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14. ABSTRACT

The mechanism by which genes are turned "on" is driven by proteins called transcription factors (TFs) binding to certain locations in the human genome called **regulatory elements**. TFs recognize and bind to specific regulatory elements (i.e., stretches of specific DNA sequences) and this interaction can result in a gene being turned on. These regulatory elements are proving to be important in cancer biology. Regulatory elements are located throughout the 98% of the human genome that *does not code for genes*. Due to the lack of tools available for studying the noncoding region, until recently it was difficult **to identify**, **to functionally characterize**, and **to therapeutically target** these 'non-genic' genomic regions. Advances are enabling the systematic identification and testing of relevant regulatory elements as well as the ability to target their associated TFs, which were once considered 'undruggable'.

The ultimate goals of our proposal are to identify and characterize critical regulatory elements that contribute to advanced kidney cancer. Aim 1 will utilize a method called chromatin immunoprecipitation followed by sequencing (ChIP-seq) in clinically relevant patient samples to characterize the regulatory landscape during progression to metastatic disease. We will also use a novel technique called HiChIP to create a 3D model of the kidney cancer genome to determine which regions communicate with each other. Gene regulation often occurs when distal enhancers come into contact with target genes in 3D space. Finally, using an innovative technology termed genome editing, Aim 2 will identify candidate regulatory elements that are functionally relevant for mediating resistance to therapy. By focusing on epigenetics and the non-coding portions of the genome, our proposal takes a decidedly innovative approach to identifying functionally important gene regulatory elements. These regions have been understudied compared with the protein coding genome. We have designed our proposal to benefit patients with advanced kidney cancer. The study will open new areas for drug target discovery. We believe that our approach paves the way for systematically studying cancer and identifying targets through an epigenetic perspective.

15. SUBJECT TERMS

Renal cell carcinoma, massively parallel sequencing, ChIP-seq, regulatory elements, transcription factors, ATAC-seq, clear cell renal cell carcinoma.

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1. **INTRODUCTION:**

Epigenetic aberrations direct much of clear cell renal cell carcinoma (RCC) pathogenesis. Indeed, genes fundamental to epigenetic programming are recurrently mutated in the disease. Despite the clear dependency on epigenetic transcriptional regulation, large-scale investigation into the epigenetic mechanisms underlying RCC have lagged behind genetic studies. Our ability to now robustly perform chromatin immunoprecipitation (ChIP) in primary kidney tumor specimens is enabling the first generation of comprehensive epigenomic studies. These tools are enabling the production of large-scale epigenomic datasets in clinically relevant samples, just as next generation sequencing facilitated tumor sequencing over the past decade. There is an increasing appreciation of the role of the non-coding genome in cancer biology and we believe that one of the next frontiers in treating cancer will be in the realm of epigenetics.

Please note that this year minimal funds were spent on the project as we applied for a no-cost extension. In the application for the extension, we described the personnel changes and technical challenges that were largely related to the COVID pandemic. We are poised to begin work on Aim 3 of the proposal after making the necessary adjustments. In the past year, we have added to the discoveries made with support from this grant. These new discoveries are in bold. Otherwise, we have largely kept intact the narrative of our work to date.

2. **KEYWORDS:**

Renal cell carcinoma, massively parallel sequencing, ChIP-seq, regulatory elements, transcription factors, ATAC-seq, chromophobe renal cell carcinoma, papillary renal cell carcinoma, clear cell renal cell carcinoma.

3. **ACCOMPLISHMENTS:**

- **What were the major goals of the project?**
  - The ultimate goals of this proposal are to comprehensively characterize the epigenetic landscape in advanced RCC in order to gain insights into key mechanisms driving lethal disease, which can then be rationally targeted. The anticipated outcomes are that we will identify **areas of vulnerability (Aims 1)** that are **functionally relevant (Aim 2)** and ultimately can be rationally targeted. The aims of the proposal require specific domains of expertise and to address this point, we have assembled an outstanding team with the appropriate scientific depth to go from target identification to analysis of function.
  - Aim 1 will generate the most comprehensive epigenomic ChIP-seq datasets to date in advanced RCC. Aim 1 will also use HiChIP, combining chromosome conformation analysis with ChIP to link the regulatory elements with their target genes. Aim 2 will utilize the powerful tools of genome editing to identify regulatory elements that are functionally relevant in the development of metastatic RCC. The outcome of this study will be a compendium of candidate regulatory elements that influence RCC progression.
- **What was accomplished under these goals?**
  - 1) Major Activities:
  - To examine the cistrome and to identify master TFs across the histologic subtypes of RCC, we performed histone (ChIP-seq, the Assay for Transposase-Accessible Chromatin using sequencing (ATAC-seq), and RNA sequencing (RNA-seq) on 42 fresh frozen RCC tumor samples (24 clear cell (cc) RCC, 6 papillary (p) RCC, 12 chromophobe (ch) RCC). Thirty-eight of 40 (95%) tissues were derived from radical nephrectomies.Patients were

grouped into two cohorts (1 and 2). We conducted H3K4me2 ChIP-seq to map both active and poised enhancers and H3K27ac ChIP-seq to identify active promoters and enhancers. ATAC-seq was performed to define the chromatin accessibility landscape, and RNA-seq was performed to capture the transcriptional programs of each RCC subtype[1]. A total of 153 libraries were generated across the different datatypes. We generated RNA seq, DNA-seq through targeted panel sequencing, and SNP profiling through SNP arrays on 28, 28, and 21 patient-derived samples, respectively.

The resulting data set represents, to our knowledge, the most extensive interrogation to date of RCC epigenomes in primary human specimens.

- 2) Specific Objectives:

- Profile the regulatory element landscape, including enhancers and promoters, across the different RCC histologies

- Characterize epigenetic difference between different RCC histologies

- Identify histotype specific transcription factors governing cellular identity and transcriptional programs

- Characterize reprogramming of regulatory elements during oncogenesis and distant metastasis

- 3) Significant Results:

Using cohort 1 to assess the regulatory landscape across the different RCC histologies, a total of 153,321 promoter-distal (enhancer) H3K27ac ChIP-Seq peaks were identified across all the samples, most of which (n=136,469) were common to two or more histologies (Figure 1A, 1B). For example, the PAX8 locus is marked by H3K27ac in all samples. PAX8 is a transcription factor (TF) involved in early kidney embryogenesis and oncogenesis in RCC[2,3] and is a clinical diagnostic tool to help differentiate RCC from other malignancies[4] (Figure 1C). Unsupervised hierarchical clustering (Figure 1D) and principal component analysis (PCA) of H3K27ac peaks clearly segregated the three histologic types of RCC, and both analyses demonstrated that the H3K27ac landscape in chRCC was more distinct than either pRCC or ccRCC. 16,852 peaks were significantly increased or decreased in one histology compared to the other two (e.g. chromophobe versus clear cell and papillary) (false discovery rate (FDR) of 0.001 and least a 4-fold difference in mean peak intensity between groups). 12,908 sites were up-regulated in one histology: 8,939 were chRCC-specific, 3,653 were pRCC-specific, and 316 were ccRCC-specific (Figure 1E). These subtype-specific H3K27ac peaks were differentially marked by H3K4me2 ChIP-Seq and were associated with open chromatin, strongly suggesting that they were histology-specific active enhancers. Moreover, differential epigenetic sites correlated with the nearest gene expression difference (p-value < 2x10-16, chi-square test) (Figure 1F-H). GREAT[5] analysis of the 8,939 chRCC-specific H3K27ac peaks revealed enrichment for genes involved in actin regulation, fatty acid oxidation, and ion transmembrane transporter activity (Figure 1I), consistent with previously reported mRNA signature analysis showing an increased ion transmembrane transport signature in chRCC[6]. Similar analysis of the 3,653 pRCC-specific sites showed enrichment for genes involved in renal system development (Figure 1J). This is consistent with the notion that pRCC arises from embryonic nephrogenic rest precursor lesions, which persist during adult life[7]. Since there was a relatively small number of ccRCC-specific sites (n=316) in comparison to chRCC and pRCC, and the enhancer landscape of ccRCC resembled that of pRCC more than chRCC, we compared H3K27ac sites between ccRCC and pRCC only. The majority of H3K27ac sites were common to the two histologies (n=113,786), while 1,265 sites were ccRCC-specific, and 2,661 sites were pRCC-specific. The 1,265 ccRCC-enriched peaks were associated with genes involved in circulatory system development and angiogenesis (Figure 1K), while the 2,661 pRCC-enriched peak genes were again enriched for genes involved in kidney embryogenesis. Similar analysis of the H3K4me2 pe demonstrated clear separation of chRCC from the other two RCC subtypes with comparable histology-specific and common “poised” sites.

H3K27ac and H3K4me2 ChIP-seq signals for all samples were strongly correlated (Pearson correlation, r=0.73) (Fig. 1L).

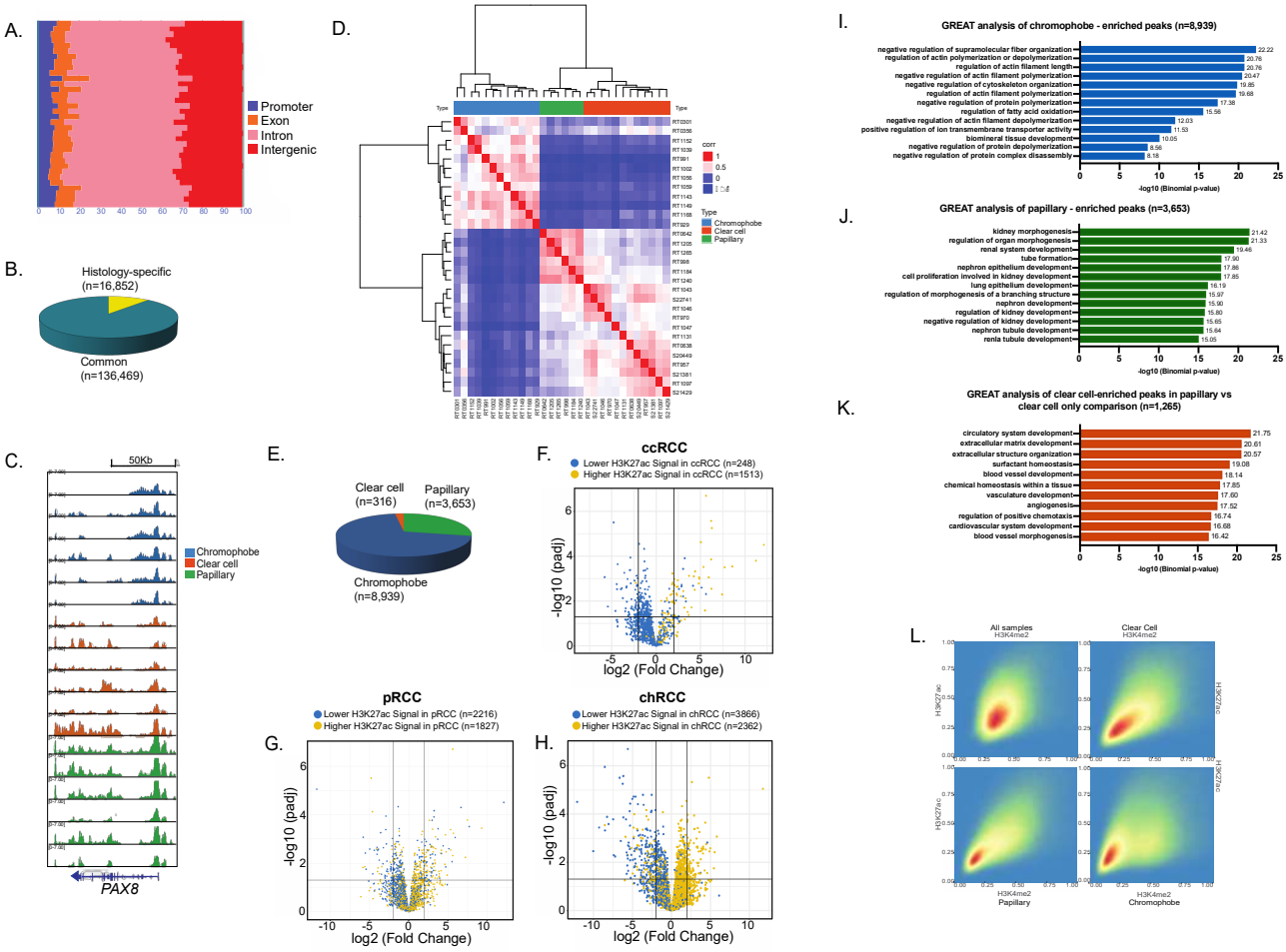

Figure 1. Landscape of H3K27ac signals across RCC histologies. A. Distribution of RCC H3K27ac peaks according to genomic region for 30 fresh frozen RCC tumor samples (12 chRCC, 6 pRCC, 12ccRCC). **B.** Numbers of histology-specific and common H3K27ac peaks. **C.** H3K27ac profiles at PAX8 in six representative samples from each RCC histology . **D.** Hierarchical clustering of chRCC, ccRCC, and pRCC based on sample-to-sample pairwise correlation of the H3K27ac ChIP-seq peaks. **E.** Distribution of histology-specific H3K27ac peaks among RCC subtypes. **FGH.** Volcano plots with the log change of gene expression (FP KM) in one histology compared to the other two and p-values from RNA-seq at genes associated with dif ferential H3K27ac peaks (ccRCC vs others, pRCC vs others, chRCC vs others). **I.** GREAT analysis of chromophobe-enriched peaks (n=8,939). **J.** GREAT analysis of papillary-enriched peaks (n=3,653). **K.** GREAT analysis of clear cell-enriched peaks in clear cell vs. papillary only comparis on (n=1,265). **L.** Density map of correlation between H3K27ac versus H3K4me2 ChIP-seq peaks across subtypes.

De novo motif analysis of H3K27ac peaks enriched in each subtype identified four motifs that were highly enriched in chRCC, including one resembling a forkhead motif, and another resembling the motif associated with TFCP2 (Figure 2A). FOXI1, a forkhead family TF, and TFCP2L1, closely related to TFCP2, have both been implicated in the development of intercalated cells of the kidney, the putative cell of origin of chRCC[8]. FOXI1 and TFCP2L1 gene loci were characterized by high H3K27ac signal in chRCC compared to absent or markedly lower signal in ccRCC and pRCC (Figures 2BC). Motif enrichment analysis of putative ccRCC-specific enhancers identified a motif resembling the basic Leucine Zipper (bZIP) BATF motif, and another resembling the basic helix-loop-helix (bHLH) TF family member HIF-2a (also known as EPAS1) (Figure 2D). ETS1, in the bZIP family with BATF, has been implicated in von-Hippel Lindau (VHL)-dependent ccRCC tumorigenesis[9], and was highly marked by H3K27ac in ccRCC, less so in pRCC, and not in chRCC (Figure 2E). HIF-2a is well known to be dysregulated in ccRCC due to the loss of the VHL protein function. It is the main driver of ccRCC and is upstream of multiple critical oncogenic pathways. Recent clinical trials with HIF2 inhibitors have shown clinical activity in advanced ccRCC patients[10,11]. The top scoring pRCC-specific motif corresponded to HNF1B (Figure 2F, 2G). HNF1B is a member of the homeodomain-containing superfamily of TFs, which is involved in nephrogenesis[12], and was highly marked by H3K27ac specifically in pRCC (Figure 2F).

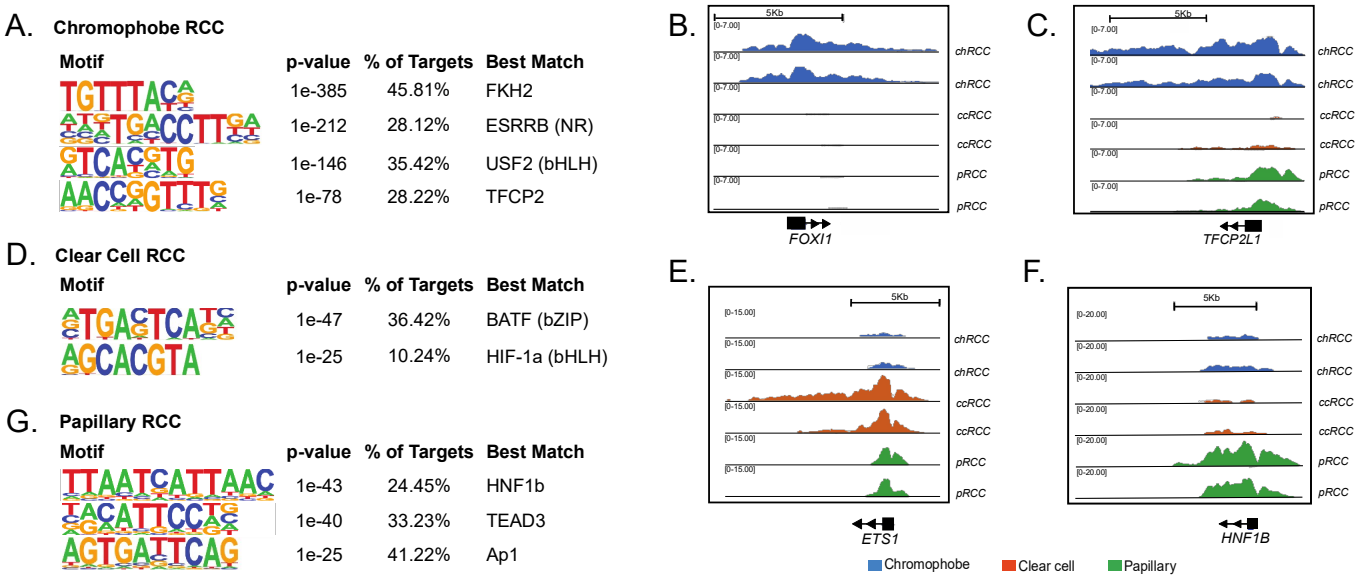

Figure 2. De novo motif analysis of differential H3K27ac peaks to identify differential TF activity . A. Four most significantly enriched nucleotide motifs present in chRCC-specific sites by de novo motif analysis, limited by ATAC peaks. **BC.** H3K27ac profiles near FOXI1 and TFCP2L1, respectively, in two representative samples of each histology (chRCC, ccRCC, pRCC). **D.** Two most significantly enriched nucleotide motifs present in ccRCC specific sites by denovo motif analysis. **EF.** H3K27ac profiles near ETS1, and HNF1B, respectively, in two representative samples of RCC histology. **G.** Three most significantly enriched nucleotide motifs present in pRCC specific sites by denovo motif analysis.

We next sought to systematically identify candidate histology-specific master TFs that define the three subtypes of RCC. Master TFs typically bind within superenhancers (SEs)[13,14], are often regulated by SEs, and regulate one another in a transcriptional core regulatory circuit (CRC) [44]. We employed an integrative approach[15,16], leveraging the RNA-Seq, ChIP-Seq, and ATAC-Seq data to identify candidate histotype-specific master TFs (Figure 3A). This approach aims to utilize orthogonal information to identify a consensus set of master TFs. We combined 1) expression data of differentially expressed TFs across RCC histologic subtypes; 2) TFs specific to RCC histologic subtypes relative to other cancer types (CaCTS; http://dx.doi.org/10.1101/839142do); 3) differential SE-associated TFs among RCC histologic subtypes; and 4) TFs with histology-specific connectivity in regulatory cliques. These four analyses identified more than 200 candidate TFs showing a histology-specific association in one or more analysis. We prioritized candidates for downstream validation by selecting those that were identified in more than one analysis. This analysis highlighted 50 candidate histotype-specific master TFs (N=20, chRCC; N=14, pRCC; N=16, ccRCC) including FOXI1, TFCP2L1, and DMRT2 for chRCC; EPAS1, ETS1, BARX2, ZNF395 for ccRCC, and HNF1B and NR2F2 for pRCC (Figure 3B). SE ranking, gene expression, and CES of the 50 histotype-specific TFs selected using this meta-analysis approach clustered the samples tightly according to their respective histologies.

To provide proof of concept validation of the nominated master TFs in driving the transcriptional identity of the different RCC histologies, we manipulated TF expression in a ccRCC cell line. We hypothesized that overexpression of chRCC-specific TFs and suppression of ccRCC-specific TFs can shift the transcriptional landscape of the ccRCC cell line 786-O to become more chromophobe-like. FOXI1 scored as a chromophobe-specific TF in 3/4 master TF analyses (Figure 3B). Furthermore, FOXI1 is selectively expressed in intercalated cells (ICs), the putative cellular origin of chRCC[8,17] and is more highly expressed in chRCC than other cancer subtype in the TCGA dataset. We also manipulated the expression of EPAS1 as a second target as it was highly specific for ccRCC in our integrative analysis, and prior studies have characterized its role in the pathogenesis

of ccRCC[18]. We overexpressed FOXI1 in the ccRCC cell line 786-O (FOXI1 OE); suppressed EPAS1 (EPAS1 KD) and simultaneously perturbed both genes in the same cell line (FOXI1 OE/EPAS1 KD). Gene set enrichment analysis (GSEA) of down-regulated genes with FOXI1 OE/EPAS1 KD in 786-O showed enrichment of genes involved in the immune responses (Figure 3C). Concomitant FOXI1 OE and EPAS1 KD in 786-O cells resulted in upregulation of chRCC-specific master TFs such as TFCP2L1 and NR3C2 and down-regulation of ccRCC-specific master TFs such as ETS1 and ZNF395 compared to control cells. (Figure 3D). Of note, the FOXI1 OE/EPAS1 KD cell line did not have significant expression differences compared to FOXI1 OE only.

We then compared differentially expressed genes between our experimental conditions in 786-O and our RCC tumor expression data (Figure 3E). Defining chRCC and ccRCC gene sets as the top 100 upregulated genes for each histology relative to the other, we showed that the chRCC gene set is enriched among upregulated genes in 786-O FOXI1 OE/EPAS1 KD cell line vs control (Figure 3F), and the ccRCC gene set is enriched among upregulated genes in 786-O control vs FOXI1 OE/EPAS1 KD cell line (Figure 3G). The FOXI1 OE/EPAS1 KD 786-O cell line demonstrated significantly higher expression of chRCC-specific candidate master TFs, TFCP2L1, GATA2, DDIT3, NKX6-1, and lower expression of ccRCC-specific candidate master TFs, ZNF395 and TSC22D3. Differential TFs in the 786-O FOXI1 OE/EPAS1 KD vs. 786O CTRL comparison overlapped more significantly with differential TFs from the chRCC vs ccRCC human sample comparison than from the ccRCC vs pRCC human sample comparison. In summary, these data show that overexpression of a single chRCC master transcription factor candidate, FOXI1, in the ccRCC cell line 786-O, with or without knockdown of EPAS1, led to marked expression changes driving the cell line to be more like a chRCC cell line without any modification to the set of mutations present in 786-O cells.

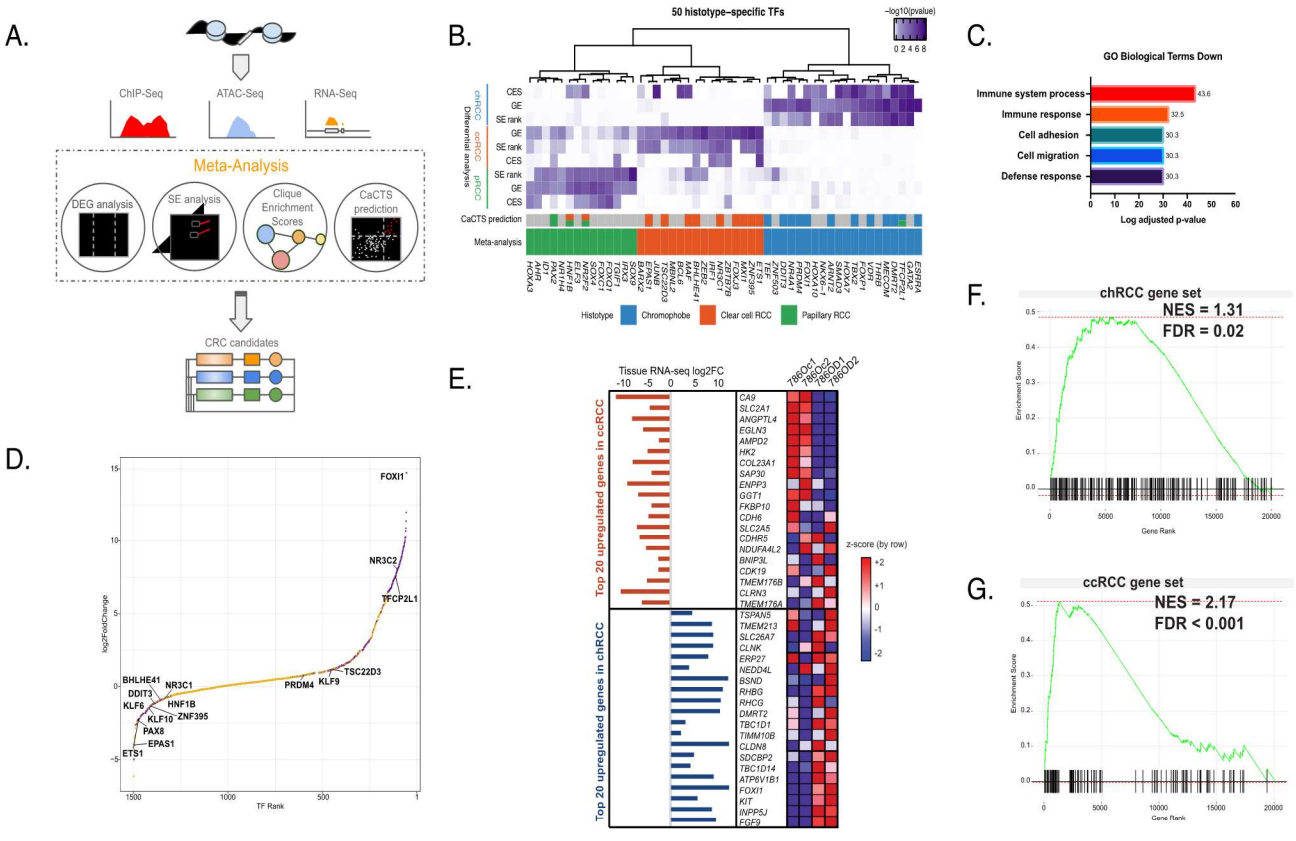

Figure 3. Multi-dimensional integrative analysis identifies histology-specific master TFs. A. Overview of the approach used to nominate histology-specific master TFs participating in core regulatory circuitries (CRC). **B.** Heatmap integrating the 50 histology-specific TFs identified by the meta-analysis approach (CES, differential expression, SE rank analysis, and CaCTS). **C.** Downregulated GO biological terms in the cell line 786-O FOXI1 OE/EPAS1 KD. **D.** Rank order of differentially expressed TFs between 786-O CTRL and 786-O FOXI1 OE/EPAS1 KD by expression levels. Each TF dot represents one TF. Purple dots indicate adjusted p-value < 0.001 by DESeq Expression. **E.** Heatmap showing the relative expression of the top 20 upregulated genes in ccRCC vs. chRCC and vice versa in the different cell line conditions. **F.** GSEA analysis showing the top 100 upregulated genes from chRCC vs. ccRCC dataset in 786-O FOXI1 OE/EPAS1 KD vs. 786-O CTRL comparison. **G.** GSEA analysis of the top 100 upregulated genes in ccRCC vs. chRCC in 786-O CTRL vs. 786-O FOXI1 OE/EPAS1 KD. CTRL: control; KD: knock-down; OE: over-expression; TF: Transcription Factor; GSEA: Gene Set Enrichment Analysis; 786Oc1: CTRL1; 786Oc2: CTRL2; 786OD1: 786-O FOXI1 OE/EPAS1 KD1; 786OD2: 786-O FOXI1 OE/EPAS1 KD2. NES: Normalized Enrichment Score. FDR: False Discovery Rate.

Allelic imbalance, the differential allelic representation of heterozygous SNPs in ChIP-seq reads, provides an in vivo comparison of cis-regulatory activity between two haplotypes (Figure 4A). As such, allelic imbalance can highlight the functional relevance of candidate causal variants[19-22] at loci that have been associated with RCC risk through GWAS. A key advantage of profiling epigenomes from many individuals is the ability to capture heterozygous sites and to measure the effect of TF binding on regulatory elements through analysis of allelic imbalance. To this end, we assessed allelic imbalance in ChIP-seq reads from these RCC samples in order to nominate causal risk SNPs from a genome-wide association study of RCC. We applied stratAS52 to H3K27ac ChIP-seq data from 20 ccRCC and 6 pRCC. We identified 10,605 imbalanced H3K27ac peaks – which we defined as peaks with one or more imbalanced SNPs after correction for multiple hypothesis testing – across 183,099 peaks tested in the combined ccRCC and pRCC sample sets (Figure 4B).

We hypothesized that AI peaks correspond to regulatory elements that are bound by master TFs. At these elements, the presence of trans-acting factors (i.e. master TFs) may result in regulatory element activity that are observable as H3K27ac AI peaks. One example is rs4903064 (chr14:73279420; 14q24.2), an eQTL for DPF355 (Figure 4C). rs4903064 is allelicly imbalanced in our dataset, has been shown to be a GWAS risk variant in all 3 histologic subtypes (ccRCC, chRCC, pRCC), and the altered C-allele of this SNP has been suggested to create a HIF binding motif (Figure 4D)[23]. To confirm this, we used HIF-2a transcription factor ChIP-Seq data from an ongoing project in an independent cohort of 43 samples derived from 23 patients with ccRCC to study the effect of the GWAS risk variant rs4903064 on HIF-2a binding. Analysis of 10 primary, 23 metastatic ccRCC samples, and 10 normal renal tissue samples showed that tumors with homozygous C/C alleles were significantly

enriched for HIF-2a peaks (Figures 4E, 4F) in both tumors and normal tissue and that rs4903064 is a HIF-2a cQTL. This confirms prior literature that the C-allele creates a HIF binding site[23].

We applied our AI analysis to annotate risk SNPs identified by a GWAS for RCC. AI peaks were highly enriched (16.7-fold) for RCC GWAS risk variants as assessed by LD score regression analysis (p = 1.9 x 10-4)[24]. Subsetting imbalanced H3K27ac peaks to regions of accessible chromatin where TFs are likely to bind (as assessed by ATAC-Seq from RCC tissues59) (Figure 4G) resulted in substantial additional enrichment (43.2-fold; p = 1.2 x 10-4) . This enrichment represents approximately twice that of the total set of H3K27ac peaks, which are themselves enriched 8.1-fold (p = 1.2 x 10-4). By contrast, multiple other GWAS phenotypes from the UK Biobank 60 showed substantially less enrichment compared to RCC GWAS SNPs, indicating the specificity of this enrichment for RCC (Figure 4H).

Using this method, we were able to fine-map a total of 30 risk SNPs, with some examples highlighted here. rs7132434, located on chr 12, has been characterized as a functional variant that alters AP-1 binding leading to upregulation of BHLHE41, thus promoting tumor growth through induction of IL-1161 (Figure 4I). Another example is rs4733579 which is located on chr 8 and is in LD with rs35252396 (Figure 4J). The latter has been shown to contribute to RCC susceptibility through regulating MYC and PVT1 expression[25]. Of note, rs35252396 is an indel and therefore cannot assessed by stratAS. Our analysis also highlighted rs46552601 (Figure 4K) and rs46541176 (Figure 4L), both located on chr2p21 within the EPAS1 gene, where at least 59 SNPs have been fine mapped. These two SNPs are imbalanced only in ccRCC but not in pRCC, consistent with the specific role of EPAS1 (HIF2-alpha) in ccRCC pathogenesis (Figures 4M, 4N). As part of this project, we plan to functionally validate the mechanism of these two SNPs in ccRCC pathogenesis.

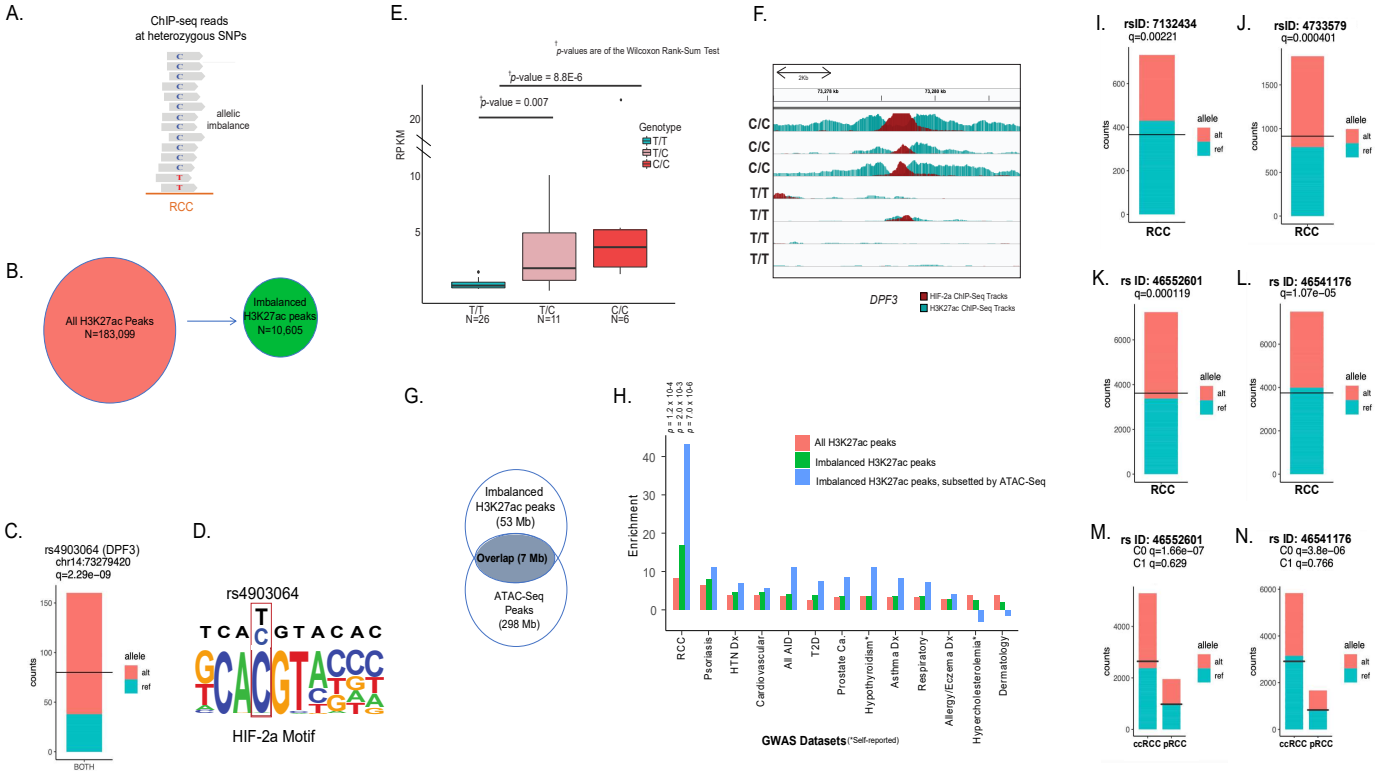

Figure 4. Allelically imbalanced H3K27ac peaks in ccRCC and pRCC. A. Schematic of allelic imbalance at heteroz ygous SNPs. **B.** Schematic showing subset of allelicaly imbalanced H3K27ac peaks from total H3K27ac peaks in all RCC samples. **C.** AI at rs4903064 (chr14:73279420; DPF3) in RCC. **D.** rs4903064 C allele sequence context creates a HIF-2a binding site. **E.** RPKM values for the HIF-2α ChIP signal in the peaks around rs4903064 in 43 samples. Genotype is shown on the X-axis. **F.** Overlayed HIF2-α and H3K27ac ChIP-Seq tracks for 7 samples within ~10Kb of genomic coordinates of rs4903064. Individual sample genotypes are sh own. **G.** Venn diagram showing overlap of imbalanced H3K27ac peaks with ATAC seq peaks in RCC. **H.** Enrichment of risk SNPs from GWAS for RCC and other diseases in H3K27ac peaks with differential allelic imbalance, compared to all H3K27ac peaks. Empiric one- sided p value is indicated. **IJ.** GWAS risk SNPs rs7132434 and rs4733579 demonstrating allelic imbalance in RCC. **KL.** AI at Chr2 SNPs within EPAS1. rs 46552601 and rs46541176 shown. **MN.** Chr2 SNPs within EPAS1, with imbalance plots split by histology. Adjusted Q values for imbalance are indicated.HTN: Hypertension. Dx: Diagnosis. T2D: Type 2 Diabetes Mellitus. AID: Autoimmune Disease. C0: Reference allele. C1: Alternate allele. AI: Allelic Imbalance.

Based on these findings, we next sought to characterize sarcomatoid RCC (sRCC). Sarcomatoid differentiation (SD) of RCC is associated with poor prognosis. Recent studies showed promising response rates of sRCC to immune checkpoint blockade (ICB). Although distinct patterns of gene expression in sRCC have been documented, the gene regulatory programs that drive sarcomatoid differentiation remain unknown. We aimed to nominate TFs driving SD and to test the association of expression levels of these TFs with clinical outcomes in patients with RCC.

We performed ChIP-seq for H3K27ac on sRCC and non-sRCC fresh frozen tissue samples collected by pur team at DFCI, with the goal of identifying differentially active regulatory elements. Regulatory elements that were activated in sRCC were assessed for enrichment of specific TF binding motifs using HOMER. Differential gene expression analysis was performed using DESeq2 on RNA-seq data from TCGA to identify differentially expressed TFs between sRCC and non-sarcomatoid RCC. RNA-seq data from patients enrolled in the IMmotion151 trial were divided into quartiles based on gene expression levels of candidate TFs. Progression-free survival (PFS) was compared between patients' quartiles using a multivariable Cox regression analysis accounting for age and IMDC risk score. Association of TF expression with objective radiographic response (ORR) was analyzed using a logistic regression model.

We identified 278 candidate regulatory elements with increased H3K27ac levels in sRCC vs. non-sRCC. Nucleotide motifs bound by the TFs FOSL1 and E2F7 were enriched in these regulatory elements. Differential expression analysis between 48 sRCC vs. 493 non-sRCC samples and showed that FOSL1 & E2F7 were significantly upregulated in sRCC vs. non-sRCC (LFC=1.7, adjusted p=5e-11; LFC=1.8, adjusted p=1.3e-20; respectively). Mean TPMs of FOSL1 & E2F7 were significantly increased in sRCC vs. non-sRCC (2.5&2.9 vs.1.5&1.9TPM, all p<0.001; resp.). Increased expression of FOSL1 & E2F7 was associated with shorter PFS in IMmotion151 patients who received sunitinib alone (HR=1.6, 95%CI=1.3-2.2, p=0.008 & HR=2.6, 95%CI=1.8-3.7, p<0.001; resp.). We observed similar results even after controlling for the presence of SD. Increased expression of E2F7 was associated with worse ORR, even after controlling for the presence of SD (OR= -1.4±0.4, p<0.001).

This is the first study to analyze epigenomic landscape of sRCC by integrating ChIP-seq and RNA-seq data. Our findings implicated FOSL1 & E2F7 as transcriptional regulators of SD with prognostic relevance. We are validating these intriguing results in a second cohort.

- **What opportunities for training and professional development has the project provided?**
  - The project has provided the opportunity to mentor three post-docs in the lab – Sylvan Baca, M.D., Sarah Alaiwi, M.D. and Amin Nassar, M.D. Dr. Baca is a medical oncology fellow at Dana-Farber Cancer Institute. He has completed post-doctoral training in genomics and bioinformatics and is now faculty at DFCI. He conducted much of the bioinformatics work described above under the mentorship of the PIs on this project. Drs. Abou Alaiwi and Nassar are both internal medicine residents at Brigham and Women's Hospital. As part of this project, they mastered ChIP-seq in human tissue specimens and worked with the bioinformatics team to analyze the data. Our team – the PIs on this project plus Drs. Abou Alaiwi and Nassar presented our work at the International Kidney Cancer Symposium and will be presenting at the upcoming American Association for Cancer Research annual conference in 2023.
- **How were the results disseminated to communities of interest?**
  - The data were initially presented at the International Kidney Cancer Symposium 2020 and updated at the 2021 meeting.
- **What do you plan to do during the next reporting period to accomplish the goals?**

- Now that we have defined the universe of enhancers associated with RCC pathogenesis and identified master TFs and downstream target genes, we will functionally characterize these potential non-coding drivers of RCC progression. Our H3K27ac ChIP seq data (above) revealed the enhancer landscape in RCC tumorigenesis, with both loss and gain of enhancers. We will use these enhancer profiles to perform an in vitro screen renal cell carcinoma cell lines. We will focus on the A498 and 798-O cell lines, which have been used as models for localized and metastatic ccRCC, respectively. To determine the functional significance of these epigenetic alterations, we will perform an unbiased pooled CRISPR/Cas9-based screen to systematically suppress each differentially activated enhancer. Recruitment of a nuclease dead version of Cas9 fused to the Kruppel-associated box repressor domain (dCas9-KRAB) results in loss of H3K27ac and silencing of the enhancer. We will clone a pool of gRNAs targeting each enhancer into a lentiviral expression system. We will use five gRNAs per enhancer targeting the center of each H3K27Ac ChIP-seq peak. We anticipate approximately 4000 enhancers to be differentially activated in tumors versus normal tissue based on our preliminary results, which is well within the range typically done with genome-scale screens involving 20,000 genes and libraries consisting of 100,000 gRNAs. As a result, we will have generated the first comprehensive functional enhancer landscape required for proliferation of primary and metastatic RCC.

4. **IMPACT:**

   - **What was the impact on the development of the principal discipline(s) of the project?**

     - To date, the majority of RCC epigenomic studies have focused on promoter DNA methylation in the context of tumor suppressor gene silencing. On the other hand, little is currently known about distal regulatory elements in the various subtypes of RCC. While some studies have begun interrogating the epigenetic landscape of RCC, the majority of epigenetic analyses have been conducted in immortalized or malignant cancer cell lines. **In this project, we have directly addressed this void in our understanding of RCC by generating a comprehensive compendium of ATAC-Seq, RNA-Seq, and histone modification data across different RCC histologies with the goal of identifying histology-specific epigenetic mechanisms driving RCC pathogenesis** and potentially explaining its diverse clinical behavior.

     - In line with histological, expression and mutational data, the regulatory element landscapes of ccRCC, pRCC and chRCC are distinct. While the majority of gene regulatory elements were common across all three histologies, large sets of epigentic marks were unique to each histology. Interestingly, within individual histologies, **mutations in commonly altered chromatin modifying genes did not have a significant impact on the regulatory element landscape**. This suggests that the highly prevalent genomic alterations discovered in large-scale genomic sequencing projects such as TCGA may not be the key players in driving the regulatory element landscape heterogeneity and, hence, the transcriptional diversity in RCC.

     - Prior work in medulloblastoma and ependymoma demonstrated how molecularly-defined cancer subgroups exhibit specific core regulatory circuitries. These studies showed that subgroup-specific TFs are faithful tracers of cell-of-origin and important regulators of tumor dependencies. In attempt to first understand the epigenetic orchestrators of cellular identity and second to highlight potential drivers of carcinogenesis in RCC, we mapped putative master TFs in each RCC histology. **Using an integrative approach, we identified 50 histotype-specific TFs**.

- **This project characterized a novel master TF, ETS-1, in ccRCC pathogenesis**. ETS proto-oncogene 1 (ETS-1) is a transcription factor that is involved in differentiation of hematopoetic cells and angiogenesis. ETS-1 also plays a crucial role in carcinogenesis. As a proto-oncogenic factor, ETS-1 is capable of activating genes associated with angiogenesis, metastasis and invasive behavior in multiple tumor types. Additionally, ETS-1 expression correlates with microvessel density in some non-glial tumors and is an independent negative prognostic marker in different tumor entities such as breast, ovarian, pancreatic and colorectal cancer. Nonetheless, the role of ETS-1 in RCC has mainly focused on its interaction with HIF2-alpha. Here, we show that ETS-1 may play a role as a master TF specific to ccRCC and not chRCC or pRCC. Prospective studies should focus on understanding the global epigenomic effect of ETS1 in driving ccRCC pathogenesis. Furthermore, HIF2-alpha, a known driver in ccRCC, was found to be a ccRCC-specific SE.

- GWAS have identified hundreds of putative cancer-risk loci. These studies have led to the conclusion that cancer is driven by thousands of variants with individual small effects, and that cancer-risk variants predominantly lie in non-coding regions of the genome. More recently, it has been realized that GWAS heritability is enriched at variants that lie in tissue-relevant epigenomic features. Emerging evidence suggests that many GWAS variants function by altering an existing or creating a new TF binding site. This in turn regulates the local chromatin regulatory landscape by altering the enhancer landscape and thus modulating the expression of target genes. **We used our population-scale epigenomic data to further empower the discovery of cis-regulatory elements that may mediate between the risk variant and target gene and thus constrain putative targets for experimental validation**. We showed that RCC GWAS risk SNPs are enriched in regulatory elements, consistent with prior studies. Profiling these regulatory elements across mutltiple (genetically diverse) individuals allowed us to identify regions of allelic imbalance, which are further enriched for GWAS SNPs, and subsetting these regions to ATAC-seq peaks where TFs are likely to bind identifies regions, showed even more enrichment for GWAS SNPs. In RCC, there are 13 recognized risk loci, but the causal variants have been identified for few, and underlying mechanisms of risk remain elusive for most regions. **Our approach can help pinpoint the SNPs associated with RCC risk and provide candidate mechanisms by identifying small numbers of SNPs that my alter binding sites for specific TFs**.

- **What was the impact on other disciplines?**

  This work combines and integrates ChIP-Seq, ATAC-seq and RNA-seq data into a unified analysis to capture the deep complexity of the epigenomic landscape of RCC. The majority of epigenetic analyses have been conducted in immortalized or malignant cancer cell lines, which inevitably have diverged significantly in many respects from primary tumor tissues, as seen in prior studies from medulloblastoma and diffuse large B-cell lymphoma. In addition, studies have demonstrated that the vast majority of available RCC cell lines are derived from ccRCC, with very few pRCC lines, and relative absence of any chRCC lines. To date, most of our knowledge in the epigenomic space of RCC primary tissue is derived from older DNA methylation analysis, limited to one specific subtype of RCC, or confined to only one epigenetic dimension. By integrating several epigenetic technologies, we discovered master TFs that drive histology-specific transcriptional programs in RCC and showed that regulatory elements with allelic imbalance are specifically enriched for RCC GWAS risk SNPs relative to GWAS risk SNPs for other disease features. Overall, our work highlights the histology-specific nature of these regulatory elements and the opportunity of cataloging the epigenetic landscapes in expanded patient cohorts and across various tumor types.

- **What was the impact on technology transfer?**
  - Nothing to Report.
- **What was the impact on society beyond science and technology?**
  - Nothing to Report

5. **CHANGES/PROBLEMS:**
   - **Changes in approach and reasons for change**
     - Nothing to Report
   - **Actual or anticipated problems or delays and actions or plans to resolve them**
     - The COVID-19 pandemic had a significant impact on the launch of this project and maintaining the intended timeline, particularly the planned functional work outlined in Aim 2 of the project, as our laboratories at Dana-Farber Cancer Institute and the National Cancer Institute were closed for most of 2020 and were limited in 2021. Both institutions placed strict restrictions on access to the laboratory for activities unrelated to direct patient care. Nonetheless, we have been able to generate the considerable amount of data described above and continue to process data generated earlier this year.
   - **Changes that had a significant impact on expenditures**
     - Nothing to Report
   - **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
     - Nothing to Report
   - **Significant changes in use or care of human subjects**
     - Nothing to Report
   - **Significant changes in use or care of vertebrate animals.**
     - Nothing to Report
   - **Significant changes in use of biohazards and/or select agents**
     - Nothing to Report

6. **PRODUCTS:**
   - **Publications, conference papers, and presentations**
     - **Journal publications.** "Leveraging the Epigenomic Landscape to identify Histology-Specific Master transcription factors and to functionally annotate risk loci in renal cell carcinoma" is under review at *Cancer Cell* and we have provided acknowledgement of federal support and specifically cited this grant.

       Integrative molecular characterization of sarcomatoid and rhabdoid renal cell carcinoma. Bakouny Z, Braun DA, Shukla SA, Pan W, Gao X, Hou Y, Flaifel A, Tang S, Bosma-Moody A, He MX, Vokes N, Nyman J, Xie W, Nassar AH, Abou Alaiwi S, Flippot R, Bouchard G, Steinharter JA, Nuzzo PV, Ficial M, Sant'Angelo M, Forman J, Berchuck

JE, Dudani S, Bi K, Park J, Camp S, Sticco-Ivins M, Hirsch L, Baca SC, Wind-Rotolo M, Ross-Macdonald P, Sun M, Lee GM, Chang SL, Wei XX, McGregor BA, Harshman LC, Genovese G, Ellis L, Pomerantz M, Hirsch MS, Freedman ML, Atkins MB, Wu CJ, Ho TH, Linehan WM, McDermott DF, Heng DYC, Viswanathan SR, Signoretti S, Van Allen EM, Choueiri TK. Nat Commun. 2021 Feb 5;12(1):808. doi: 10.1038/s41467-021-21068-9.

- **Books or other non-periodical, one-time publications.** Nothing to Report
- **Other publications, conference papers, and presentations.** "Mapping the epigenetic landscape of renal cell carcinoma", presented at the International Kidney Cancer Symposium 2020

  "Epigenomic profiling nominates master transcription factors (TFs) driving sarcomatoid differentiation of renal cell carcinoma (RCC)", submitted to American Association for Cancer Research conference in 2023

- **Website(s) or other Internet site(s)**
  Nothing to Report

- **Technologies or techniques**
  Nothing to Report. Techniques developed in our lab for performing epigenetic analysis in human specimens has been reported in previous publications and updated in our current manuscript.

- **Inventions, patent applications, and/or licenses**
  Nothing to Report

- **Other Products**
  The epigenetics data generated by the project thus far will be made publicly available via the NCBI Gene Expression Omnibus upon publication of our current manuscript.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

Name:	Mark Pomerantz, M.D.
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	ORCID ID 0000-0003-4914-1157
Nearest person month worked:	0.60 CM
Contribution to Project:	Dr. Pomerantz has led overall study design, data generation and data analysis. He directs the database and biobank that provides the materials for the project.
Funding Support:	

Name:	Matthew Freedman, M.D.
Project Role:	Co-Investigator

Researcher Identifier (e.g. ORCID ID):	ORCID ID 0000-0002-0151-1238
Nearest person month worked:	0.33 CM
Contribution to Project:	Dr. Freedman is involved in overall study design, data generation and data analysis
Funding Support:	

Name:	Toni Choueiri, M.D.
Project Role:	Co-Investigator
Researcher Identifier (e.g. ORCID ID):	ORCID ID 0000-0002-9201-3217
Nearest person month worked:	0.44 CM
Contribution to Project:	Dr. Choueiri is involved in overall study design, data generation and data analysis
Funding Support:	

Name:	Dory Freeman
Project Role:	Clinical Research Coordinator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1.48 CM
Contribution to Project:	Dory has led data acquisition from and curation of our renal cell carcinoma data base and biobank. She also consents patients to our banking protocol and prepares all tumor tissue specimens for planned experiments.
Funding Support:	

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

POMERANTZ, MARK

PREVIOUS:
Nothing to report

CURRENT:

AWARDED
W81XWH-22-1-0696 (Fox) 08/01/22 – 07/31/25 0.30 CM
DoD
Ceramide NanLiposomes: a novel therapeutic option for castration resistant prostate cancer
Specific Aims: 1) Determine the regulatory and transcriptional mechanism by which AR regulates SPTSSB and de novo ceramide synthesis in PCa; 2) Determine the translational potential of CNL in preclinical mouse models of PCa
POC: Grants Management Specialist (GMS): Jennifer Shankle; jennifer.e.shankle.civ@mail.mil; 301-619-2193

AWARDED
21CHAL04 (Freedman) 11/19/21 - 11/19/23 2.40 CM
Prostate Cancer Foundation
Leveraging Epigenomics to Target Acquired Vulnerabilities in Treatment Resistant Prostate Cancer
Specific Aims: 1) Chemical and genetic perturbation of candidate transcriptional regulators to abrogate resistance to AR inhibition and selectively block NEPC cell proliferation, in vitro and in vivo; and 2) Creation of the metastatic prostate cistrome project.
Role: Co-Investigator
POC: Manager, Program Administration: Audrey Gardener; agardner@pcf.org; 310-570-4792

FREEDMAN, MATTHEW

PREVIOUS:

COMPLETED
R01CA211707 (Gayther) 01/0/17-12/31/22
NIH/NCI
Title: Functional Effects of Ovarian Cancer Risk Variants
Role: Co-Investigator
Goal: Goal of this proposal is to study causal variants and genes. Dr. Freedman and his group will be responsible for causal variant identification using epigenome- and genome- editing. They will perform the circular chromosome conformation capture (4C).
Specific Aims:
Aim 1: To identify and fine map common variant ovarian cancer risk loci
Aim 2: To identify candidate susceptibility genes associated with ovarian cancer risk loci and establish their role in disease pathogenesis
Aim 3: To identify causal variants and regulatory mechanisms underlying ovarian cancer risk loci

POC: Colleen M. Loew, MBA; Sr Grant and Contract Officer

COMPLETED
W81XWH-19-0553/KC180206 (Choueiri) 09/15/19-09/14/22
DOD / KCRP TDA
Title: Development and testing of circulating-free methylation DNA as a prognostic biomarker for recurrent kidney cancer
Role: Co-Investigator
Goal: Methylation of cfDNA analyses (extraction, profiling/optimization, validation, correlation.
Specific Aims:
Aim 1: To evaluate the relationship between cfmeDNA and recurrence in patients with early-stage RCC treated with placebo.
Aim 2: To evaluate the relationship between cfmeDNA and recurrence in patients with early-stage RCC treated

with pazopanib.
POC: Michelle Cromwell, Grant Specialist

COMPLETED
R01CA204954 (Freedman) 04/15/16 - 03/31/22
NIH/NCI
Title: Identifying causal variants and genes underlying breast cancer risk loci
Role: Principal Investigator
Goal: The overall goal of this proposal is to outline a rigorous strategy to discover functionally causal variants and genes underlying complex traits.
Specific Aims:
Aim 1: identify enhancer-target gene interactions (using eQTL).
Aim 2: identify candidate causal variants using case-control fine mapping data intersected with epigenetic profiling and perform genome editing on candidate causal variants.
Aim 3: test the target genes in cell-based models to understand their influence on cancer-related phenotypes, such as proliferation and invasion.
POC: Jacquelyn Boudjeda, Grant Management Specialist

CURRENT:

NEW
R01CA26990 (Baylor) 07/01/22-06/30/27 1.80 CM
NIH/NCI
Title: Notification of access to targeting castration resistant prostate cancer via potent inhibition of signaling lipids
Role: PI: Kaochar; Co-PI: Freedman
Goals: Dr. Freedman's lab will be responsible for all of the chromatin immunoprecipitation (ChIP) and RNA sequencing work based on 28 samples (paired normal/tumor from 14 cases).
Specific Aims:
1) Each sample will be ChIP'd for five antibodies (AR, FOXA1, SREBP1, SREBP2, and H3K27ac).
2) The 28 samples will also undergo RNA-seq. All samples will be performed in duplicate.
POC: Chryll Batiste

NEW
R01CA259058 07/01/22-06/30/27 1.20 CM
NIH/NCI
Title: Identifying common mechanisms underlying the biology of pleiotropic risk loci
Role: MPI Gayther
Goals: To generate and analyze HiChIP data in breast, prostate, and ovarian cell lines. To perform CRISPR-based editing at high-priority regulatory elements that confer increased risk of breast, prostate, and ovarian cancers.
Specific Aims:
2) To identify and validate candidate susceptibility genes at BPO cancer risk loci; and
3) To determine causality for BPO cancer risk variants, their regulatory targets and candidate susceptibility genes at pleiotropic risk loci.
POC: Ian Fingerman, Program Director

NEW
R01CA262577 07/01/22-06/30/27 1.20 CM
NIH/NCI
Title: Elucidating prostate cancer risk mechanisms through large-scale cistrome wide association studies
Role: MPI Freedman, Gusev, Gimelbrant

Goals: At the completion of this project, we fully anticipate that we will have begun to unravel the causal (i.e., pathogenic) variants that initiate human prostate cancer. Discovering the mechanisms underlying human traits will not only inform the biology of disease but may also reveal opportunities to more rationally intervene in treatment and prevention.
Specific Aims:
1) To generate large-scale genetic and epigenetic datasets enabling identification of candidate causal regulatory variants and target genes;
2) To apply and develop statistical tools based on AI and QTL signals for causal variant fine mapping; and
3) To experimentally and mechanistically validate candidate causal variants
POC: Jacquelyn Saval, GMO

NEW
W81XWH-22-1-0951/KC210259 04/01/22-03/31/26 0.60 CM
DoD (Xu) (Salary support only)
Title: Circulating Biomarkers of Renal Cell Carcinoma
Role: Mentor
Goal: To prove that plasma KIM-1 can 1) distinguish patients with benign versus malignant renal masses either alone or in combination with cfMeDIP-seq, 2) identify patients likely to benefit from adjuvant therapy, and 3) prognosticate patients with advanced RCC.
Specific Aims:
Aim 1: Evaluate plasma KIM-1 with or without cfMeDIP-seq as a diagnostic aid in patients with renal masses;
Aim 2: Validate KIM-1 to predict clinical outcomes in the adjuvant setting; and
Aim 3: Assess KIM-1 as a biomarker for mortality risk and likelihood of response to initial treatment in metastatic RCC.
POC: Michael Hall, SO

CHOUEIRI, TONI:

PREVIOUS:

COMPLETED
W81XWH-19-1-0553/KC180206 09/15/19-09/14/22
DOD / KCRP TDA
Development and testing of circulating-free methylation DNA as a prognostic biomarker for recurrent kidney cancer
Aim 1: To evaluate the relationship between cfmeDNA and recurrence in patients with early-stage RCC treated with placebo.
Aim 2: To evaluate the relationship between cfmeDNA and recurrence in patients with early-stage RCC treated with pazopanib.
Role: Principal Investigator
Funding Agency POC:
Joshua D. McKean, Grants Officer,
Department Of The Army
Us Army Medical Research Acquisition Activity

COMPLETED
W81XWH-19-1-0551/KC180129 09/15/19-09/14/22
DOD / KCRP IDA

Host Immune Signatures as Therapy Response Biomarkers in Metastatic Renal Cell Carcinoma
Aim 1: Identify immune response factors by characterizing the tumor immune microenvironment from pre-treatment mRCC tumor RNA-seq data.
Aim 2: Evaluate whether observing an expansion of tumor-infiltrating immune repertoire in post treatment blood is associated with patient response to VEGF-TT, ICB and ICB+VEGF-TT.
Aim 3: Validate the results using other mRCC cohorts with VEGF-TT, ICB and ICB+VEGF-TT treatment.
Role: Principal Investigator
Funding Agency POC:
Michelle Cromwell, Grants Specialist,
Assistance Agreements Group
U.S. Army Medical Research Acquisition Activity

CURRENT:

NEW
R01CA266424 07/01/22-06/30/27 1.80 CM
NIH/NCI
Tissue-based biomarkers of anti-PD-1-based therapy in metastatic renal cell carcinoma
Major Goals: 1) To identify and evaluate the impact of ERVs on clinical outcomes following ICI-based combination therapy in mccRCC; 2) To determine the immune cell composition and phenotypes in the ccRCC microenvironment that contribute to response and resistance to ICI-based combination therapy; and 3) To uncover novel immune biomarkers and develop an integrated model of ICI response (exploratory).
Role: Principal Investigator
Funding Agency POC: Tawnya Mckee

NEW
W81XWH-22-1-0518 | KC210127 07/01/22-06/30/25 0.42 CM
Department of Defense
Biomarkers of response to combination Cabozantinib+Nivolumab in Advanced Renal Cell Cancinoma
Aims: To identify immune cell populations in the ccRCC microenvironment that predict response or resistance to ICI+VEGF-TKI combination therapy; 2) To identify gene signatures and genomic alterations that predict response to ICI+VEGF-TKI combination therapy in metastatic ccRCC; and 3) To develop and externally validate a model for prediction of PFS using biomarkers and conventional clinico-pathological variables in mccRCC.
Role: Principal Investigator
Funding Agency POC: Medha Darshan

- **What other organizations were involved as partners?**
  - Nothing to Report

8. **SPECIAL REPORTING REQUIREMENTS**
   - **COLLABORATIVE AWARDS:** Nothing to Report
   - **QUAD CHARTS:** Nothing to Report
9. **APPENDICES:**

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