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## Transcriptional Networks in the Human Epididymis

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### Abstract

The epithelial lining of the human epididymis is critical for sperm maturation. This process requires distinct specialized functions in the head, body and tail of the duct. These region-specific properties are maintained by distinct gene expression profiles which are governed by transcription factor networks, non-coding RNAs and other factors. Using RNA-seq we characterized the transcriptomes of the caput, corpus and cauda segments of adult human epididymis tissue and primary human epididymis epithelial (HEE) cell cultures derived from them. Among the many differentially expressed transcripts were epithelial-selective transcription factors (TFs), microRNAs, and genes involved in antiviral responses. Caput-enriched TFs, included hepatocyte nuclear factor 1 (HNF1) and the androgen receptor (AR), both of which were also predicted to occupy *cis*-regulatory elements identified as open chromatin in HEE cells. HNF1 targets were identified genome-wide using CHIP-seq, in HEE cells. Next the impact of HNF1 on the HEE cell transcriptional network was determined by siRNA-mediated depletion of the TF. The results showed that HNF1 coordinates epithelial water and solute transport in caput epithelium. The importance of AR in HEE cells was revealed by AR CHIP-seq, and by RNA-seq after synthetic androgen (R1881) treatment. AR has a distinct transcriptional program in the HEE cells and likely recruits different co-factors (RUNX1 and CEBP $\beta$  in comparison to those used in prostate epithelium. Our data identify many other transcription factors that also regulate the development and differentiation of HEE cells. Moreover, a comparison of genome-wide open chromatin from immature and adult HEE cells showed key TFs in the transition to fully differentiated function of this epithelium. These data may help identify new targets to treat male infertility and have the potential to open new avenues for male contraception.

### Keywords

human epididymis epithelium; caput; corpus; cauda; RNA-seq; differential gene expression; sperm maturation; antimicrobial and antiviral; transcriptional network

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Author contributions.

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## 1) The human epididymis epithelium

The epithelial lining of the human epididymis ensures spermatozoa acquire full motility and the ability to fertilize an egg. The epididymis consists of three regions; the caput and corpus direct early and late sperm maturation, respectively, and the cauda provides a storage compartment. The efferent ducts, which connect the testis to the epididymis are also functionally specialized, but will not be considered further here. The transcriptional networks responsible for the regional specialization in human epididymis are poorly characterized. Studies in rodents (Turner, et al., 2003) and small mammals (Guyonnet, et al., 2009) have greatly enhanced our understanding of epididymis biology. However, well-established differences in epididymis anatomy and physiology between species (Cornwall, 2009, Sullivan and Mieusset, 2016) necessitate the investigation of human epididymis tissues and cells to reveal critical aspects of their function.

## 2) Combining deep sequencing protocols to investigate transcriptional networks

Next generation sequencing (NGS) protocols have the capacity to provide precise, in-depth insights into gene regulatory networks in the human epididymis. To investigate human epididymis epithelial function we used tissue segments and also established differentiated epididymis epithelial cell cultures from the same tissue donors (Leir, et al., 2015). Tissue was obtained with IRB approval from patients undergoing inguinal radical orchiectomy for a diagnosis of testicular cancer (ages 19 – 55 years). None of the epididymides have extension of the testicular cancer. Figure 1 illustrates the pipeline whereby we combine data from different NGS protocols to build a regulatory map of a given transcription factor (TF). First DNase-seq (or ATAC-seq) reveals open (active) chromatin regions in human epididymis epithelial (HEE) cells that likely contain *cis*-regulatory elements coordinating gene expression in these cells. Subsequent *in silico* analysis identifies transcription factor-binding sites (TFBS) that are over-represented in HEE open chromatin (Bischof, et al., 2013, Browne, et al., 2016, Browne, et al., 2014). CHIP-seq using well-validated antibodies specific for individual TFs identifies their sites of occupancy genome-wide, and determines their target genes (Browne, et al., 2016, Yang, et al., 2018). Furthermore, the contribution of the TF to the transcriptome can be determined by RNA-seq following siRNA-mediated TF depletion, or in the case of ligand-activated TFs such as hormone receptors, upon their activation (Figure 1). We used this approach to map tissue-specific transcriptional networks in HEE cells, which were then validated by relevant functional assays (Browne, et al., 2016, Fossum, et al., 2017, Fossum, et al., 2014, Gosalia, et al., 2015, Yang, et al., 2016).

## 3) The transcriptomes of human caput, corpus and cauda tissues and derivative HEE cells.

### i) Tissue Regions.

We explored the transcriptome of caput, corpus and cauda tissues from human epididymis using RNA-seq (Browne, et al., 2016). Consistent with the microarray analysis of human epididymis tissue done by others, we observed that caput tissue is functionally distinct from

the corpus and cauda tissues, which have similar transcriptomes (Dube, et al., 2007, Thimon, et al., 2007, Zhang, et al., 2006). RNA-seq has a higher sensitivity than is provided by microarrays. Hence, it was to be expected that our RNA-seq analysis revealed many more differentially expressed genes (DEGs) and associated gene ontology processes that diverged significantly between caput and corpus/cauda (Browne, et al., 2016).

#### ii) Caput cell-enriched genes and processes.

Similarly, we used RNA-seq to define the transcriptome of cultured HEE cells from the caput, corpus and cauda regions. These cells, which are described in more detail elsewhere (Leir, et al., 2015), include many epithelial cell types that are present in the intact epididymis. They include principal cells, with high expression of the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, together with basal cells expressing keratin 5 (*KRT5*) among other populations. A more detailed analysis of individual cell types in these cultures will be revealed by single cell RNA sequencing (scRNA-seq). As noted in the tissue analysis, very few transcripts were differentially expressed between the corpus and cauda HEE cells, while there were many DEGs between cells from these regions and from the caput. This demonstrated that caput HEE cells are functionally divergent from those of the corpus and cauda, which have similar transcriptomes (Browne, et al., 2016). Several ion and solute transport-related processes are predominant in caput epithelial cells. These include channels and transporters for potassium, chloride, bicarbonate and water. Also, highly over-represented in caput cells are pathways of response to hormone stimulus, and inspection of the genes within these pathways revealed a number of relevant hormone receptor genes including the androgen receptor (*AR*), the estrogen receptor (*ESR*) and the glucagon receptor (*GCGR*) (Browne, et al., 2016). The enrichment of the *AR* in the proximal epididymis is consistent with *AR* immunostaining of adult human epididymis sections (Ungefroren, et al., 1997). Also enriched in caput epithelial cells are the transcripts of several genes with a key role in urogenital tract development. These include paired-box-2 and -8 (*PAX2* and *PAX8*), which are known to regulate kidney development (Bouchard, et al., 2002, Torres, et al., 1995) and hepatocyte nuclear factor 1 beta (*HNF1 $\beta$* ). *HNF1 $\beta$*  is also important for kidney development, as demonstrated by urogenital tract abnormalities in both kidney-specific *Hnf1 $\beta$*  null mice (Igarashi, et al., 2005) and humans with *HNF1 $\beta$*  mutations (Bellanne-Chantelot, et al., 2004).

#### iii) Corpus/cauda cell-enriched genes and processes.

Our RNA-seq analysis showed that relatively few genes are differentially expressed between corpus and cauda HEE cells. However, of note pathways of defense response were significantly more important in the corpus/cauda (Browne, et al., 2016).

### 4) Regional distribution of antibacterial and antiviral responses in the epididymis.

The differential expression of defense response genes warrants further discussion in part due to its complexity and also because of the marked regional distribution of the innate immune response.

### i) Defensins.

Key components of the innate immune response in humans include  $\alpha$ - and  $\beta$ -defensins, which are small cationic and cysteine-rich peptides that share similar tertiary structures but have different cellular distributions.  $\beta$ -defensins are produced by many mucosal epithelial cells in the body, though within the male reproductive tract the majority are abundant only in the testis (Hollox and Abujaber, 2017) and epididymis (Dorin and Barratt, 2014). In addition to their direct antimicrobial properties, defensins also possess chemotactic activity towards neutrophils, immature dendritic cells and memory T cells (Niyonsaba, et al., 2004, Yang, et al., 1999).  $\beta$ -defensins were reported to play important roles in human and rodent sperm function (Dorin and Barratt, 2014) however, there are species-specific differences (Bjorkgren, et al., 2016, Zhang, et al., 2018, Zhou, et al., 2013). In humans, several  $\beta$ -defensins including DEFB1 and DEFB126 are associated with male fertility (reviewed in (Dorin and Barratt, 2014). However, mutation of their mouse homologues (Defb1 and Defb22), respectively) did not impair fertility. Although  $\beta$ -defensins have intraregional segment-specific expression patterns in the rodent epididymis (Jelinsky, et al., 2007), we did not see marked regional distribution the human epididymis using RNA-seq or quantitative RT-PCR. This may in part be due to humans lacking the connective tissue septa that demarcate segments of the mouse epididymis (Jelinsky, et al., 2007). Human caput and corpus express multiple  $\beta$ -defensins, while the cauda mainly expresses DEFB1 and DEFB4 and low levels of DEFB108 and DEFB131 (Figure 2A). Also more abundant in caput and corpus are Sperm-Associated Antigens (SPAG) 11A and 11B, which show some regions of similarity with the  $\beta$ -defensins, and have antimicrobial properties. Of note, sperm isolated from within epididymis tissue express significantly higher levels of  $\beta$ -defensins than the tissue itself (Figure 2B). Interestingly DEFB4 transcripts, which are at very low abundance in epididymis tissue, are highly expressed in HEE cells from all three epididymis regions (Figure 2A).

Several  $\beta$ -defensins are known to inhibit the activity of lipopolysaccharide (LPS) (Ribeiro, et al., 2016), We found that in HEE cells from 5 of 9 donors, LPS or lipoteichoic acid (LTA) treatment of caput and corpus HEE cells increased (~ 65% for LPS and ~30% for LTA) DEFB1 and DEFB4 levels though cells from the remaining 4 donors were not responsive. Additionally, LPS or LTA exposure did not induce expression of the other  $\beta$ -defensins tested (DEFB104, DEFB108, DEFB118, DEFB121, DEFB126 and DEFB131), all of which were abundant in epididymis tissues.

### ii) Antiviral responses.

Though epididymitis (inflammation of the epididymis) is more commonly caused by bacteria, viruses such as rubulavirus, Coxsackie-B, Herpesvirus (HSV) and Human papilloma viruses (HPV) are likely viral pathogens in the epididymis (Dejucq and Jegou, 2001, Emerson, et al., 2007, Kapranos, et al., 2003, Vuorinen, et al., 2014). While investigating the antiviral defense response mechanisms of HEE cells (Browne, et al., 2018), we found that the toll-like receptor (TLR) 3 and retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) are enriched in cells from the corpus and cauda regions. HEE cells from corpus and cauda, are substantially responsive to antiviral ligands (poly(I:C) and HSV-60), as shown by increased IFN- $\beta$  mRNA expression and IFN- $\beta$  secretion, while caput cells show

little response. Nuclear translocation of phosphorylated p65 occurs after poly(I:C) exposure. However, PAX2, which was implicated in regulating antiviral response pathways, is required for basal expression of the DNA sensor, Z-DNA binding protein (ZBP1) and type I interferon, in caput but not in cauda cells (Browne, et al., 2018).

## 5) Regional distribution of transcription factors and their role in the human epididymis epithelium

Consistent with the regional differences in epididymis function, we observed differential expression of TFs that establish and maintain unique transcriptional networks in the caput and corpus/cauda. Thirty-two TFs were significantly more abundant in caput cells and 58 TFs were more enriched in corpus and cauda cells (Browne, et al., 2016). Among caput cell-enriched TFs was the AR, consistent with data showing that caput epithelium plays a predominant role in AR-regulated processes of sperm maturation (Krutskikh, et al., 2011, O'Hara, et al., 2011). Mutations in PAX2, which is also differentially expressed in caput cells, are associated with kidney and urogenital tract abnormalities (Bower, et al., 2012, Eccles and Schimmenti, 1999). Other TFs of interest that were differentially expressed in caput cells include SRY (Sex Determining Region Y)-Box 17 and Spalt-like TF 1 (SALL1). Mutations in SOX17 cause autosomal dominant vesicoureteral reflux syndrome (OMIM: 61374) (Gimelli, et al., 2010), which has a phenotype of functional abnormalities in the kidneys, bladder and ureters. SALL1 (previously named Epididymis Secretory Protein Li 89) mutations are associated with two inherited disorders, Townes–Brocks syndrome (TBS) and branchio-oto-renal syndrome (BOR) (Engels, et al., 2000, Kohlhase, et al., 1998), which are developmental defects affecting multiple organ systems including the anus (TBS) and the kidneys (BOR). Analysis of open chromatin data identified TFs that are predicted to occupy *cis*-regulatory elements in HEE cells (Yang, et al., 2016). Of those that were enriched in caput cells, we further investigated the role of the androgen receptor (AR) and hepatocyte nuclear factor 1 (HNF1).

### i) The androgen receptor (AR) transcriptional network in HEE cells.

The transcriptional regulation of the AR in the human epididymis is poorly studied. We used our primary adult caput HEE cells (Leir, et al., 2015) to perform ChIP-seq for AR following activation by synthetic androgen (R1881). We also intersected these data with RNA-seq results from R1881-treated HEE cells to identify direct targets of AR. Inspection of over-represented motifs in AR ChIP-seq peaks identified several new potential AR cofactors including CCAAT/Enhancer binding protein-beta (CEBP $\beta$ ) and Runt-related transcription factor-1 (RUNX1) (Yang, et al., 2018). Moreover, we showed that CEBP $\beta$  occupancy is required for AR binding at a subset of sites in HEE cells. In normal prostate tissue and the LNCaP prostate cancer cell line, FOXA1 is the key TF interacting with AR at peaks of occupancy (Jin, et al., 2014, Pomerantz, et al., 2015) suggesting a distinct AR transcriptional program in the epididymis and prostate epithelium. The importance of AR in the caput epididymis is consistent with knockout mouse models which demonstrate that AR is required for normal epididymis function (Krutskikh, et al., 2011, O'Hara, et al., 2011).

## ii) The HNF1 $\beta$ transcriptional network in HEE cells.

We also investigated the role of another caput enriched TF, HNF1. HNF1 $\alpha$  and HNF1 $\beta$  TFs recognize the same DNA consensus sequence, which they occupy as homodimers or heterodimers. As noted above (section 3) HNF1 $\beta$  is important for kidney development in mice (Igarashi, et al., 2005) and humans (Bellanne-Chantelot, et al., 2004) and is the predominant form of HNF1 in HEE cells. RNA-seq analysis of the impact of repression of HNF1 $\alpha$  and  $\beta$  showed differential expression of 1892 transcripts (902 were downregulated and 990 upregulated) in comparison to a non-targeting siRNA (Browne, et al., 2016). Combining HNF1 $\beta$  ChIP-seq data in HEE cells with the RNA-seq data enabled a gene ontology process enrichment analysis of DEGs with HNF1 ChIP-seq peaks within 20kb. Among the most significant pathways associated with down-regulated genes were transport of water, phosphate and bicarbonate, all critical processes in epididymis epithelial function. Measurements of intracellular pH confirmed a role for HNF1 in regulating the epididymis luminal environment (Browne, et al., 2016). HNF1 is also part of a transcription factor complex that regulates expression of the *CFTR* gene in intestinal epithelial cells (Kerschner and Harris, 2012, Mouchel, et al., 2004) and likely plays a similar role in the caput epithelium, since we observe HNF1 ChIP-seq peaks overlapping known *cis*-regulatory elements in the locus.

## 6) Regional expression of microRNA

Region-specific expression of small noncoding RNAs can also regulate segment-specific gene expression in the epididymis epithelium. Tissue-derived microRNA (miRNA) signatures from human epididymis were reported by others (Belleannee, et al., 2012). We recently documented the microRNA (miRNA) repertoire of caput, corpus and cauda tissues and HEE cells (Browne, et al., 2018). We identified 324 epithelial cell-derived and 259 tissue-derived miRNAs, some of which were region-specific. MiRNAs that were abundant in the caput cells included miR-573 and miR-155 while miR-1204 and miR-770 were enriched in cauda cells. In the caput tissue abundant miRNAs included miR-1247 and miR-4461 while miR-146a, miR-135b and miR-3074 were enriched in the cauda tissue. These epithelial cell- and tissue-derived miRNAs may contribute to the regulation of regionalized gene expression and function of the human epididymis epithelium (Browne, et al., 2018). It is also probable that as in rodent epididymis, vesicles released from the human epididymis epithelial cells (epididymosomes, (Sullivan, et al., 2007)) contain specific miRNAs that may be recruited by sperm as part of their maturation process (Sharma, et al., 2018).

## 7) Development and differentiation of HEE cells

We previously mapped open chromatin in immature primary HEE cells (Bischof, et al., 2013, Harris and Coleman, 1989) and more recently in caput HEE cells from adult donors (Leir, et al., 2015, Yang, et al., 2016). A similar number of DHS were identified in immature cells (132,814 sites) and adult cells (128,573 sites). To identify *cis*-regulatory elements that were selective for immature HEE cells, DHS from five different cell types, generated by the ENCODE consortium (Song, et al., 2011) were subtracted from the immature HEE cell signature and non-overlapping sites recorded. The five ENCODE data sets were from skin

fibroblasts (FibroP), a lymphoblastoid cell line (GM12878), an erythroleukemia cell line (K562), a liver carcinoma cell line (HepG2) and human umbilical vein endothelial cells (HUVEC). An identical subtraction was performed to identify DHS that were selective for adult HEE cells. Of the total HEE cell DHS 25.8% (34,327/132,814) were selective to immature HEE cells and 30.1% (38,778/128,573) were selective to adult HEE cells, and about 50% were shared between the two.

## 8) *In silico* analysis of Transcription Factor Binding Sites (TFBS) enriched in DHS within immature and adult HEE cells

To search for enriched TFBS predicted in open chromatin peaks in immature and adult HEE cells, we performed *de novo* HOMER motif analyses (Heinz, et al., 2010) on both immature-selective and adult-selective DHS (Supplementary data, Tables SI-II). *In silico* prediction of TFBS enriched in immature- compared to adult-selective DHS include Basic Leucine Zipper ATF-Like Transcription Factor (BATF) and B Cell CLL/Lymphoma 6 (BCL6). In follicular T-helper cells, BATF regulates BCL6 expression (Ise, et al., 2011), though the relevance of this factor to HEE cell function is unclear. Among TFBS enriched in both immature- and adult-selective DHS is the TEA Domain Transcription Factor (TEAD) motif. Of note, TEAD motifs are also found within peaks of AR occupancy in caput HEE cells (Yang, et al., 2018). The TEAD TF family members (TEAD1–4) participate in diverse cellular processes such as cell growth, proliferation, homeostasis and also development (Johnson and Halder, 2014, Mo, et al., 2014). Of note, TEAD2 and TEAD4 are enriched in caput cells while TEAD3 is enriched in the corpus and cauda cells. The motif for the nuclear receptor 2E1 (*NR2E1*, also known as *TLX*) is also enriched in both immature- and adult-selective DHS. In mice, Nr2e1 can repress expression of Pax2 (Yu, et al., 2000), a TF that we have shown to play important roles in both immature and adult HEE cells (Browne, et al., 2018, Browne, et al., 2014). In addition, NR2E1 regulates the PAX6 expression and the PAX6 binding motif is also enriched in immature-selective DHS.

*In silico* prediction of TFBS enriched in adult- compared to immature-selective DHS identified multiple TFs relevant to the differentiated function of the adult epididymis epithelium. These include Fos-Related Antigen 1 (FRA1, also known as FOSL1), which is enriched in corpus and cauda cells. FRA1 is a component of the AP-1 transcription factor complex. Both AR ChIP-seq and HNF1ChIP-seq peaks identified AP-1 components as potential co-factors in adult HEE cells (Browne, et al., 2016, Yang, et al., 2018). The SRY-Box 9 (SOX9) motif was also enriched in adult-selective DHS. Sox9 regulates anti-Müllerian hormone gene expression (De Santa Barbara, et al., 1998).

## 9) Conclusions

In conclusion in this review we illustrate how a combination of NGS protocols, supported by relevant bioinformatics tools, can be used to advance our understanding of the regional differentiated properties of a highly complex organ, the human epididymis. Further mechanistic studies are underway to integrate these genome-wide analyses of the transcriptional networks, with a detailed understanding of the biology of the epididymis epithelium in man.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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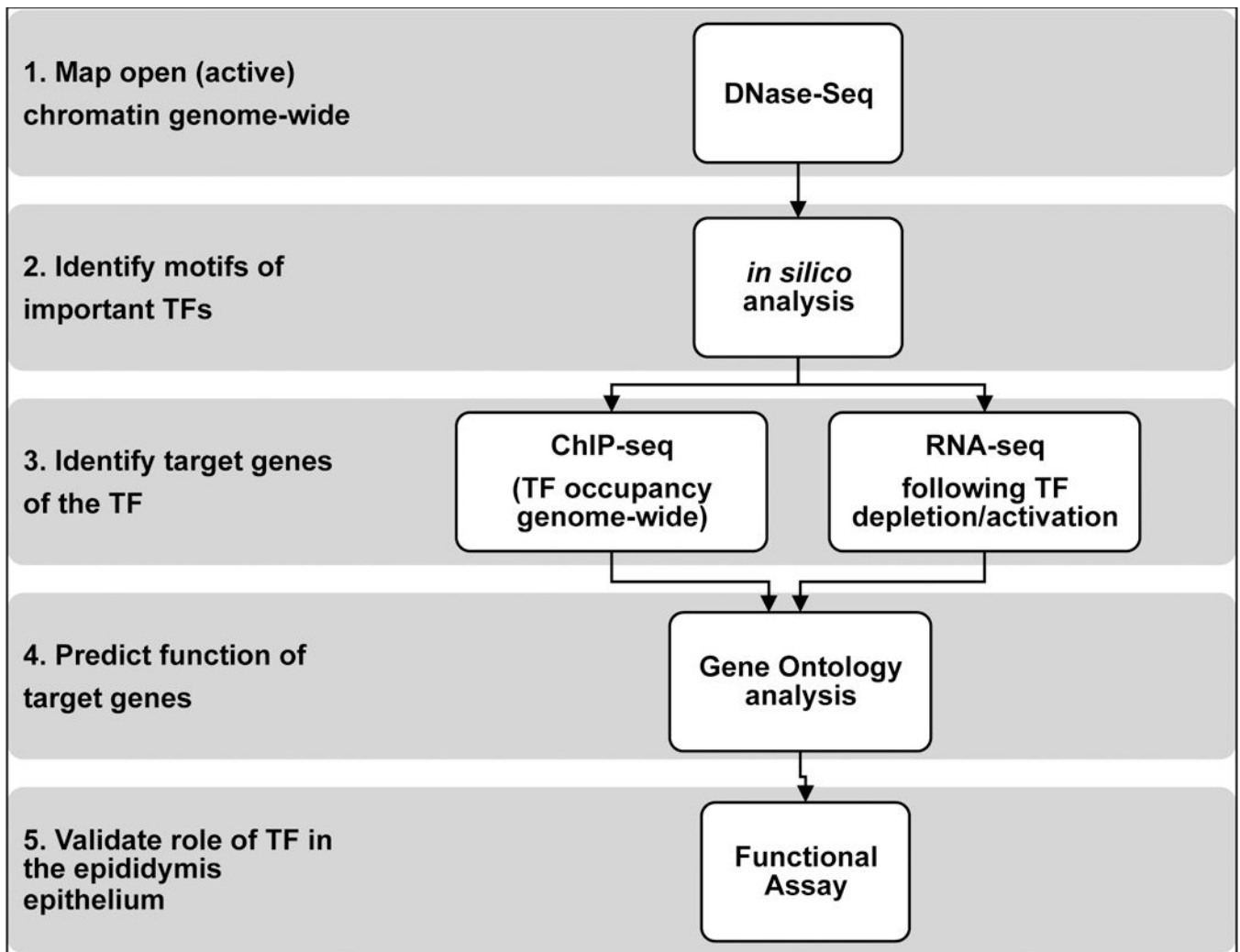
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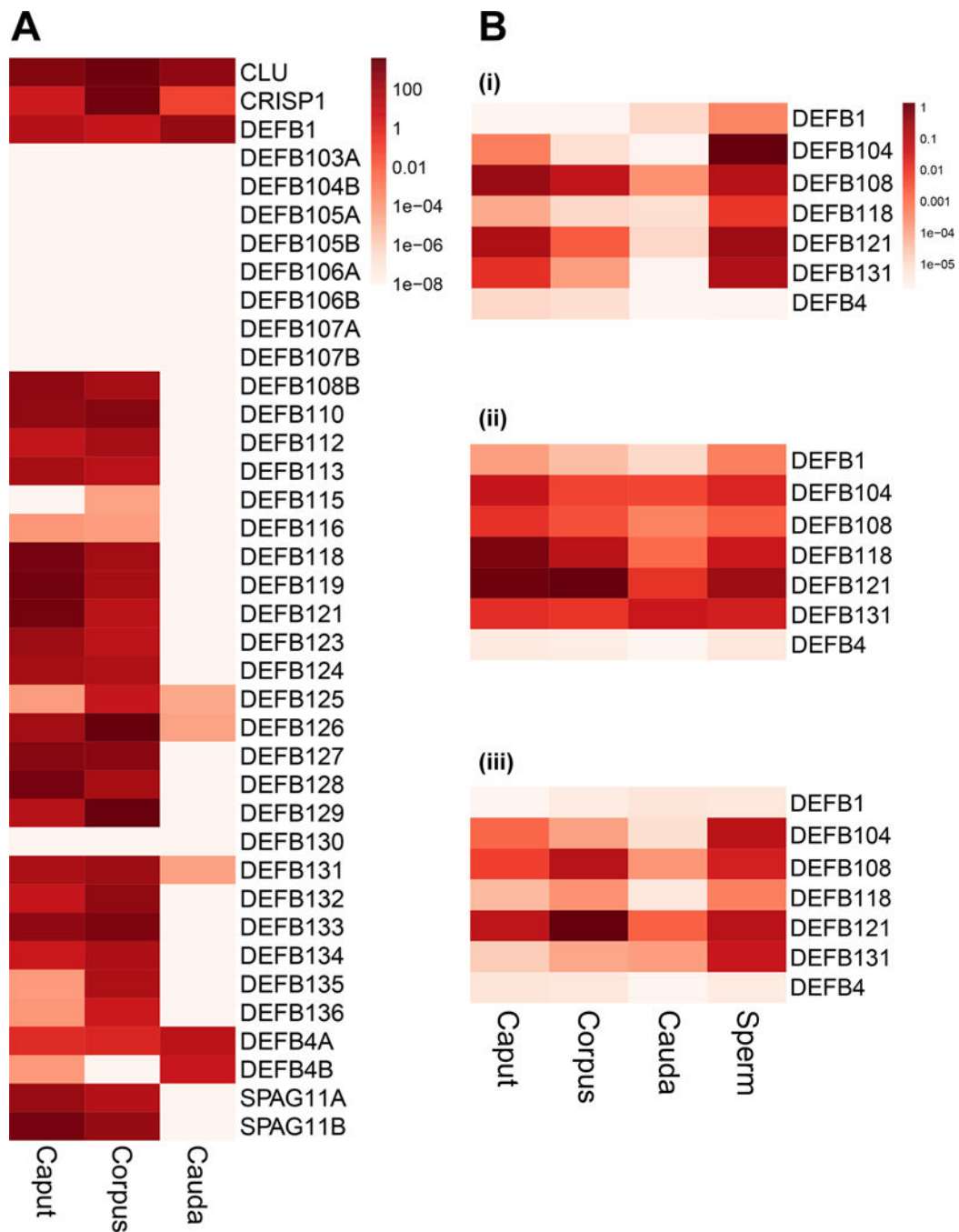
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**Figure 1.** Combining different NGS-based experiments to build transcriptional regulatory networks in the epididymis epithelium. 1. DNase-seq maps open (active) regions of chromatin to identify potential regulatory elements. 2. ChIP-seq identifies transcription factor occupancy genome-wide and its target genes. 3. RNA-seq following TF activation/depletion determines the contribution of the TF to the transcriptome. 4. Gene ontology analysis to predict the function of target genes. 5. Validate identified role of TF with a functional assay.



**Figure 2.**

$\beta$ -defensin expression in different regions (caput, corpus and cauda) of the human epididymis. (A) Heatmap showing  $\beta$ -defensin levels in RNA-seq data from human epididymis tissues (Log scale; 2 donors) (Browne, et al., 2016). Epididymis positive control markers: CLU= clusterin; CRISP1= cysteine rich secretory protein 1. (B) Expression levels of 6  $\beta$ -defensins (DEFB1, DEFB104, DEFB108, DEFB121, DEFB131 and DEFB4) assayed

by RT-qPCR relative to  $\alpha$ -2 microglobulin in epididymis tissue and sperm isolated from 3 different donors (i-iii) (none of the donors overlap with those shown in panel A).

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