificity/distribution. For all three testis panels, a fixed order of OPAL fluorophores was used for rapid panel development. The OPALs were used as follows; first cycle OPAL690, second cycle OPAL620, fourth cycle OPAL570, fifth cycle OPAL480, and sixth cycle OPAL-DIG+780. The third cycle was always used with the candidate protein which was iteratively changed for each new slide stained. Low-protein expressors of panel markers were assigned to brighter fluorophores when

possible and more abundant markers were in general used with the dimmer fluorophores. Further, each antibody was tested at all different cycles to ensure optimal position and manually inspected to prevent apparent reduction and secure a well-represented staining distribution and overall pattern.

Slides were directly taken from bleaching and light treatment and subjected to mIHC staining protocol at RT. Staining was done using the IHC HRP reagent kit from Epredia (Epredia UltraVision LP HRP kit) and one cycle of staining was done as follows: blocking, primary antibody incubation, a secondary antibody with horseradish peroxidase (HRP) polymer, and an OPAL fluorophore (Akoya Biosciences, Marlborough, Massachusetts, USA), and antibody stripping in retrieval buffer in a decloaking chamber. After the last cycle, slides were incubated with the OPAL 780 fluorophore-conjugated anti-DIG antibody and 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen, D1306, Thermo Fisher Scientific). Slides were then mounted using Prolong Glass Antifade mounting medium and left to cure overnight in RT. Slides were scanned using PhenoImager (Akoya Biosciences) according to the manufacturer’s instructions. Acquired images were spectrally unmixed and image export was performed using the built-in synthetic spectral library of the inForm software (Akoya Biosciences). For the SPG panel, a specific library was created by following the manufacturer's instructions due to the use of OPAL650 for fractions of stainings. See Supple-mentary Table 6 for detailed staining cycle infor-mation regarding panel marker antibodies, dilutions, reagents, incubation times, and OPAL fluorophores.

Selection of candidate proteins for staining in mIHC panels

Candidate proteins were selected to be stained primarily with one panel and a main emphasis to select proteins with cell type-specific staining patterns from the extended annotation of testis IHC images. We assumed high compatibility between standard chromogenic and multiplex stainings as both stainings are similar in pretreatment and staining protocol, and therefore the dilution factor of the primary candidate protein was based on previous staining results. In general, the dilution factor used in IHC was multiplied 1.5x which was deemed an appropriate approach to stain primary antibodies of candidate proteins in mIHC setting. The resulting staining was evaluated to have high comparability with chromogenic IHC staining without apparent non-specific staining patterns vastly dissimilar to the previous stainings. Slides were restained if the staining for any of the panel markers was incomplete or dissimilar to a set of representative images after manual inspection. The same antibody/clone was, to the best of our ability, used if a protein was stained in multiple panels. However, for some proteins, antibody availability and compatibility with the current panel forced us to use alternative rabbit pAbs directed toward the same protein.

Image Analysis

The fluorescence images were analyzed using a custom Python script that processes each channel separately to define cells and retrieves the mean staining intensity value of all OPAL flours in each cell. Briefly explained the script segments cells and determines the phenotype of cells based on the panel marker fluorescence signal. Lastly, the candidate protein is overlaid on all phenotype cells, but also unstained/non-phenotyped cells. Each processed slide corresponds to one protein being processed for one panel and the resulting data frame is used for all subsequent downstream analyses. In detail, the scanned slides were batch exported using the Akoya Phenochart software (Akoya Biosciences) and using a synthetic library or panel-specific library, all according to the manufacturer's instructions. Each of the six TMA cores was exported as a separate tif-file with the channels and OPAL fluorophores in the following order: 1-DAPI; 2-OPAL480; 3-OPAL520; 4-OPAL570; 5-OPAL620; 6-OPAL690; 7-OPAL780; 8-Autofluorescence. The candidate protein channel for every slide (OPAL520-channel image) was individually extracted as a grayscale tiff file and used for segmenting positive signals by training a pixel-based algorithm in Ilastik image analysis software. By defining three classes for drawing (positive, negative, and back-ground), and using brush stroke labels on 11% of the proteins, the algorithm predicts which class a pixel belongs to for all pixels (55). The output segmentation image was used as a mask for specifying which cells were positive for each candidate protein. Next, the panel OPAL-channel images were individually extracted as grayscale and subjected to automated thresholding by Otsu/multi-otsu segmentation with the rationale that the marker stainings had a high signal-to-noise-ratio and the automated approach captured the marker staining with high certainty. In the case of multi-otsu segmentation, the upper thresholded pixels were always regarded as the true positive signal for the panel marker, and the other levels below were considered negative/background staining. The level of multi-otsu segmentation was as follows: for SPG panel images, all OPAL-channel images were set to 3-level segmentation. For SPC panel images, a regular Otsu segmentation was applied, and for SPT panel images, a 3-level multi-otsu segmentation was used for OPAL480, OPAL570, OPAL620, OPAL690, and 4-level multi-otsu segmentation for OPAL780-channel images. Binary masks for all OPAL-channel images were produced and in some cases, after manual inspection of a fraction of images, the Otsu/multi-otsu was increased or decreased to better fit the staining distribution of that specific slide/protein. Cells were defined with a DAPI channel image by using the pretrained StarDist 2D cell segmentation model and the following input parameters: nms_threshold=0.7, probability_threshold=0.486166. The mean intensity values for all OPAL fluorophores in all cells (objects) were saved in a data frame and the mean intensity of all cells above the threshold after Otsu/multi-otsu thresholding

were saved in a data frame. Further, we phenotyped the cells based on panel OPAL positivi-ty. For the candidate protein OPAL520-channel values, we applied a min-max scaling to rescale and normalize the values to allow comparison between proteins stained with the same panel marker.

Downstream-descriptive analysis

Image analysis data from SPG, SPC and SPT panels were processed using R (version 4.3.2). Data Frames corresponding to each panel were imported and organized into structured lists. Metadata from the Laboratory Information Management System (LIMS) was integrated to filter out cells from low-quality tissue cores, such as those lacking seminiferous ducts or showing insufficient panel marker representation. Phenotypes were assigned to each cell based on the panel markers' OPAL intensity, as described in Supplementary Table 7. Cells without an assigned phenotype were excluded from subsequent analyses. Summary tables were created to calculate the mean OPAL520 intensity (ranging from 0 to 1) for different cell types, and cell frequency tables were also generated. Hierarchical clustering was performed on the mean OPAL520 intensity data for each panel, and clusters were visualized using heatmaps generated with the Pheatmap R package. The gene clusters identified through hierarchical clustering were subjected to Gene Ontology (GO) enrichment analysis using the clusterProfiler Bioconductor package. Enrichment was evaluated for the Biological Process, Molecular Function, and Cellular Component ontologies. GO terms with an adjusted p-value < 0.05 were considered statistically significant, and a redundancy simplification step was applied with a cutoff of 0.7 for the adjusted p-value. The top five most significant GO terms for each gene cluster were visualized using a lollipop plot displaying the rich factor (ratio of genes in the cluster associated with a GO term to the total number of genes associated with that term), adjusted p-value (color-coded), and gene count (represented by point size) for each term.

Transcript and protein expression dynamics

The correlation analysis aimed to explore the linear relationship between RNA and protein expression. scRNA-seq data were obtained from the Seurat objects with each dataset corresponding to distinct cell populations of interest (SPG, SPC, and SPT). Protein expression data from multiplexed immuno-fluorescence (OPAL) assays were retrieved from the pre-processed data frames. Cells states were classified based on the lineage differentiation stages. RNA expression values were normalized using a min-max scaling approach, resulting in a 0-1 range for each gene. OPAL protein expression values were utilized in their pre-scaled form as described. For both RNA and protein datasets, we computed summary statistics including mean, median, standard deviation, minimum, and maximum expression levels. These calcu-

lations were performed for each gene across the defined cell states. Gene expression patterns were visualized using line plots generated with the ggplot2 R package, where mean expression levels were shown alongside standard deviation ribbons. To quantify the relationship between RNA and protein expression, z-score normalization was applied to both datasets, standardizing expression values for each gene across all cell types. The normalized RNA and protein datasets were merged by gene identifiers and cell type classifications. Pearson correlation coefficients were calculated between RNA and protein expression profiles.
Scatter plots were created using ggplot2 to depict the relationship between the correlation coefficient and the highest mean RNA expression level. Density plots were also created using ggplot2 to visualize the distribution of the correlation coefficient and RNA peak intensity values.

Quantification and statistical analysis

Detailed statistical methods for each stage of the study are provided can in the respective sections of the Method details.

Main figure legends

Figure 1

Overview workflow of the study. A) The complex microstructure of the testis includes a multitude of germ cells positioned within the seminiferous tubules of the testis. A schematic representation of the main cell types observed in testis histology is shown in the cropped image. B) IHC-based annotation of human testis stainings and single-cell RNA-seq (scRNA-seq) datasets from two sources were used to build a multiplex IHC (mIHC) antibody panel for spatial characterization of germ-cell associated proteins. C) Using the established antibody panels, candidate proteins were iteratively stained by using constant panel markers for cell phenotyping for each slide and replacing the primary antibody towards the candidate protein to be characterized in the third staining cycle, here depicted with green pseudo-color (view Methods for more details). D) The cells were classified by a custom-built image analysis pipeline, and cell profiling was based on automatic intensity thresholding of the mIHC panel stainings. The candidate protein data was defined and used to determine the quantitative expression of each candidate protein at the cell/state-specific level (green overlays). E) The high-resolution single-cell level data can be used in different downstream analyses such as rudimentary analysis (protein quantification in specific cell populations or clustering based on in situ protein expression data) or to explore functional and correlative associations across the characterized cell types.

Figure 2

The tissue-based testis proteome as defined by antibody profiling and single-cell transcriptomics. A) All IHC-annotation intensity values were binarized (negative = 0 and weak, moderate, strong =1) and displayed as an Upset plot to view the presence of intensity in any germ cell type count frequency of intensity in horizontal bars and the count frequency of specific combinations of intensity in one cell type regardless of intensity in other in vertical bars. The total count is displayed on top of each bar. B) A heatmap with hierarchical clustering showing the intensity value (negative, weak, moderate, and strong) in germ cells (columns) is visualized for each protein (rows) with cell-type specificity group annotation for each protein. The number of proteins in each cell-type specificity group is seen within parenthesis in the heatmap labels. C) Spearman correlation of the data presented in the heatmap in B, with a color ramp to show the correlation coefficient. D) UMAP projections of scRNA-seq sub-clustering of spermatogonia (left), spermatocytes (middle), and spermatids (right). E) Dot-plot of the selected genes used for each panel (x-axis) and their mean expres-sion level and distribution in the defined sub-clusters (y-axis). The order of the genes is displayed as they appear in the below schematic figure, with the genes UTF1, IRF2BPL, DMRT1, CTCFL, and BEND2 being used in the spermatogonia panel as SPG.0, SPG.1, SPG.2-3, SPG.4 and SPC.1. HELLS, SCML1, TCFL5, SUN5 and PRM1 were used in the spermatocyte panel to label SPC.1, SPC.2, SPC.3, SPT.early and SPT.late cells. Lastly, LYAR, OLAH, C3, SPATA24, and TPPP2 were included in the spermatids panel to define SPT.early.1, SPT.early.2, SPT.late.1, SPT.late.2 and SPT.late.3 cells.

Figure 3

Deep spermatogenic profiling by high-throughput multiplex imaging. A) Representative images of each testis mIHC panel to showcase the panel markers in seminiferous tubules of normal human testis samples in the top pane with schematic depictions of each marker and the relative abundance and position of cells highlighted in each panel. B) Examples of proteins stained at various states during spermatogenesis performed with the three different antibody panels. In each staining, the candidate protein is visualized in green with the protein name depicted on top. C) Venn diagram showing the number of unique candidate proteins stained with each panel.

Figure 4

High-resolution quantification and single-cell characterization based on bioimage analysis. A) The iteratively stained testis slides were scanned on a slide scanner (Akoya Phenoimager) by using recommended settings and batch-extracted with panel-specific algorithms for proper multispectral

separa-tion of each fluorescent signal. The top right image showcases the protein LBR (green) stained with the SPG panel. The slide is scanned in 7-channel mode, and images are exported after a visual inspection of successful staining. Protein = candidate protein. The nuclear DAPI signal is used for cell identification and segmentation. In contrast, the panel ab fluores-cent signals in the middle right image are used for cell phenotyping by automated thresholding methods (gray outlines show cell boundaries from cell segmentation). The fluorescent signal for each candidate protein is thresholded using an image classifier that outputs positive (yellow), negative (blue), and background (red) pixels and creates overlay masks using the positive signal only. Candidate proteins are quantified in single cells with known differentiation state/phenotype. B) A stacked percentage bar chart showing the percentage distribution of each cell state for all stained proteins defined by the automated cell phenotyping using the custom-built image analysis platform.

Figure 5

Hierarchical clustering and functional analysis of proteins expressed in different phases and states in the A) SPG, B) SPC, and C) SPT panels. For heatmaps, scaled intensity values were z-score nor-malized and visualized per row. Clustering analysis using cut-tree separated the proteins according to their staining in each cell state. The clusters are numbered according to their order from top to bot-tom. For functional analysis, cluster-wise functional analysis was performed by GO enrichment analysis for proteins expressed in different phases and states in spermatogenesis in all three panels.

Figure 6

Protein expression across spermatogenic states. Representative examples of proteins from each cluster, as well as a line expression plot depicting the normalized protein expression across cell states, are visualized for SPG (A), SPC (B), and SPT (C) proteins. Staining images display all markers in the left pane, and crop-in only shows the candidate protein staining (green). Clusters were defined by the heatmap expression profile in Figure 5.

Figure 7

The spatiotemporal expression dynamics of RNAs and proteins during spermatogenesis. A) Pearson's correlation of mRNA and protein levels at single cell level for each panel. Each dot represents the correla-tion coefficients (-1 to 1) based on scaled mRNA and protein levels in SPG, SPC, and SPT panels (y-axis). The Highest Mean RNA Expression (x-axis) is derived from the normalized scRNA-seq levels and the peak mean RNA for each dot is displayed in the plot. The correlations were calculated in the direction of the first, most undifferentiated cell in each panel and towards the direction of

the more differentiated cell state. Density plots show the distribution of correlation coefficient (in grey) and peak mean RNA (colors). B) Representative concord-ant and discordant stainings (candidate protein in green pseudocolor) with line expression plots for selected proteins stained with SPG, SPC, and SPT panels. The gene and protein expression is presented as mean scaled expression data from scRNA-seq and scaled quantitative levels from image analysis by mIHC staining. The highlights for RNA (yellow) and protein (green) represent the standard error.

Supplementary figure legends

Supplementary Figure 1

Single-cell RNA-seq quality control data. A) UMAP projection of the entire integrated scRNA-seq dataset, and B) grouped by individual dataset. C) Cluster and cell types distribution per dataset. D) Hallmark genes were used to define and annotate cell clusters. E) Umap showing the manually annotated (grouped) cell clusters, and F) their cell type distribution per dataset. G) Pseudo-time analysis only applied to the extracted germ cells visualized in E.

Supplementary Figure 2

Re-clustering of spermatogonia, spermatocytes and spermatid main clusters. Trajectory analysis was performed on unannotated clusters (left) and on annotated clusters (right). Cluster annotation was done by the use of established Hallmark genes in spermatogenesis and their expression in each cluster is shown in a dotplot (middle).

Supplementary Figure 3

Panel antibodies stained with conventional IHC-DAB using HPA standard protocol. The cell state name, gene symbol, and antibody ID are shown for all stainings. Each side of the crop-in is 125 µm.

Supplementary Figure 4

IHC annotation for antibodies stained in each specif-ic mIHC testis panel. For proteins stained with spermatogonia (top), spermatocytes (middle), and spermatids (bottom), the presence of intensity in any germ cell type in IHC is displayed as upset plots with the count frequency of any intensity (weak, moderate, or strong) in cell types depicted in the horizontal bars and the count frequency of specific combinations of intensity in one or more cell types presented in vertical bars with the total count at the top of each bar.

Supplementary Figure 5

Positive mask generation in ilastik for candidate proteins. A) Stained slides are scanned and for all candidate proteins, the OPAL520 fluorescence signal channels were extracted. B) In total, 69 OPAL520-images were used to cover representative stainings from each panel. Approximately 11% of all available images were selected for drawing. C) Using ilastik image analysis software, positive, negative, and background signals were drawn to train a pixel-based algorithm for segmenting positive OPAL520-signal. D) The remaining images were processed with the pixel classifier to generate positive signal masks for all OPAL520 images.

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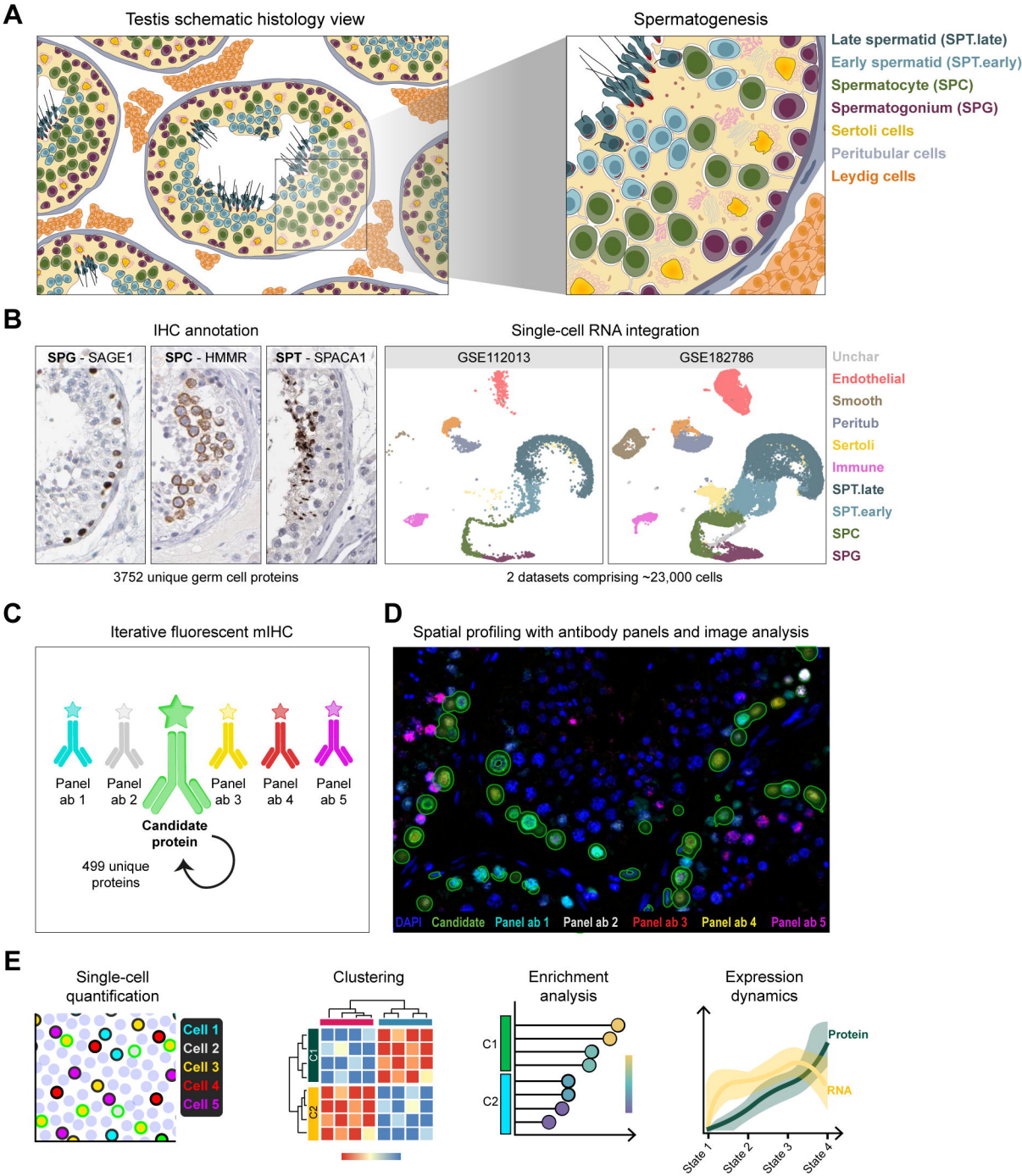
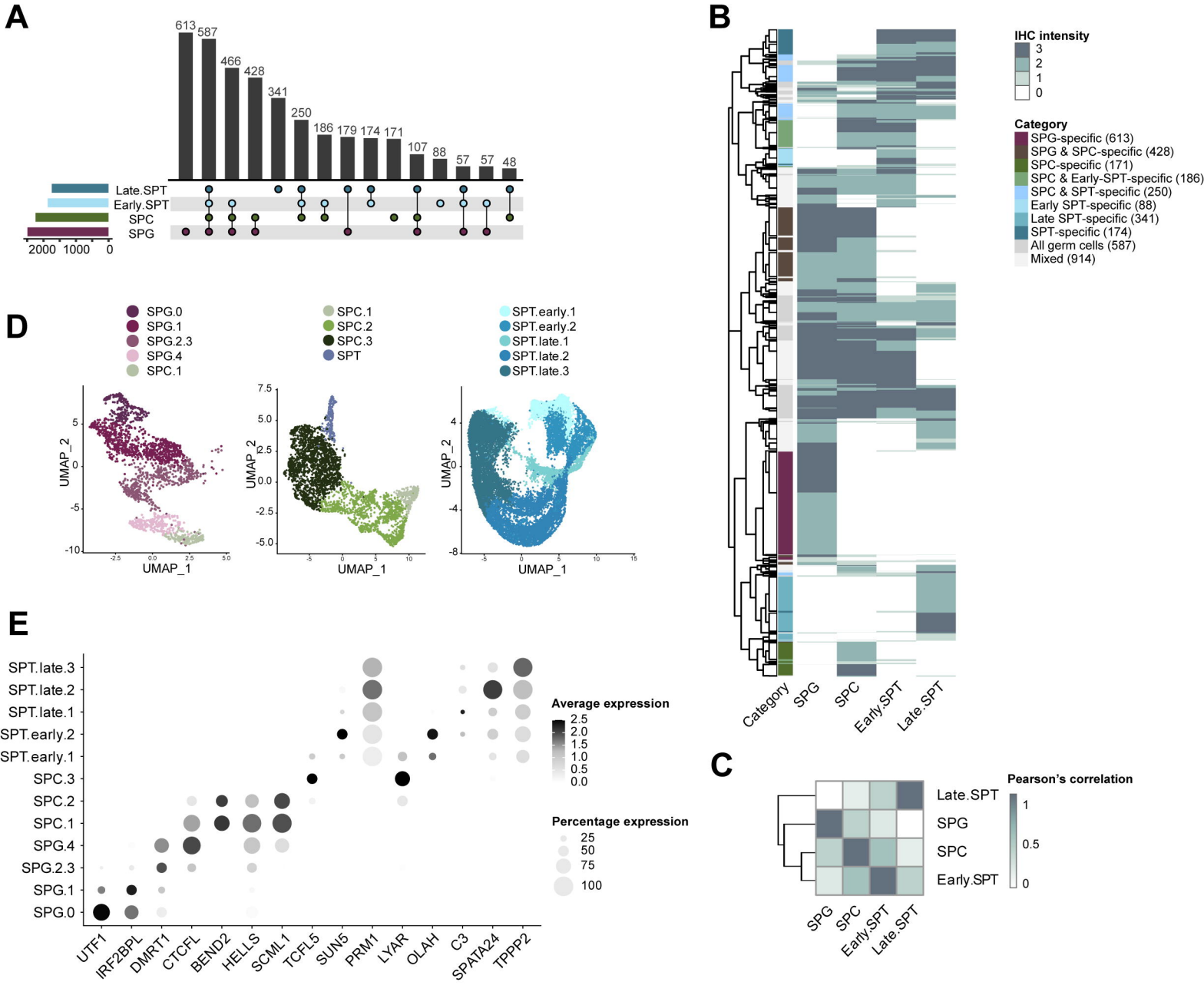

A
Testis schematic histology view
Spermatogenesis
Late spermatid (SPT.late)
Early spermatid (SPT.early)
Spermatocyte (SPC)
Spermatogonium (SPG)
Sertoli cells
Peritubular cells
Leydig cells
B
IHC annotation
Single-cell RNA integration
SPG - SAGE1
SPC - HMMR
SPT - SPACA1
GSE112013
GSE182786
Unchar
Endothelial
Smooth
Peritub
Sertoli
Immune
SPT.late
SPT.early
SPC
SPG
3752 unique germ cell proteins
2 datasets comprising ~23,000 cells
C
Iterative fluorescent mIHC
Panel ab 1
Panel ab 2
Panel ab 3
Panel ab 4
Panel ab 5
Candidate protein
499 unique proteins
D
Spatial profiling with antibody panels and image analysis
DAPI
Candidate
Panel ab 1
Panel ab 2
Panel ab 3
Panel ab 4
Panel ab 5
E
Single-cell quantification
Cell 1
Cell 2
Cell 3
Cell 4
Cell 5
Clustering
C1
C2
Enrichment analysis
C1
C2
Expression dynamics
Protein
RNA
State 1
State 2
State 3
State 4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613
587
466
428
341
250
186
179
174
171
107
88
57
57
48
Late.SPT
Early.SPT
SPC
SPG
2000 1000 0
B
IHC intensity
3
2
1
0
Category
SPG-specific (613)
SPG & SPC-specific (428)
SPC-specific (171)
SPC & Early-SPT-specific (186)
SPC & SPT-specific (250)
Early SPT-specific (88)
Late SPT-specific (341)
SPT-specific (174)
All germ cells (587)
Mixed (914)
Category
SPG
SPC
Early.SPT
Late.SPT
C
Pearson's correlation
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0.5
0
Late.SPT
SPG
SPC
Early.SPT
SPG
SPC
Early.SPT
Late.SPT
D
SPG.0
SPG.1
SPG.2.3
SPG.4
SPC.1
SPC.1
SPC.2
SPC.3
SPT
SPT.early.1
SPT.early.2
SPT.late.1
SPT.late.2
SPT.late.3
UMAP_2
UMAP_1
E
SPT.late.3
SPT.late.2
SPT.late.1
SPT.early.2
SPT.early.1
SPC.3
SPC.2
SPC.1
SPG.4
SPG.2.3
SPG.1
SPG.0
UTF1
IRF2BPL
DMRT1
CTCFL
BEND2
HELLS
SCML1
TCFL5
SUN5
PRM1
LYAR
OLAH
C3
SPATA24
TPPP2
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1.5
1.0
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Percentage expression
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50
75
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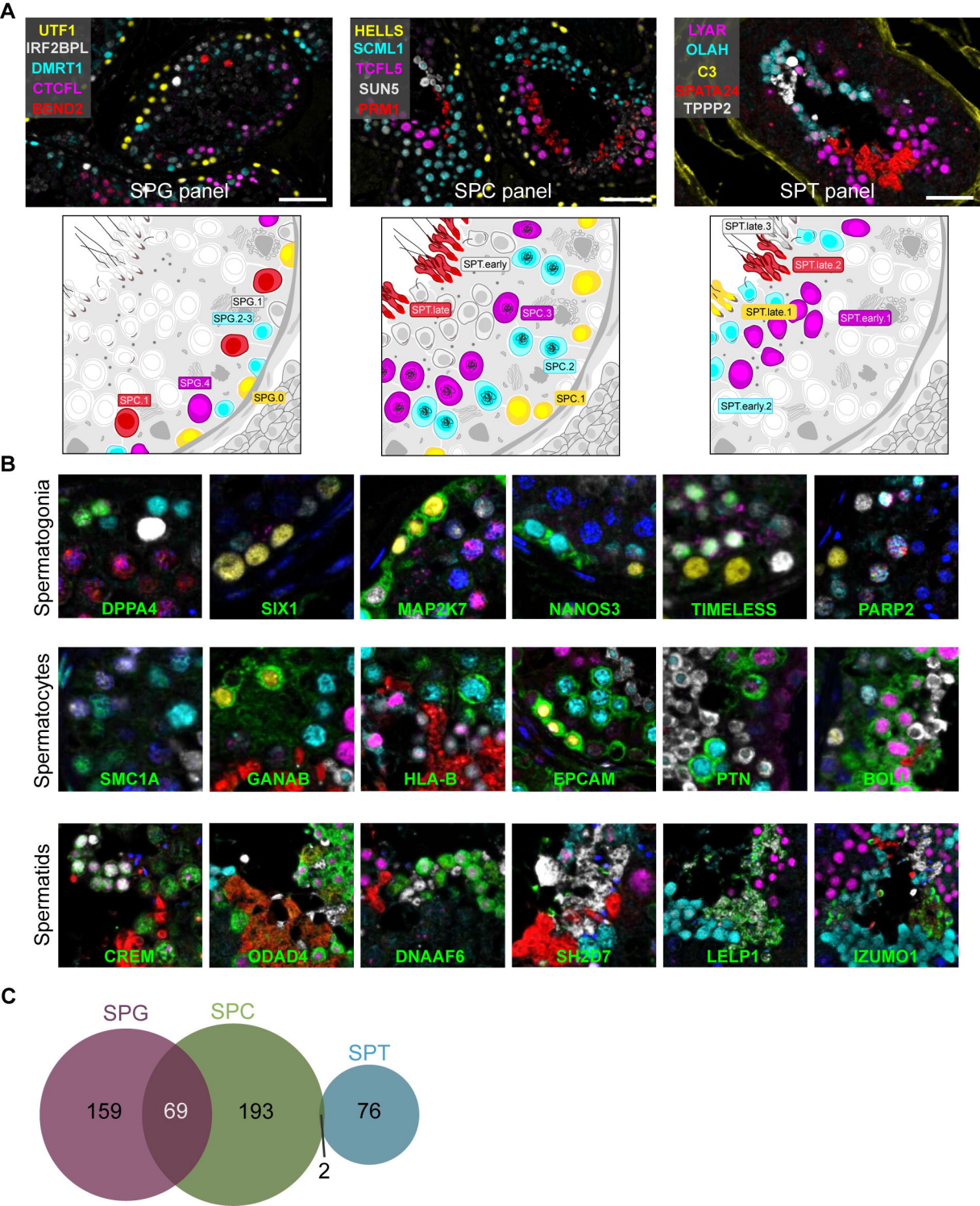
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TCFL5
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LYAR
OLAH
C3
SPATA24
TPPP2
SPT panel
SPG.1
SPG.2-3
SPG.4
SPC.1
SPG.0
SPT.early
SPT.late
SPC.3
SPC.2
SPC.1
SPT.late.3
SPT.late.2
SPT.late.1
SPT.early.1
SPT.early.2
B
Spermatogonia
DPPA4
SIX1
MAP2K7
NANOS3
TIMELESS
PARP2
Spermatocytes
SMC1A
GANAB
HLA-B
EPCAM
PTN
BOLL
Spermatids
CREM
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SH2D7
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IZUMO1
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SPG
SPC
SPT
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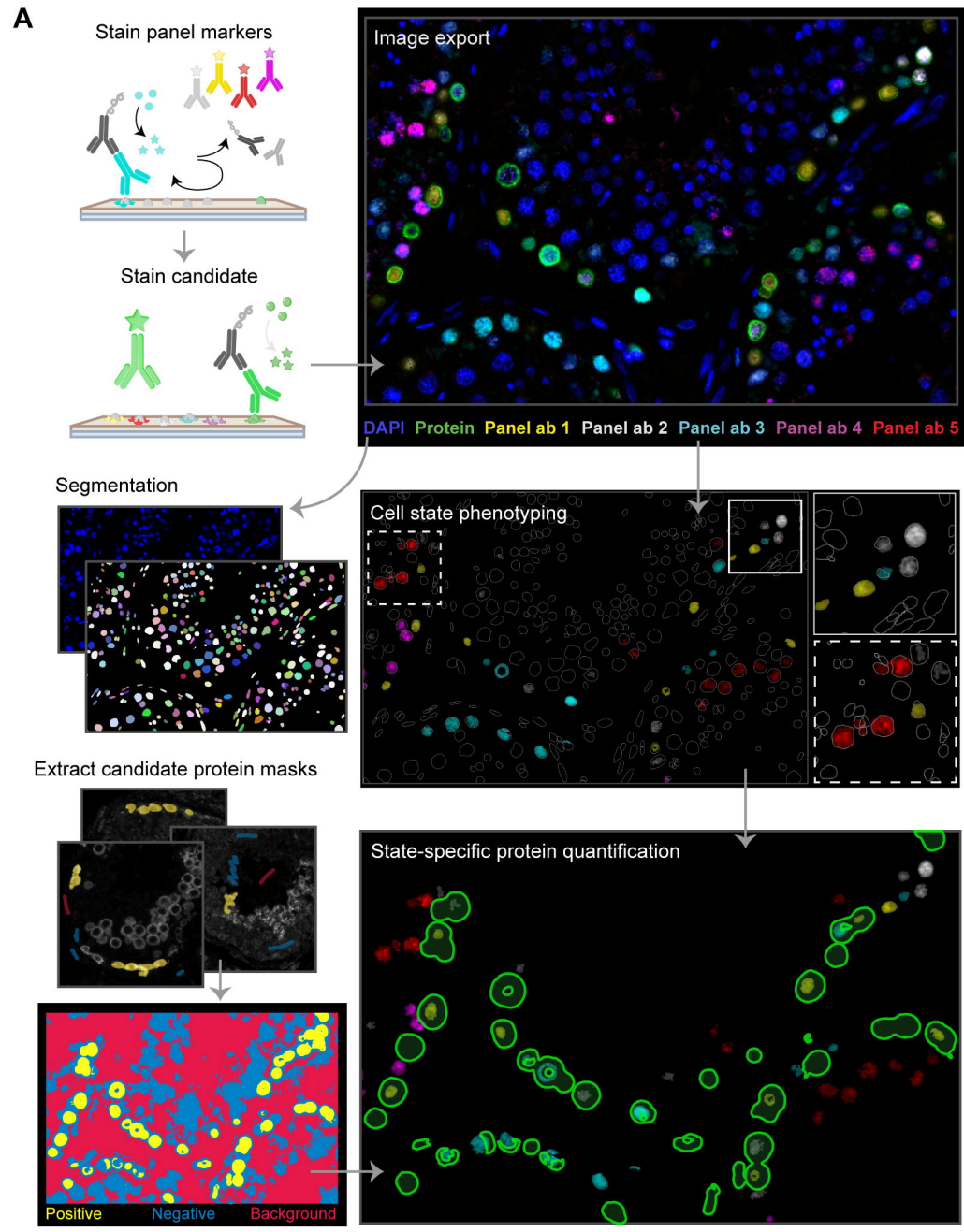
69
193
76
2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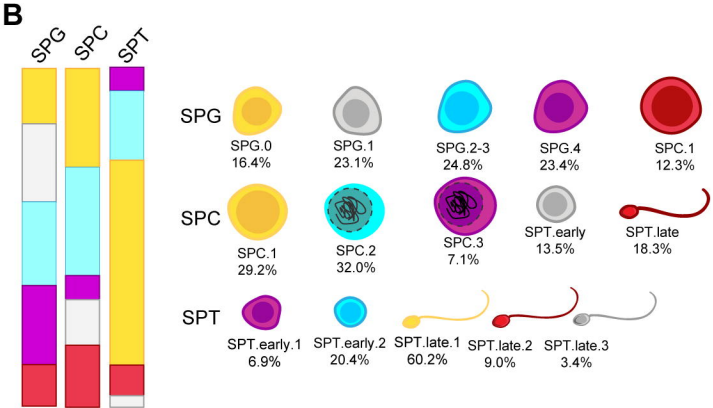
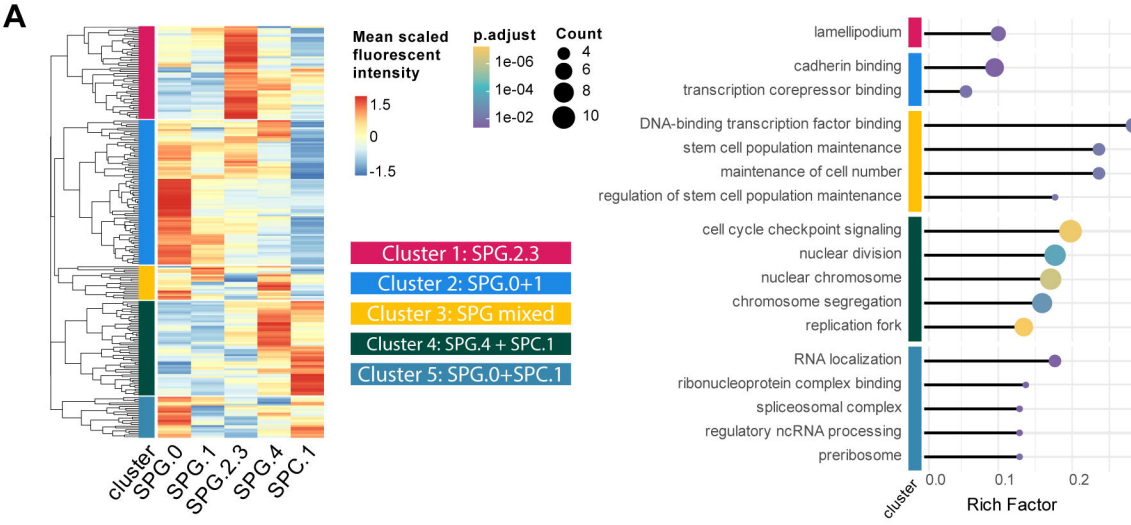
Stain panel markers
Stain candidate
Image export
DAPI Protein Panel ab 1 Panel ab 2 Panel ab 3 Panel ab 4 Panel ab 5
Segmentation
Cell state phenotyping
Extract candidate protein masks
State-specific protein quantification
Positive
Negative
Background


B
SPG
SPC
SPT
SPG
SPG.0 16.4%
SPG.1 23.1%
SPG.2-3 24.8%
SPG.4 23.4%
SPC.1 12.3%
SPC
SPC.1 29.2%
SPC.2 32.0%
SPC.3 7.1%
SPT.early 13.5%
SPT.late 18.3%
SPT
SPT.early.1 6.9%
SPT.early.2 20.4%
SPT.late.1 60.2%
SPT.late.2 9.0%
SPT.late.3 3.4%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Mean scaled fluorescent intensity
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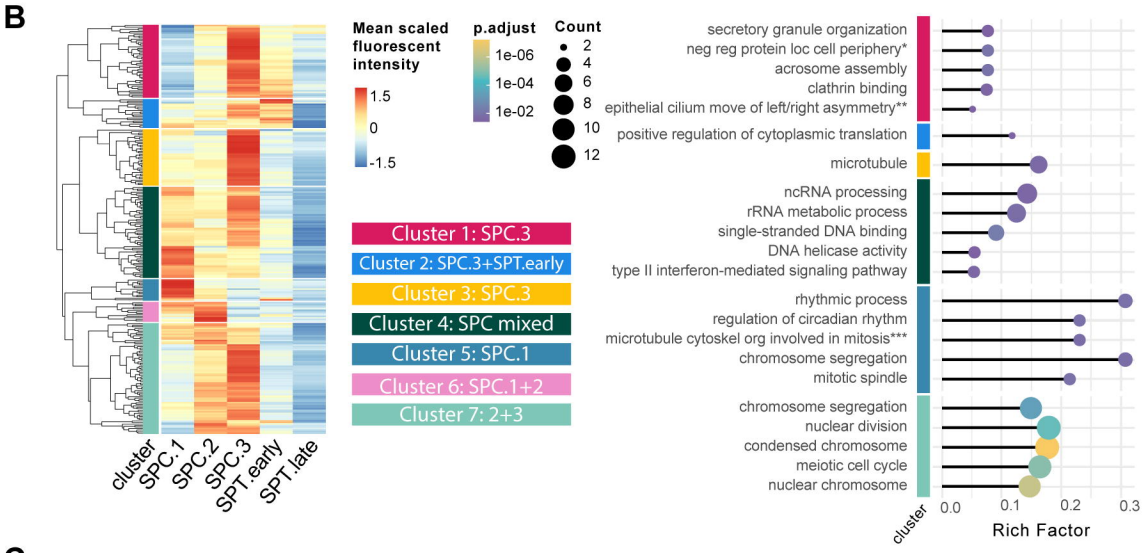
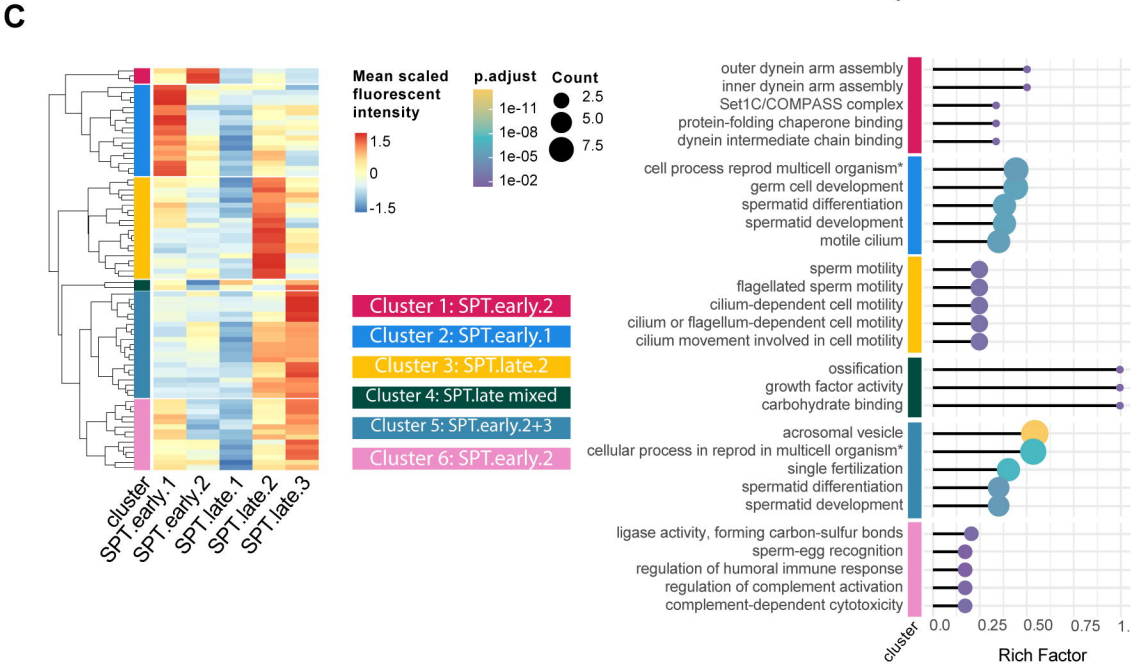
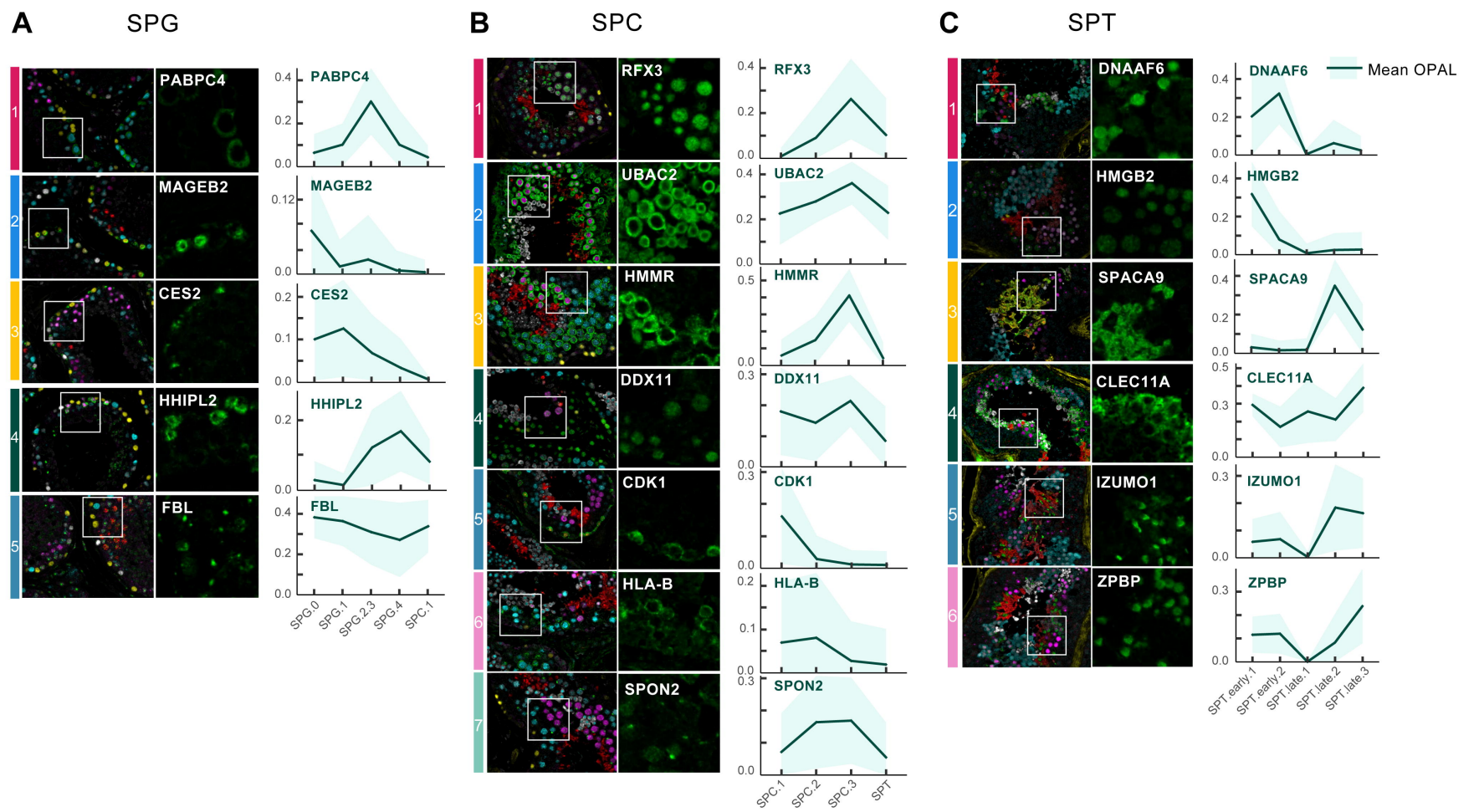
0
-1.5
p.adjust
1e-06
1e-04
1e-02
Count
4
6
8
10
Cluster 1: SPG.2.3
Cluster 2: SPG.0+1
Cluster 3: SPG mixed
Cluster 4: SPG.4 + SPC.1
Cluster 5: SPG.0+SPC.1
cluster
SPG.0
SPG.1
SPG.2.3
SPG.4
SPC.1
lamellipodium
cadherin binding
transcription corepressor binding
DNA-binding transcription factor binding
stem cell population maintenance
maintenance of cell number
regulation of stem cell population maintenance
cell cycle checkpoint signaling
nuclear division
nuclear chromosome
chromosome segregation
replication fork
RNA localization
ribonucleoprotein complex binding
spliceosomal complex
regulatory ncRNA processing
preribosome
0.0
0.1
0.2
Rich Factor


B
Mean scaled fluorescent intensity
1.5
0
-1.5
p.adjust
1e-06
1e-04
1e-02
Count
2
4
6
8
10
12
Cluster 1: SPC.3
Cluster 2: SPC.3+SPT.early
Cluster 3: SPC.3
Cluster 4: SPC mixed
Cluster 5: SPC.1
Cluster 6: SPC.1+2
Cluster 7: 2+3
cluster
SPC.1
SPC.2
SPC.3
SPT.early
SPT.late
secretory granule organization
neg reg protein loc cell periphery*
acrosome assembly
clathrin binding
epithelial cilium move of left/right asymmetry**
positive regulation of cytoplasmic translation
microtubule
ncRNA processing
rRNA metabolic process
single-stranded DNA binding
DNA helicase activity
type II interferon-mediated signaling pathway
rhythmic process
regulation of circadian rhythm
microtubule cytoskel org involved in mitosis***
chromosome segregation
mitotic spindle
chromosome segregation
nuclear division
condensed chromosome
meiotic cell cycle
nuclear chromosome
0.0
0.1
0.2
0.3
Rich Factor


C
Mean scaled fluorescent intensity
1.5
0
-1.5
p.adjust
1e-11
1e-08
1e-05
1e-02
Count
2.5
5.0
7.5
Cluster 1: SPT.early.2
Cluster 2: SPT.early.1
Cluster 3: SPT.late.2
Cluster 4: SPT.late mixed
Cluster 5: SPT.early.2+3
Cluster 6: SPT.early.2
cluster
SPT.early.1
SPT.early.2
SPT.late.1
SPT.late.2
SPT.late.3
outer dynein arm assembly
inner dynein arm assembly
Set1C/COMPASS complex
protein-folding chaperone binding
dynein intermediate chain binding
cell process reprod multicell organism*
germ cell development
spermatid differentiation
spermatid development
motile cilium
sperm motility
flagellated sperm motility
cilium-dependent cell motility
cilium or flagellum-dependent cell motility
cilium movement involved in cell motility
ossification
growth factor activity
carbohydrate binding
acrosomal vesicle
cellular process in reprod in multicell organism*
single fertilization
spermatid differentiation
spermatid development
ligase activity, forming carbon-sulfur bonds
sperm-egg recognition
regulation of humoral immune response
regulation of complement activation
complement-dependent cytotoxicity
0.0
0.25
0.50
0.75
1.00
Rich Factor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SPG
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MAGEB2
CES2
HHIPL2
FBL
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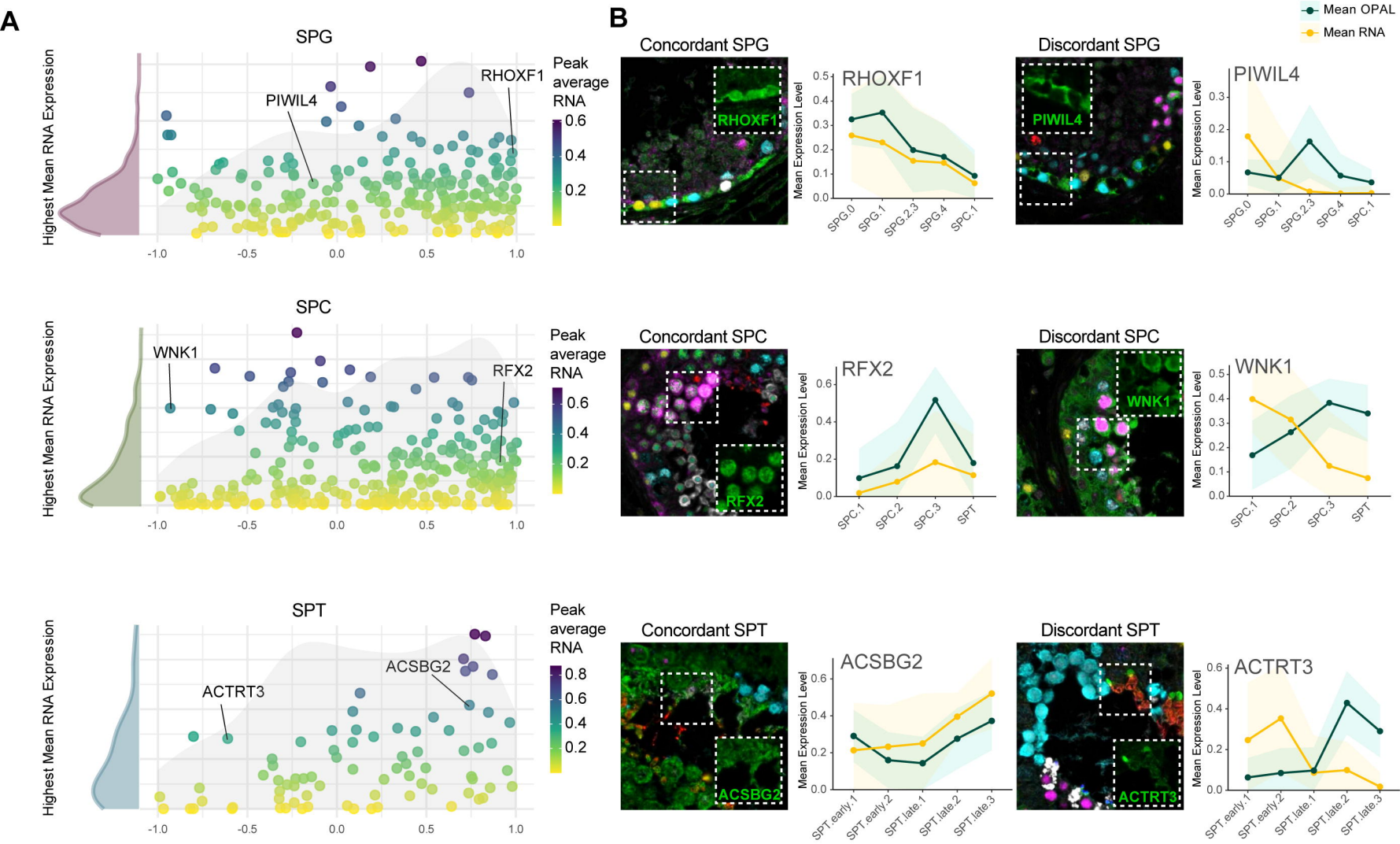
SPG.1
SPG.2,3
SPG.4
SPC.1
B
SPC
RFX3
UBAC2
HMMR
DDX11
CDK1
HLA-B
SPON2
SPC.1
SPC.2
SPC.3
SPT
C
SPT
DNAAF6
HMGB2
SPACA9
CLEC11A
IZUMO1
ZPBP
Mean OPAL
SPT.early.1
SPT.early.2
SPT.late.1
SPT.late.2
SPT.late.3


A
B
Mean OPAL
Mean RNA
SPG
Highest Mean RNA Expression
PIWIL4
RHOXF1
Peak average RNA
0.6
0.4
0.2
-1.0
-0.5
0.0
0.5
1.0
Concordant SPG
RHOXF1
Mean Expression Level
SPG.0
SPG.1
SPG.2.3
SPG.4
SPC.1
Discordant SPG
PIWIL4
SPC
WNK1
RFX2
Peak average RNA
Concordant SPC
RFX2
SPC.1
SPC.2
SPC.3
SPT
Discordant SPC
WNK1
SPT
ACTRT3
ACSBG2
Peak average RNA
0.8
Concordant SPT
ACSBG2
SPT.early.1
SPT.early.2
SPT.late.1
SPT.late.2
SPT.late.3
Discordant SPT
ACTRT3