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DATASET BRIEF

# Immortalized mouse caput epididymal epithelial (mECap18) cell line recapitulates the in-vivo environment

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## Abstract

Residing between the testes and the vas deferens, the epididymis is a highly convoluted tubule whose unique luminal microenvironment is crucial for the functional maturation of spermatozoa. This microenvironment is created by the combined secretory and resorptive activity of the lining epididymal epithelium, including the release of extracellular vesicles (epididymosomes), which encapsulate fertility modulating proteins and a myriad of small non-coding RNAs (sncRNAs) that are destined for delivery to recipient sperm cells. To enable investigation of this intercellular communication nexus, we have previously developed an immortalized mouse caput epididymal epithelial cell line (mECap18). Here, we describe the application of label-free mass spectrometry to characterize the mECap18 cell proteome and compare this to the proteome of native mouse caput epididymal epithelial cells. We report the identification of 5,313 mECap18 proteins, as many as 75.8% of which were also identified in caput epithelial cells wherein they mapped to broadly similar protein classification groupings. Furthermore, key pathways associated with protein synthesis (e.g., EIF2 signaling) and cellular

**Abbreviations:** EVs, line extracellular vesicles; IPA, Ingenuity Pathway Analysis; mECap18, mouse caput epididymal epithelial cell line; NR3C1, glucocorticoid receptor; sncRNA, small non-coding RNA.

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protection in the male reproductive tract (e.g., sirtuin signaling) were enriched in both proteomes. This comparison supports the utility of the mECap18 cell line as a tractable in-vitro model for studying caput epididymal epithelial cell function.

**KEYWORDS**
epididymis, fertility, male reproduction, proteomics, sperm maturation

The production of functionally competent mammalian spermatozoa is an exceptionally complex and carefully orchestrated biological process [1]. The first phase of sperm development occurs within the seminiferous tubules of the testes and encompasses successive waves of mitotic and meiotic divisions before the cells differentiate to assume the specialized architecture of the mature spermatozoon [2]. Despite their morphological appearance, these germ cells lack the capacity for progressive motility and to initiate productive interactions with the ovum [3, 4]. Indeed, this functional maturation is only achieved following two sequential phases of post-testicular development. The first of these occurs as spermatozoa transverse the epididymis (epididymal maturation), and the second, during their ascension of the female reproductive tract (capacitation) [5]. Supporting the first of these maturation steps is the epididymis, a highly regionalized tubule situated between the testis and the vas deferens, and one that fulfils a significant role in male fertility. The mouse epididymis is broadly divided into four anatomical segments with spermatozoa entering via the initial segment prior to descending sequentially through the caput (head), corpus (body), and cauda epididymis (tail), wherein they are stored prior to ejaculation (Figure 1A) [5]. A defining feature of epididymal maturation is that it is completed in the complete absence of *de-novo* gene transcription and protein translation in the sperm cells themselves, and thus reliant on the surrounding luminal milieu in which they are bathed [5, 6].

Accompanying epididymal maturation, the global protein profile and RNA composition of spermatozoa is significantly remodeled [7, 8]; processes that appear to be driven by the combined secretory and resorptive activity of the unique epididymal epithelium [9, 10]. A defining element of the epididymal secretions are epididymosomes, specialized extracellular vesicles (EVs) released from the lining epithelial cells via an apocrine secretory pathway [11]. Thereafter, epididymosomes relay their complex encapsulated payload of RNA and proteins to recipient spermatozoa and downstream epithelial cells [11]. Indeed, as a reflection of the transcriptionally inert state of the male germ cell, epididymosomes have been implicated in facilitating the bulk transfer of small non-coding RNA (sncRNA) (< 200 nucleotides) and fertility modulating proteins to spermatozoa [5, 8, 12]. In addition to this important physiological role, an emerging body of research indicates that the epididymosome cargo can be substantially altered in response to a diversity of paternal environmental and lifestyle stressors [13–16]. Such evidence identifies the epididymis as an important sensory tissue, capable of transducing changes in the sperm proteome/epigenome with pathophysiological implications extending to sperm function and downstream programming of offspring phenotype [8, 16].

In an effort to provide new molecular insight into how the epididymis is innervated by external stressors and the cascade of its downstream responses, it would be valuable to develop in-vitro models that recapitulate cellular responses to stress exposures and enable the efficacy of targeted therapeutic interventions to be tested. In recognition that the epididymis is resistant to immortalization via the expression of oncogenes, our previous work focused on the development of a mouse caput epididymal epithelial (mECap18) cell line utilizing simian virus 40 T antigen. The mECap18 cell line so generated originated from caput epididymides dissected, along with the inclusion of the initial segment, from 4 to 5-month-old mice as described (Figure 1A) [17]. This cell line has proven to be a valuable tractable model to study epididymal secretory activity and the expression of epididymal-specific genes and proteins [18]. Furthermore, EVs isolated from mECap18 cells have been shown to harbor a macromolecular cargo of proteins and sncRNA similar to that of the native mouse epididymosomes [19].

With the growing adoption of the mECap18 cell line to study epididymal function [18, 20], we considered it valuable to analyze the proteomic composition of these cells to confirm their utility as a surrogate for native caput epididymal cells. To deliver on this objective, mECap18 cells were cultured in supplemented DMEM (Thermo Fisher Scientific, Waltham, MA) as described [18, 19], until approximately 70% confluent (passage 12). Cells were detached using Trypsin EDTA (Thermo Fisher Scientific) prior to three consecutive washes and centrifugation cycles in DMEM and sterile PBS. Cell pellets ($n = 4$) were snap frozen and stored at −80°C, until resuspended in chilled 4% w/v sodium deoxycholate (SDC), 100 mM Tris-HCl (pH 8.5) and prepared for MS-based proteomic analysis as described (Figure 1A) [7, 21, 22]. In brief, lysed cell pellets were quantified and concentration equalized (200 µg/270 µL) prior to reduction, alkylation, and overnight trypsinization in a deep-well plate. Tryptic peptides were diluted 1:1 with 100 mM Tris-HCl (ensuring SDC concentration < 1%) and vortexed briefly before adding 1:1 of 99% ethylacetate /1% TFA and thoroughly mixing on a ThermoMixer (2000 rpm). Peptides (7.5 µg) were desalted using styrenedivinylbenzene-reverse phase sulfonated StageTips [23]. RP nLC-MS/MS was performed using an Orbitrap Exploris 480 MS coupled to a Dionex Ultimate 3000RSLC nanoflow high-performance LC system (Thermo Fisher Scientific). Samples were loaded onto an Acclaim PepMap 100 C18 75 µm × 20 mm trap column (Thermo Fisher Scientific) for pre-concentration and online de-salting. Separation was then achieved using an EASY-Spray PepMap C18 75 µm × 250 mm column (Thermo Fisher Scientific), employing a 125 min

liner gradient of ACN (2%–35%). Full MS/data dependent acquisition MS/MS mode was utilized on Xcalibur (Thermo Fisher Scientific; v4.2.47) using the FAIMS Pro interface with two compensation voltages (−50, −60), individually analyzed on each biological replicate. The Orbitrap mass analyzer was set at a resolution of 60,000, to acquire full MS with an m/z range of 350 to 1200, incorporating a normalized automatic gain control target of 300% and maximum fill times of 50 ms. The 20 most intense multiply charged precursors were selected for higher-energy collision dissociation fragmentation with a collisional energy of 30%. MS/MS fragments were measured at an Orbitrap resolution of 15,000 using standard mode for automatic gain control target and maximum fill times of 120 ms.

Consistent with previous studies [16, 21, 24–27], database searching of raw files were performed using Proteome Discoverer 2.5 (Thermo Fisher Scientific). SEQUEST HT was used to search against the UniProt *Mus musculus* database (25,444 sequences, downloaded November 29th, 2022). Database searching parameters included up to two missed cleavages, a precursor mass tolerance set to 10 ppm and fragment mass tolerance of 0.02 Da. Trypsin was designated as the digestion enzyme. Cysteine carbamidomethylation was set as a fixed modification, while acetylation (K, N-terminus) and oxidation (M) were assigned as dynamic modifications. Examination of the corresponding reversed database was performed to assess the FDR of peptide identification using Percolator on the basis of *q*-values, which were estimated from the target-decoy search approach. To filter out target peptide spectrum matches over decoy peptide spectrum matches, a fixed FDR of 1% was set at the peptide level. Protein lists were refined to include only those with four quantitative values and ≥ 2 unique peptides [16, 21, 24–27]. By exception, proteins with 1 unique peptide were retained if their coverage sequence was ≥ 25.5% (i.e., corresponding to the average sequence coverage of the full complement of proteins with ≥ 2 unique peptides) [7].

This pipeline returned a complex proteome of 5,313 proteins (Table S1). Using Perseus (v1.6.10.43), Pearson correlations revealed robust reproducibility between replicates (average $r^2 = 0.992$) (Figure 1B). Further evaluation of the proteome provided the level of evidence supporting the existence of each identified protein (UniProt), with 5,074 proteins (95.5%) harboring evidence at the protein level, 225 (4.2%) showcasing evidence at the transcript level, and 14 proteins (0.3%) being inferred from homology (Figure 1C). To interrogate the classification of the mECap18 proteome, Ingenuity Pathway Analysis (IPA; Qiagen, Hilden, Germany) was employed as described [7, 24–26]. These bioinformatic tools revealed that the mECap18 proteome contained an expected diversity of protein types with enzymes representing 65.5% of the proteome (1,939), followed by transcription regulators (508; 17.2%), transporters (333; 11.3%), and translation regulators (82; 2.7%). The remaining 3.3% of the proteome consisted of receptors, ion channels, cytokines, and growth factors (Figure 1D).

To determine the relative abundance of mECap18 proteins, a z-score normalization approach was applied using Perseus [28]; whereby high, average, and low abundance proteins are assigned scores of ~ 2, 0 and −2, respectively (Table S1). The z-score was calculated by subtracting the mean of each biological replicate from the corresponding quantitative value of the protein and then divided by the standard deviation of the replicate. As expected, the top five highly abundant proteins represent a range of core cellular functions, such as cytoplasmic actin (ACTB), vital for the structural integrity of epithelial cells [29]. By contrast, the lowly abundant proteins generally fulfilled more nuanced functions, such as the transforming growth factor $\beta$ (TGF-$\beta$) receptor type-2 subunit (TGFBR2), a receptor for the TGF-$\beta$ cytokine family that has been implicated in establishing/maintaining epididymal immune privilege [30] (Figure 1E). In keeping with observations originally reported by Sipilä et al. [17], in which it was recognized that mECap18 cells display an exceptionally low level of expression of the androgen receptor (AR; *Nr3a1*) transcript, the cognate AR protein was not detected among the mECap18 proteome reported herein. At present it remains uncertain whether this is simply a reflection of the relatively low abundance of AR, rendering it below the detection limit of the MS.

Next, we directly compared the proteomic signature of native mouse caput epididymal epithelial cells (*Mo*) reported in Trigg et al. [16, 31] with that of the mECap18 proteome (*Me*) described herein (Table S2, combined filterable proteome), to determine their overall conservation. This comparison yielded an overlap of 3,341 core epididymal proteins (i.e., 75.8% of all mouse caput epididymal proteins) (Figure 2A). At present it remains to be determined to what extent the remaining ~ 24% of non-conserved proteins represent genuine proteomic novelties as opposed to being attributed to incomplete coverage of either cell proteome. In this context, it is evident that neither study achieved the full annotation of the ~ 17,000 expressed gene products predicted by previous transcriptional profiling of mouse

**Significance Statement**

Sperm's journey through the epididymis defines not only its functional competency to engage in fertilisation, but also is endowed with small non-coding RNAs (sncRNAs), conduits of epigenetic information. These sncRNAs are synthesised in the epididymal epithelium and are delivered to the maturing sperm cells via extracellular vesicles (EVs). Paternal stressors can modulate the cargo of EVs and have been linked to the alteration of the sperm epigenome, which have downstream implications in programming of offspring phenotypes.

This study introduces the first-ever proteomic characterization of an in-vitro mouse caput epididymal epithelial cell line, known as mECap18, with detailed in-silico analyses demonstrate how closely these cells recapitulate the in-vivo environment of the epididymis. This research validates the use of mECap18 cells as a reliable model for investigating the intricate mechanisms underlying somatic-to-germ cell communication, implications for sperm maturation and understanding and potentially mitigating the impact of paternal stress on future generations.

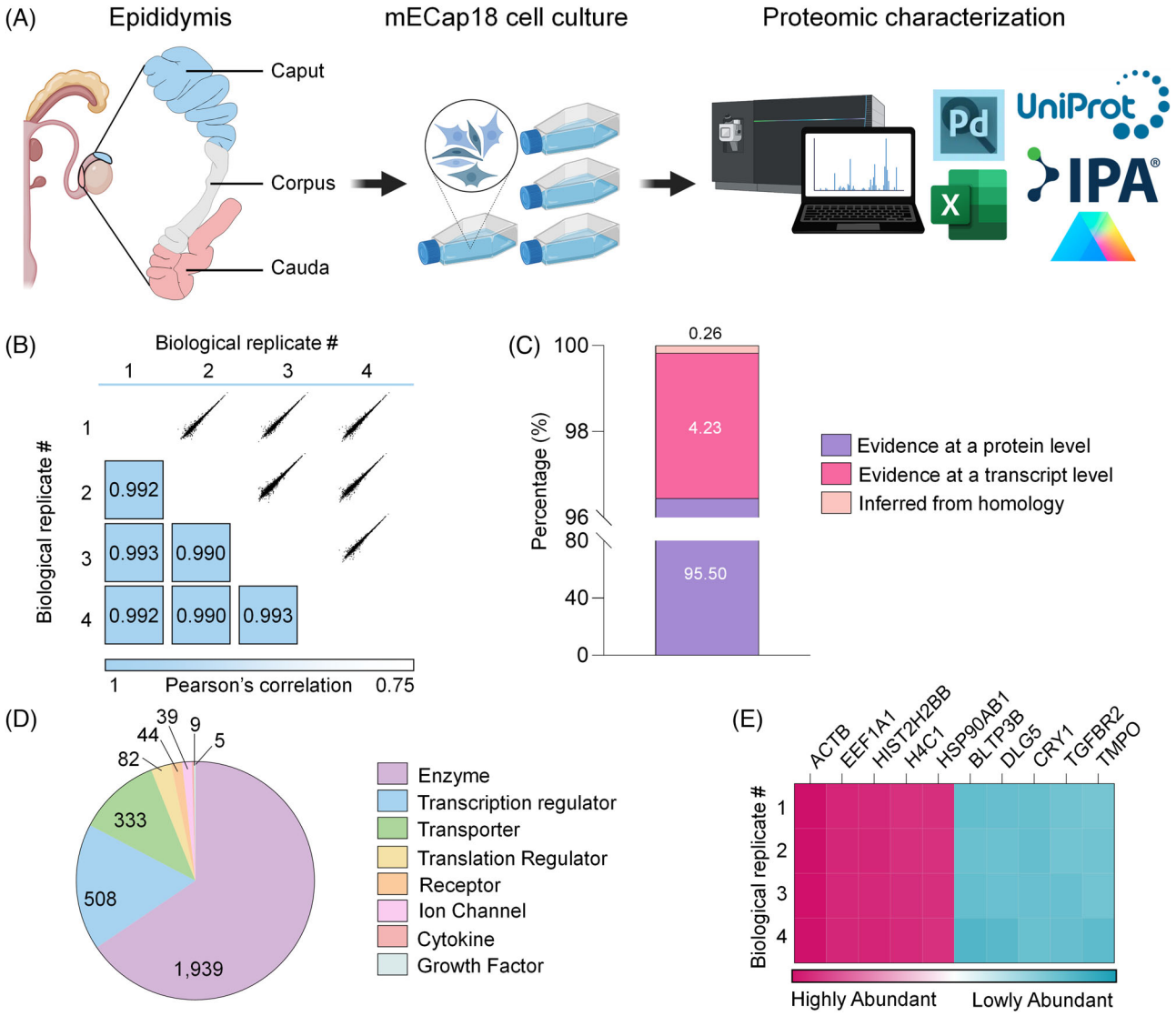


**FIGURE 1** **Characterization of the mECap18 cell proteome.** (A) Overview of the male reproductive tract and structure of the epididymis. Here, we describe the proteomic characterization of an immortalized caput epididymal epithelial cell line (mECap18) using mass spectrometry and an established bioinformatic pipeline [2–7]. (B) Pearson's correlation plots illustrating the strength of the relationship between the proteome of each of four biological replicates. (C) The level of annotated evidence available for each identified protein as annotated in UniProt. (D) Analysis of the proteome using Ingenuity Pathway Analysis categorizing the classification for each protein, with proteins classed as 'other' having been omitted (2,346). (E) Relative protein abundance was determined using z-score normalization, with the heatmap depicting the top five highly abundant (pink) and lowly abundant (teal) proteins. Full list is available in Table S1.

epididymal tissue [32]. Notwithstanding this inherent limitation, *in-silico* interrogation of both cellular proteomes confirmed they were dominated by proteins classified as enzymes, followed by transcription regulators and transporters (Figure 2B). We did, however, note a proportional increase (~ 50%) in the weighting of transcription regulator proteins and a reciprocal reduction (~ 18%) in transporter protein categories in the proteome of mECap18 cells versus that of native epididymal epithelial cells (Figure 2B; Table S2). Under the specific circumstances for immortalization, exposure to substrates in the culture media, and characteristics of adherent cell types, the mECap18 cells may be subjected to altered conditions compared to that of the complex *in-situ* microenvironment of the mouse epididymis. Owing to this, it is reasonable to expect these cells to express a different cohorts of proteins adapt to the in-vitro environment.

To extend this comparison, we elected to investigate the upstream regulators, canonical pathways and downstream functions predicted by IPA's comparative analysis function. In terms of upstream regulators significantly enriched ($p \leq 0.05$) in both proteomes (Table S3)[33], we noted that 'the guardian of the genome', tumor protein 53 (TP53), was the most significantly enriched regulator, followed by rapamycin-insensitive companion of mTOR (RICTOR), which is a known regulator of cell growth and function implicated in the mTOR pathway [34] (Figure 2C). At the level of enriched pathways, we again observed strong overlap between in-vitro and in-vivo epididymal cell proteomes

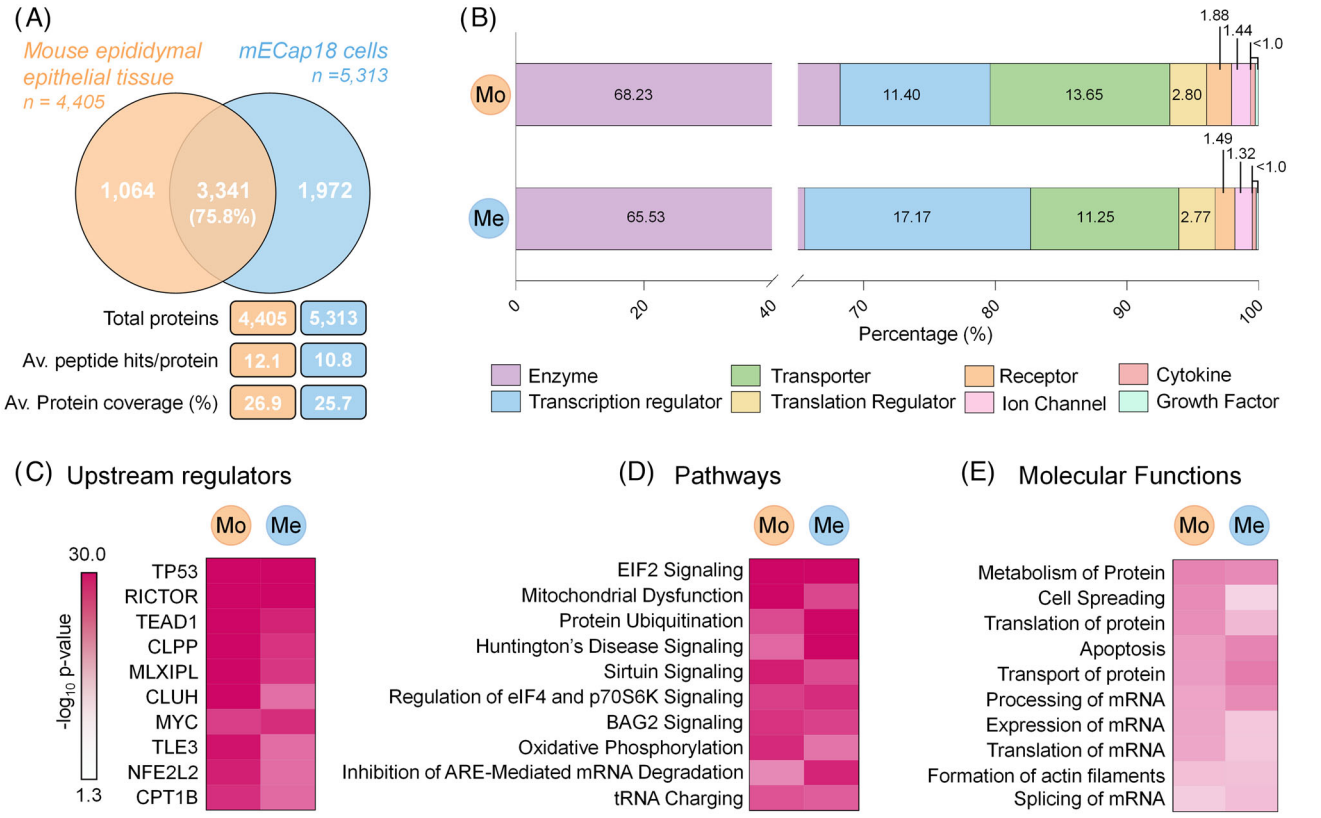


**FIGURE 2** **Conservation between the mECap18 cell proteome and that of the parent caput epididymal epithelial cells.** (A) Comparison of the proteome of the mECap18 cell line (*Me*) with that of previously published mouse caput epithelial proteome (*Mo*) [16] using a Venn Diagram to depict the overlap in identified proteins, as well as a summary of the depth of protein coverage achieved in each study. (B) Bar chart depicting Ingenuity Pathway Analysis (IPA) categorization of mECap18 and epididymal cell proteomes. Heatmaps depicting the top 10 highly enriched ($p \leq 0.05$) (C) upstream regulators, (D) pathways, and (E) their downstream molecular functions as determined by IPA of both mECap18 and epididymal cell proteomes.

(Figure 2D; Table S4), with the most enriched pathway being that of eukaryotic initiation factor 2 signaling (EIF2). EIF2 not only identifies the translation start site on a gene but completes a vital step of protein synthesis by binding to and guiding the guanosine triphosphate and methionyl-tRNA to the ribosome during initiation of protein synthesis [35]. Also prominent was the sirtuin signaling pathway, which has known roles in governing male reproductive function [36] by virtue of its ability to suppress oxidative stress cascades that might otherwise damage cellular integrity [37]. Focusing next on the subset of predicted 'molecular and cellular functions' that achieved statistical significance ($p \leq 0.05$) (Table S5), we identified a variety of mRNA processing functions as well as protein specific functions (e.g., metabolism, translation, and transport), and formation of actin filaments (Figure 2E). These *in-silico* comparisons strengthen the degree of putative functional overlap between the mECap18 and epididymal cell linages and in so doing, reinforce the applicability of the mECap18 cell line as a biological relevant model to study epididymal function.

Notwithstanding the extent of proteomic overlap described above, we also focused our analysis on the subset of proteins uniquely detected within each proteome (Figure 2A) to determine changes that may be associated with the immortalization and/or culture of epididymal cell lines. Thus, proteins uniquely detected in mECap18 cells were analyzed by IPA (Table S6). Seeking to determine what functions may be unique to the mECap18 cells, all IPA outputs that overlapped with those identified in the corresponding epididymal epithelial cell proteome were removed. Although it should be noted detection limits could account for absence of protein expression. As anticipated, this approach identified factors important for cellular immortalization and growth. In the upstream regulator analysis, we noted the significant enrichment of the upstream regulator zinc finger and BTB domain containing 17 (ZBTB17) (Figure 3A; Table S7); a multifunctional protein that acts as a binding partner of both the oncoprotein c-Myc [38] and the TP53 tumor suppressor [39]. The interplay between c-Myc and ZBTB17 has been implicated in the maintenance of cell fate [40] and, consistent with our data, has also been linked to the process of cellular immortalization [17]. Similarly, TP53, which was identified as being putatively enriched among the mECap18 proteome signaling pathways (Figure 3B) has also been causally linked with cell cycle regulation. Specifically, TP53 normally prevents uncontrolled cell proliferation by promoting cell cycle arrest [41], yet this activity is compromised by ZBTB17, which adheres to the TP53 DNA-binding motif and thereby prevents its suppression of target genes. In addition to these insights, several of the other enriched pathways identified in the mECap18 cell proteome were also associated with the regulation and integrity of

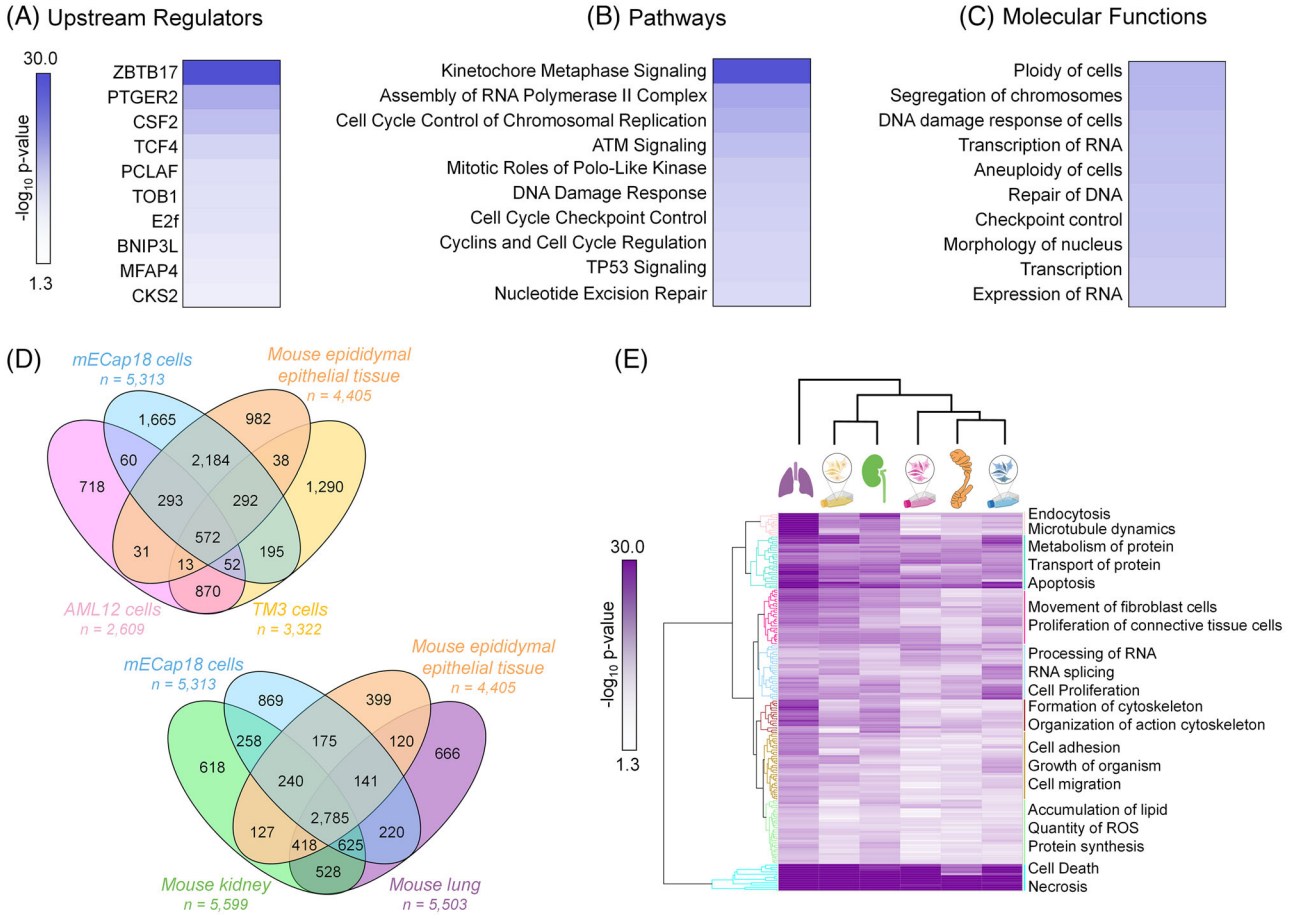


**FIGURE 3** **Molecular functions of proteins uniquely identified in mECap18 cells.** Proteins uniquely detected in mECap18 cells were assessed by Ingenuity Pathway Analysis (IPA) and the top 10 highly enriched ($p \leq 0.05$) (A) upstream regulators, (B) pathways, and (C) their downstream molecular functions are represented by heatmaps. (D) Two Venn Diagram comparisons of the mECap18 cells and mouse epididymal epithelium [16] to other immortalized epithelial cell lines originating from the liver (AML12) [43] and the testis (TM3),[44] as well as, kidney [42] and lung [25] tissue. (E) Unbiased hierarchical clustering of the shared downstream IPA molecular function outputs across of six proteomes (170 functions), depicted as a heatmap (based on the $-\log_{10}$ of the *p*-value), with clustering indicated on the left and significant functions on the right. Above is the hierarchical clustering of the samples; lung (purple), kidney (green), epididymis (orange), mECap18 (blue), AML12 (pink), and TM3 (yellow).

cell cycle processes (e.g., cyclins and cell cycle regulation, cell cycle checkpoint control) (Figure 3B; Table S8), as were the molecular function categories (e.g., segregation of chromosomes, aneuploidy of cells, checkpoint control) (Figure 3C; Table S9).

As a final analysis, we compared the proteomes of mECap18 and epididymal cells to that of tissues (i.e., mouse kidney [42] and lung [25] tissue) and alternative immortalized epithelial cell lines originating from the liver (alpha mouse liver 12 (AML12)) [43] and the testis (Leydig-like (TM3) cells) [44]; so selected on the basis of being generated using similar sequencing strategies, equivalent cell lineages, and/or embryonic origin (Table S10). As anticipated, direct comparison against immortalized cell lines confirmed the greatest overlap existed between epididymal tissue and the mECap18 cells, with 2,184 uniquely shared proteins identified (total 3,341 shared protein; Figure 3D). By comparison, only 1,111 proteins were shared in total between mECap18 and the next closest cellular proteome of the TM3 cell line. Expanding this comparison to include more complex tissue proteomes revealed that mECap18 cells, whole lung and kidney tissues have a 75.8%, 78.6%, and 81.0% overlap, respectively with epididymal tissue (Figure 3D). To refine this analysis beyond protein lists, IPA was utilized in combination with the Perseus software to conduct a hierarchical clustering analysis of shared significant downstream molecular functions identified across all six proteomes (i.e., 170 total shared molecular functions; Table S11; Figure 3E). This analysis also confirmed that the closest relationship existed between mECap18 and mouse epididymal cells. The next closest relationship was that of the AML12 cells, followed by the grouping of the TM3 cells and the kidney, the latter of which shares the same embryonic origin as the epididymis, while the most distant relation from all proteomes was the lung [45, 46] (Figure 3E). Clustering of the shared functions revealed eight distinct clusters, mapping to key processes such as microtubule dynamics, movement of proliferation of cells, RNA splicing, protein synthesis, and cell adhesion (Figure 3E; Table S11).

In summary, here we have provided a comprehensive proteomic characterization of the mECap18 cell line and through comparative analyses, have demonstrated its biological relevance as a suitable

in-vitro model with which to study the function of epididymal epithelial tissue. Owing to the importance of the epididymis in the promotion of sperm maturation [7, 47] and regulation of the sperm epigenome [16], this resource will help to facilitate improvements in our mechanistic understanding of the capacity of the epididymis sense and respond to paternal stressors and provide a platform for testing the efficacy of therapeutic interventions to protect the integrity of male germline. By way of example, the expression of the major glucocorticoid receptor (NR3C1) in mECap18 cells provides exciting opportunities to investigate the direct innervation of the male reproductive tract to the burden of increased stress hormone production and to inform effective strategies to mitigate changes to the sperm epigenome arising from such a challenge.

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## CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

## DATA AVAILABILITY STATEMENT

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository [48] with the dataset identifier PXD042082.

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Additional supporting information may be found online https://doi.org/10.1002/pmic.202300253 in the Supporting Information section at the end of the article.

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