

Figure S1

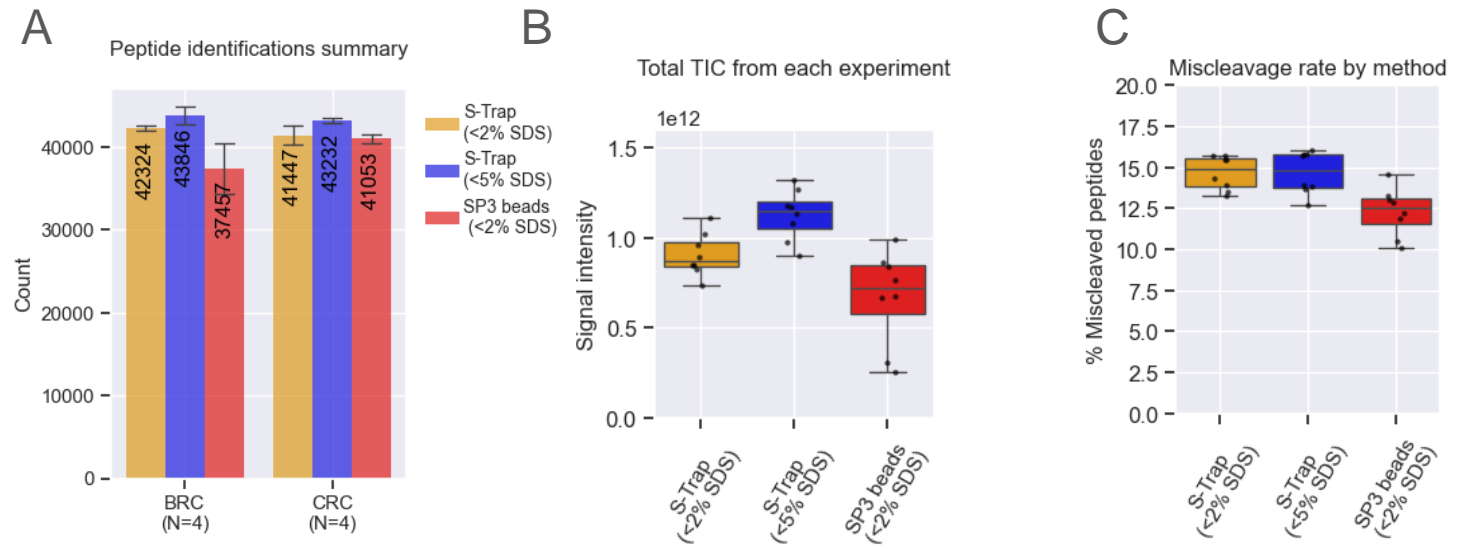


Figure S1: Method Optimization

A. Peptide summary from all workflows. Bar plots represent data from $n=4$ scrolls per block, showing means and standard deviation (SD) error bars.

B. Quantified TIC of all peptides from each experiment across $n=4$ replicates. The box represents the interquartile range (IQR), with the top and bottom edges indicating the 75th and 25th percentiles, respectively. The line inside the box denotes the median, and whiskers extend to capture data within 1.5x of the IQR.

C. Digestion efficiency of S-Trap and SP3 workflows. Bar plots represent data from $n=4$ scrolls per block, showing means and SD error bars. The box represents the IQR, with the top and bottom edges indicating the 75th and 25th percentiles, respectively. The line inside the box denotes the median, and whiskers extend to capture data within 1.5x of the IQR.

Figure S2

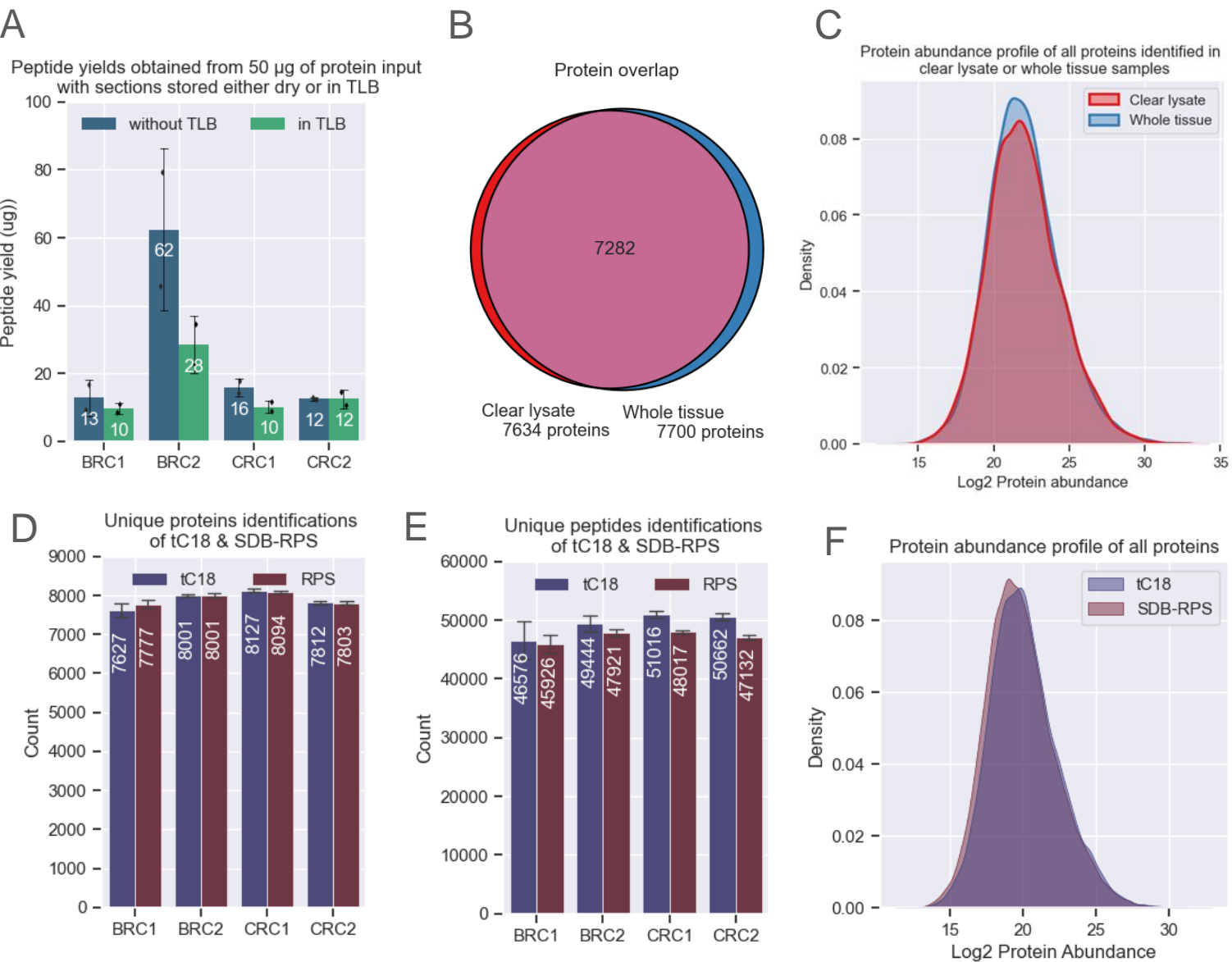


Figure S2: Comparative Evaluation of Sample Collection in Wet vs. Dry Wells and Different Stage-Tipping Materials

A. Peptide yields remain consistent across storage conditions (n=2 scrolls per condition) for each block. The BRC2 block shows the highest yields, likely due to processing the full tissue homogenate, which may increase protein/peptide yields.

B. Protein identifications and overlap between whole tissue and clear lysate samples.

C. Density plot showing the protein abundance profile, which remains consistent between whole tissue and clear lysates. The peak shape represents the kernel density estimate of all proteins after log₂ transformation and normalization.

D-E. Comparison of protein and peptide depth in samples desalted with tC18 and SDB-RPS. Each bar represents data from four independent 10 µm scrolls.

F. Density plot showing protein abundance for all identified proteins using tC18 and SDB-RPS. The peak shape represents the kernel density estimate of all proteins after log₂ transformation.

Figure S3

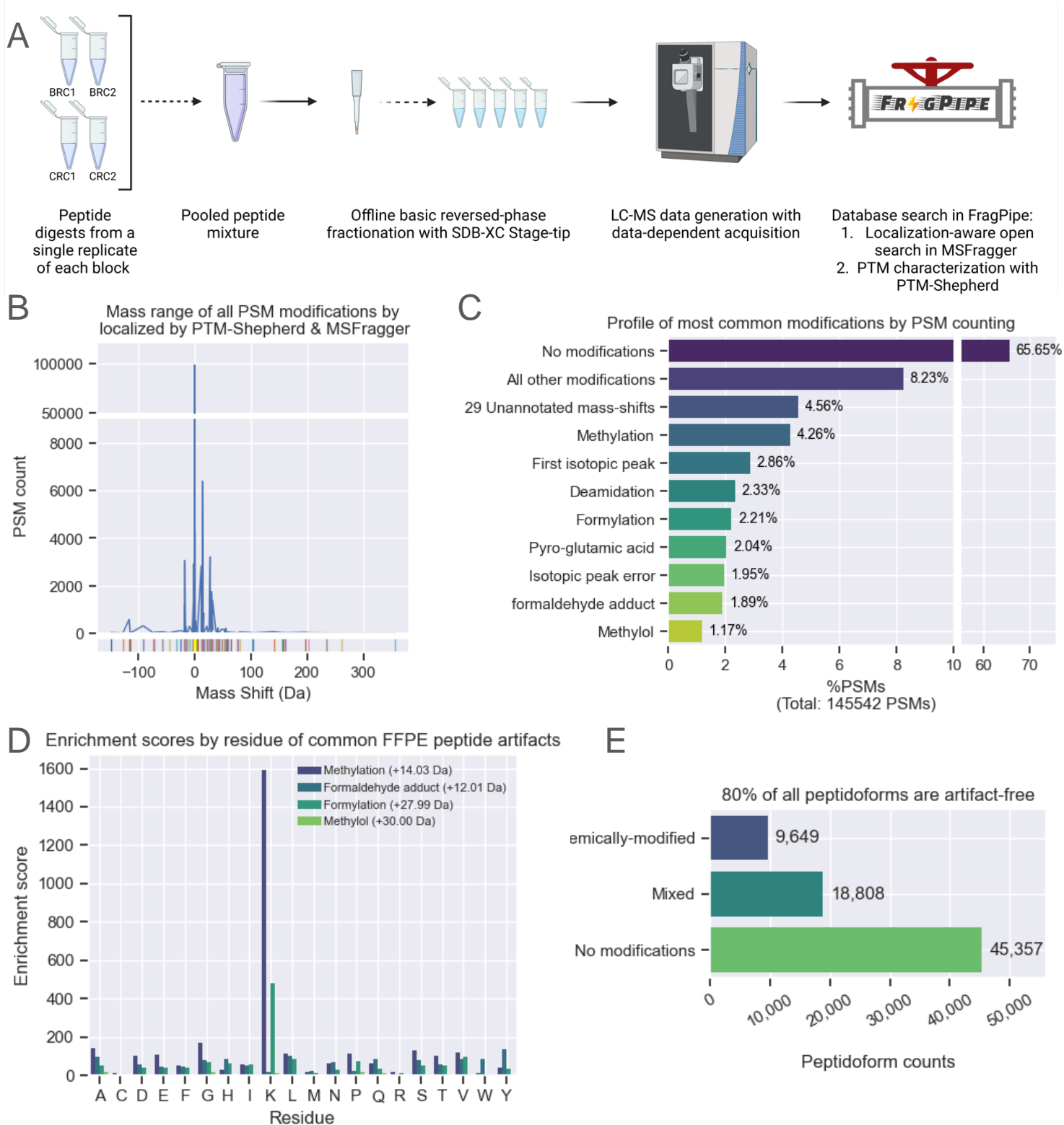


Figure S3: Evaluation of Artfactual FFPE-Derived Peptide Modifications

A. Peptide digests from single replicates of CRC and BRC blocks were pooled equally and fractionated using SDB-XC stage tips into five fractions for data-dependent acquisition (DDA). Data were analyzed using the open-search workflow in FragPipe.

B. Open searching with MSFragger and PTM-Shepherd identified 99 different modifications, with most modifications centered around 0 Da. The data were generated from DDA runs of stage-tip fractionated samples pooled from CRC and BRC groups. MSFragger was used for searching, and PTM-Shepherd for modification characterization.

C. Peptide Spectrum Match (PSM) summary of all modifications. FFPE-related modifications make up less than 10% of all PSMs.

D. Enrichment scores by residue for the most common FFPE modifications. The enrichment score represents the PSM count for each localized residue associated with a specific modification.

E. Global modification profile represented by the count of identified peptides from the fractionated pooled sample.

Figure S4

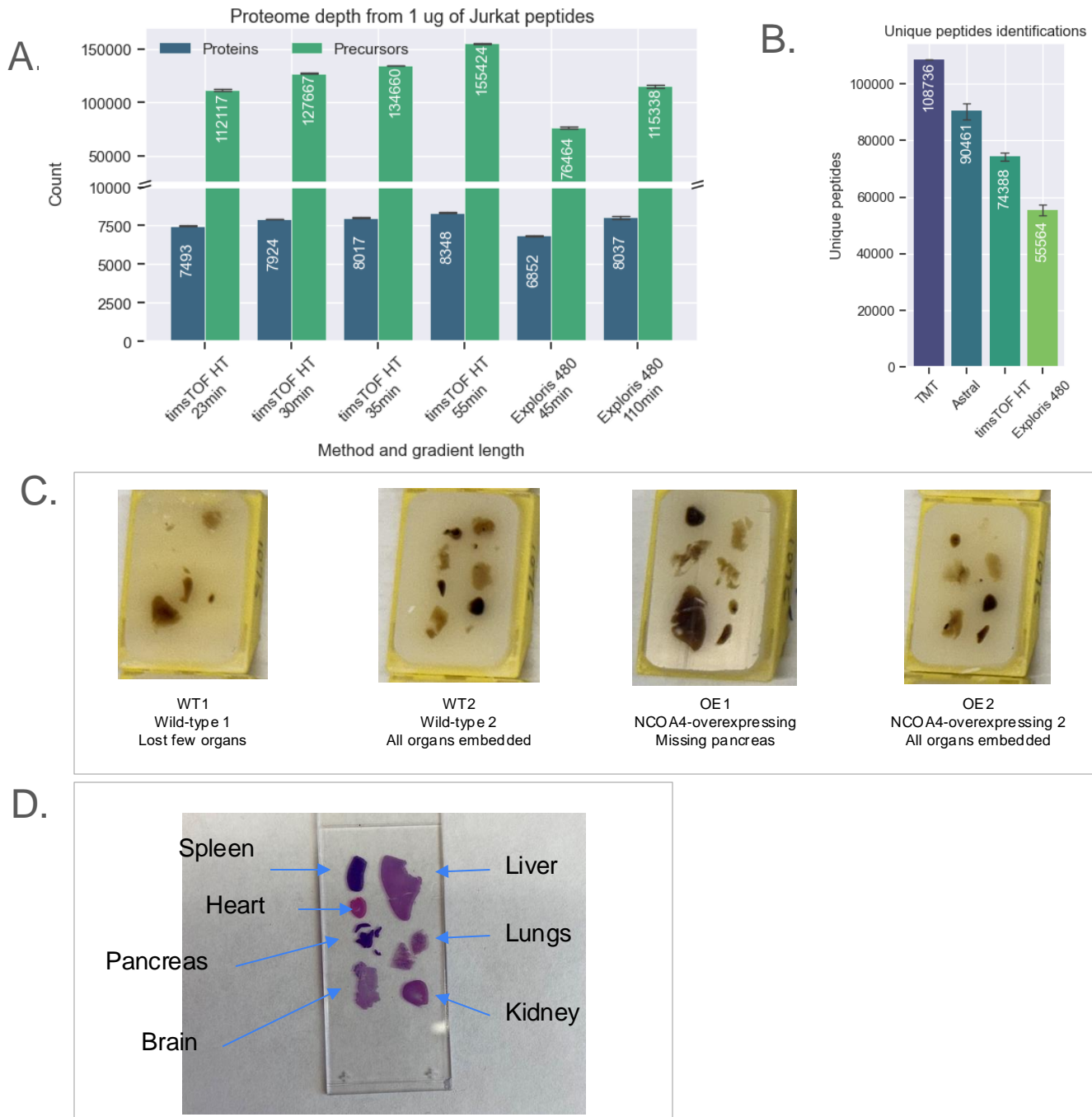


Figure S3: Evaluation of Different DIA Methods and Description of Mouse FFPE Blocks

A. Proteome depth from 1 μ g of Jurkat peptides analyzed using optimized DIA methods on the timsTOF HT and Exploris 480. Bars represent the average of (n=2 injections), with error bars showing the standard deviation. The Bruker timsTOF HT, equipped with a dual trapped ion mobility spectrometry (TIMS) tunnels, allows for parallel accumulation-serial fragmentation (PASEF), separating ions by their collisional cross-section (CCS), allowing precursor selection based on their ion mobility (IM) and mass to charge (m/z). Variable-window diaPASEF was optimized for FFPE proteomics using Jurkat peptide digests across four gradients (23, 30, 35, and 55 minutes) on a 25 cm PepSep column. The results were compared to data acquired on the Orbitrap Exploris 480 with 110-minute and 45-minute gradients using a 25 cm home-packed Reprosil C18 column. The wide-window DIA method on the Orbitrap Exploris480 utilized variable isolation windows ranging from 12 m/z to 24 m/z, adjusting to precursor density. Both methods aimed for six data points per peak (DPPP) for quantitative reproducibility. The timsTOF HT 35-minute gradient achieved ~8,000 unique proteins, comparable to the 110-minute Orbitrap gradient, with a balanced unique peptide depth (~135,000).

B. Peptide identifications across the four methods tested.

C. Four blocks from genetically engineered mouse models (GEMM) were used in the mouse FFPE experiments. Two blocks with total body Ncoa4 overexpression (OE) were compared to wild-type (WT) blocks. Blocks WT2 and OE2 included all organs, while WT1 lacked a few organs, and the OE1 block was missing the pancreas.

D. Slide representation showing all organs embedded into the GEMM FFPE blocks.

Figure S5

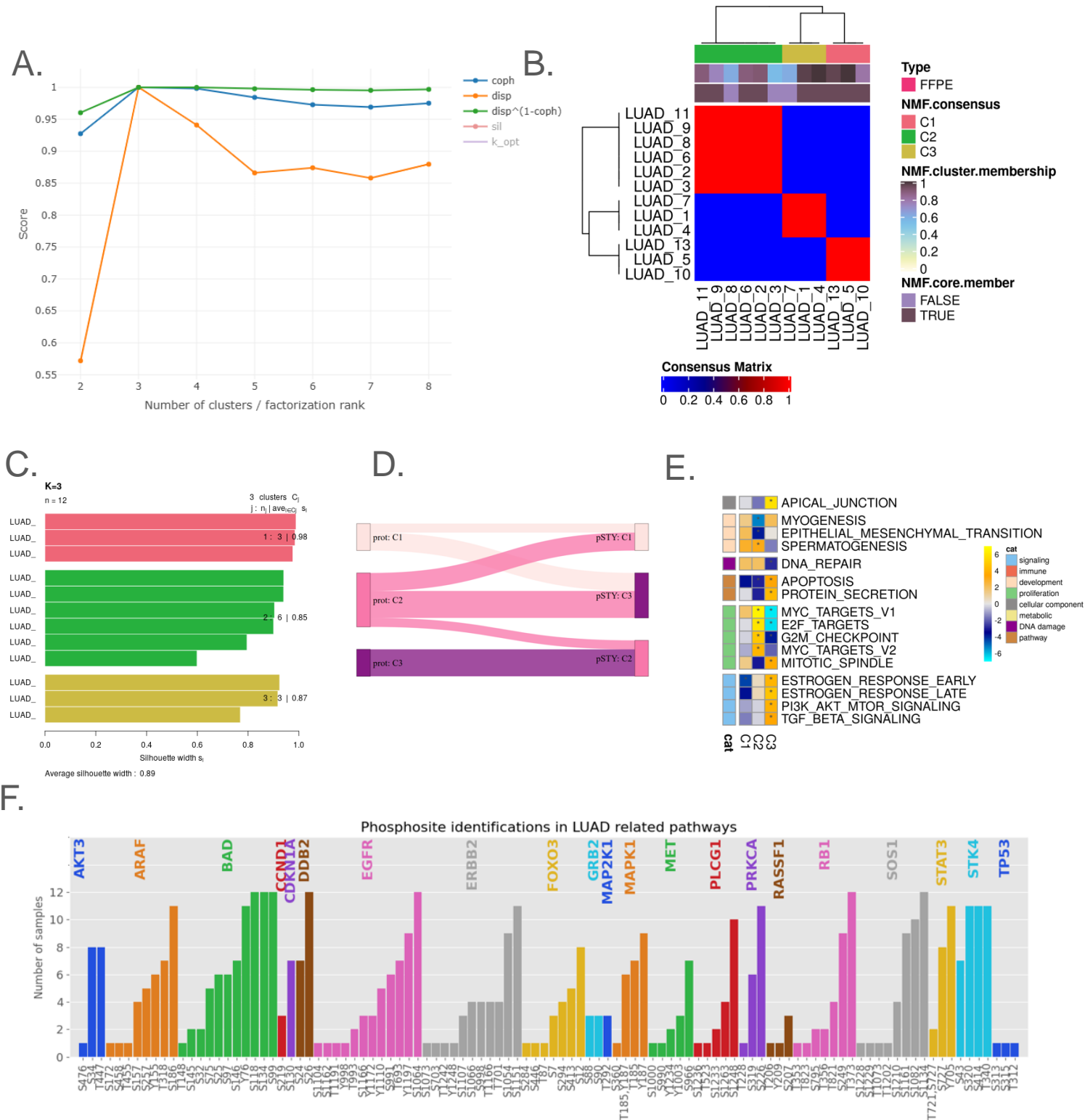


Figure S5: NMF Clustering of Proteome and Phosphoproteome

A. Cophenetic correlation and dispersion scores for each NMF proteome cluster count, with three clusters identified as optimal based on their intrinsic structure and high reproducibility.

B. Heatmap showing the clustering stability of LUAD samples into three distinct groups, as defined by NMF consensus and membership scores for each sample.

C. Silhouette scores for each LUAD sample based on NMF cluster assignment, used to evaluate the quality of assigned memberships.

D. Sankey diagram depicting NMF cluster memberships derived from either proteomic or phosphoproteomic datasets.

E. ssGSEA results from the three phosphoproteome NMF clusters.

F. Key phosphorylation events and their completeness across the LUAD 12 samples.