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TITLE: Transferrin Receptor Identifies a Comprehensive Pool of Circulating Tumor Cells with Unique Molecular Features from Metastatic Prostate Cancer Patients

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14. ABSTRACT Metastatic castration resistant prostate cancer (CRPC) is currently incurable, due to treatment resistance. Elucidation of resistance mechanisms requires frequent tumor sampling to monitor tumor evolution and tailor treatments to the individual. Circulating tumor cells (CTCs) represent a non-invasive, accessible "liquid biopsy" source of tumor cells, allowing for longitudinal molecular disease profiling. Due to limitations with existing EpCAM-based CTC isolation assays we have identified and clinically tested Transferrin Receptor (TfR) as a novel cell-surface antigen that enables capture of all CTCs across the EMT gradient from metastatic patients. Mining large datasets (TCGA, SU2C) revealed **TfR enrichment in metastatic patients,** which significantly correlated with advanced state from localized PC to CRPC to the aggressive neuroendocrine NEPC. RNA-seq analysis indicates that TfR^+-CTCs possess unique expression profile and are enriched in EMT and tumor progression pathways, as compared to $EpCAM^+$- CTCs. Expression of androgen receptor (AR) splice variants (AR-Vs) is known to drive disease progression. We have developed a highly sensitive—down to single cell—digital droplet PCR assay for the quantitation of AR-Vs in patient CTCs. Isolation of TFR^+ vs $EpCAM^+$ CTCs from metastatic patients, revealed significant AR-V **enrichment in TFR^+ CTCs,** while AR-FL expression was similar. When we analyzed **single CTCs** using the same ddPCR assay, we observed even more striking enrichment, with AR-Vs detected in 21% of single TFR^+-CTCs vs 0% in $EpCAM^+$-CTCs. These data support our **hypothesis** that *TfR can identify a comprehensive pool of CTCs (not limited to the epithelial-only phenotypes) and provide an accurate representation of metastatic disease burden*. To test this, we propose to **prospectively** collect peripheral blood from CRPC and NEPC patients to **1**. Molecularly profile TFR^+-CTCs and $EpCAM^+$-CTCs, and matching tumor biopsies, by RNA-Seq to identify the driving oncogenic pathways that correlate with clinical outcomes **2.** Characterize heterogeneity and clinical impact of AR-V expression, assessed by ddPCR, in single TFR^+- and $EpCAM^+$-CTCs from CRPC patients. In addition, we propose to **3**. Explore the functional relationship between TfR and Myc in patient-derived tumor and animal models.

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1. Introduction

Metastatic castration resistant prostate cancer (CRPC) is currently incurable, due to treatment resistance. Elucidation of resistance mechanisms requires frequent tumor sampling to monitor tumor evolution and tailor treatments to the individual. Circulating tumor cells (CTCs) represent a non-invasive, accessible "liquid biopsy" source of tumor cells, allowing for longitudinal molecular disease profiling. Due to limitations with existing EpCAM-based CTC isolation assays we have identified and clinically tested Transferrin Receptor (TfR) as a novel cell-surface antigen that enables capture of all CTCs across the EMT gradient from metastatic patients. Mining large datasets (TCGA, SU2C) revealed **TfR enrichment in metastatic patients,** which significantly correlated with advanced state from localized PC to CRPC to the aggressive neuroendocrine NEPC. RNA-seq analysis indicates that TfR^+-CTCs possess unique expression profile and are enriched in EMT and tumor progression pathways, as compared to $EpCAM^+$- CTCs. Expression of androgen receptor (AR) splice variants (AR-Vs) is known to drive disease progression. We have developed a highly sensitive—down to single cell—digital droplet PCR assay for the quantitation of AR-Vs in patient CTCs. Isolation of TFR^+ vs $EpCAM^+$ CTCs from metastatic patients, revealed significant AR-V **enrichment in TFR^+ CTCs,** while AR-FL expression was similar. Taken together these data led us formulate the following hypothesis:

Hypothesis *TfR can identify a comprehensive pool of CTCs (not limited to the epithelial-only phenotypes) and provide an accurate representation of metastatic disease burden*.

To address this hypothesis, we proposed to **prospectively** collect peripheral blood from CRPC and NEPC patients and

Specific Aim 1. Molecularly profile TFR^+-CTCs and $EpCAM^+$-CTCs, and matching tumor biopsies, by RNA-Seq to identify the driving oncogenic pathways that correlate with clinical outcomes

Specific Aim 2. Characterize heterogeneity and clinical impact of AR-V expression, assessed by ddPCR, in single TFR^+- and $EpCAM^+$-CTCs from CRPC patients.

Specific Aim 3. Explore the functional relationship between TfR and Myc in patient-derived tumor and animal organoids.

2. Keywords: Prostate Cancer, castration resistant prostate cancer (CRPC), neuroendocrine prostate cancer (NEPC), circulating tumor cells (CTCs), Transferrin Receptor (TfR), androgen receptor (AR), AR splice variants (AR-Vs), epithelial cell adhesion molecule (EpCAM)

3. **Accomplishments:**

- **What were the major goals of the project?**

Specific Aim 1. Molecularly profile TFR^+-CTCs and $EpCAM^+$-CTCs, and matching tumor biopsies, by RNA-Seq to identify the driving oncogenic pathways that correlate with clinical outcomes

Specific Aim 2. Characterize heterogeneity and clinical impact of AR-V expression, assessed by ddPCR, in single TFR^+- and $EpCAM^+$-CTCs from CRPC patients.

Specific Aim 3. Explore the functional relationship between TfR and Myc in patient-derived tumor and animal organoids.

- **What was accomplished under these goals?**

Specific Aim 1. Identify clinically meaningful genes/oncogenic pathways associated with disease progression and/or response to therapy by molecular profiling of TfR+ -CTCs and EpCAM+ -CTCs from CRPC patients and NEPC patients using serial sampling at baseline, on treatment and progression and correlate with clinical outcomes.

The *working hypothesis* of this aim is that TfR+-CTCs will provide a more accurate representation of metastatic disease burden and include a more comprehensive spectrum of CTCs, whose molecular analysis will be clinically informative. In addition, we hypothesize that TfR+-CTCs will contribute to the diagnosis and molecular phenotyping of NEPC.

In this Aim we have been collecting peripheral blood from patients with metastatic CRPC who have progressed to prior treatment with AR-targeted therapies (abiraterone/enzalutamide) (IRB0707009283, PI Tagawa) and from patients with NEPC (at Dana Farber, IRB19883, PI: Beltran) and enriching for circulating tumor cells (CTCs) by

depleting the contaminating CD45+ leukocytes (RosetteSep human CD45 depletion cocktail, STEMCELL™ technologies). CTCs were collected at baseline and at the time of progression to AR-Signaling Inhibitors (ARSI), abiraterone and enzalutamide and were molecularly profiled by RNA-Sequencing. RNA-Seq raw reads were trimmed and aligned to the human reference genome (hg38). Differential gene expression was calculated using DESeq2 and subjected to negative-binomial models analyses to identify the differentially expressed genes using a False-Discovery Rate cutoff of FDR <0.05 to identify statistical significance. Gene Set Enrichment Analysis (GSEA) identified significant enrichment of TFRC (TfR encoding gene)-related upregulated pathways at disease progression (**Figure 1**). GSEA analyses also identieid significant enrichment of Rb loss gene expression signature pathway in patients who did not respond to treatment with ARSIs (**Figure 2**). Correlations between TFRC pathways and RB-loss pathways in relation to response/resistance to ARSI treatment are currently ongoing and planned in the lab. Taken together these preliminary results are support our working hypothesis that TfR expression and related pathways will be clinically meaningful and likely actionable for prostate cancer patients.

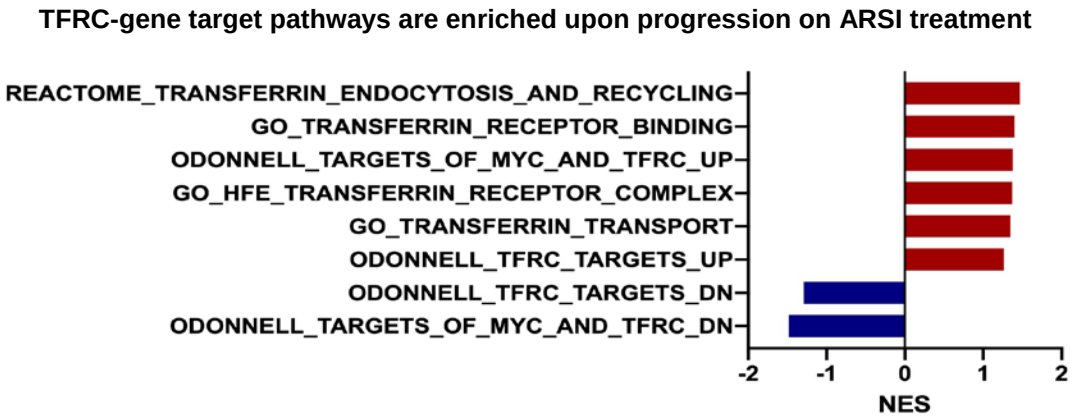


***Figure 1**. Gene Set Enrichment Analysis (GSEA) of CTCs isolate at progression over CTCs isolated at baseline from CRPC patients receiving ARSI treatment. TFRC-related pathways upregulated at progression are shown as red bars, while TFRC-related pathways downregulated at progression are shown as blue bars. NES: normalized enrichment score.*

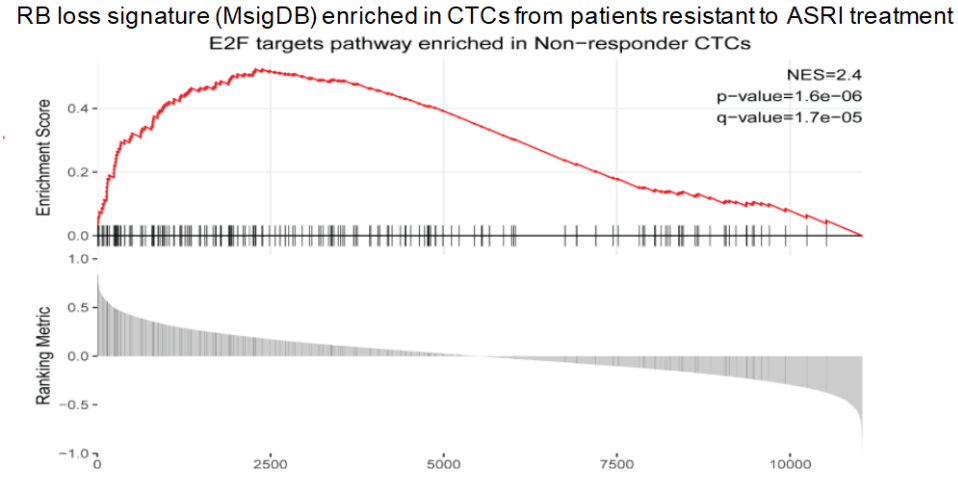


***Figure 2:** Gene Set Enrichment Analysis (GSEA) of CTCs enriched from patients with mCRPC receiving ARSI treatment, shows significant enrichment for RB loss pathway (MsigDB) in patients who did not respond to ASRI treatment.*

Specific Aim 2.

Characterize heterogeneity and clinical impact of AR-V expression, assessed by ddPCR, in single TFR^{+}- and EpCAM^{+}-CTCs from CRPC patients.

In this Aim, we first quantified the AR-V expression in pools of TFR^{+}- and EpCAM^{+}-CTCs from 20 patients with mCRPC by the AR-V ddPCR assay **(Figure 3).** The prevalence of both AR-V7 and AR-v567 was significantly lower in the EpCAM-positive fraction compared to the TFR-positive fraction, suggesting that EpCAM-based CTC enrichment does not capture the entire pool of CTCs, likely underestimating AR-variant prevalence with potentially important clinical implications. AR-V7 was expressed in 9/20(45%) TFR^{+}-CTCs and only in 7/20 (35%) EpCAM^{+} CTCs while ARv567es was expressed in 6/20(30%) TFR+-CTCs and only in 3/20 the EpCAM^{+}-CTCs. We observed no significant difference in the AR-FL prevalence between the two CTC subpopulations. (**Table 1**) Taken together these results indicate that CTC subpopulations have distinct AR-V expression pattern and that antigen agnostic CTC enrichment might provide information on a more comprehensive pool of CTCs which would be missed by antigen-specific enrichment methods.

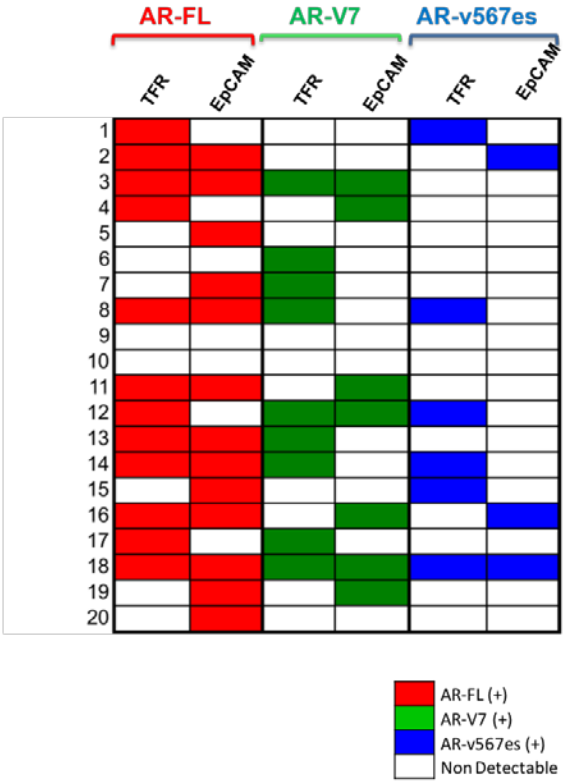


Figure 3. Expression of AR-FL, AR-V7 and AR-v567es in TFR^{pos} and EpCAM^{pos} CTC pools in patients with mCRPC *Pools of TFR+/CD45- or EpCAM+/CD45^{-} CTCs were isolated from 20 mCRPC patients and processed for quantification of each AR transcript by ddPCR. (AR-FL, red; AR-V7, green; AR-v567es, blue; not detected, white)*

Pooled CTCs from 20 patients

AR-FL		AR-V7		AR-v567es	
TFR+	EpCAM+	TfR+	EpCAM+	TfR+	EpCAM+
(12/20)	(13/20)	(9/20)	(7/20)	(6/20)	(3/20)
60%	65%	45%	35%	30%	15%

Table 1. Prevalence of each transcript in TFR+-CTC vs. EPCAM+-CTC pools

Prompted by the uneven AR-V expression in the pools of TFR^{+} vs EpCAM^{+} CTCs and given that intra tumoral heterogeneity is implicated in disease progression and treatment resistance, we investigated the **intra-patient heterogeneity analyzing AR-V expression in single CTCs**. Hence, we isolated 102 single TFR^{+}- and 63 single EpCAM^{+}-CTCs from 3 patients with mCRPC and quantified each transcript alone by the AR-V ddPCR assay. The single CTC analysis demonstrated an even more significant enrichment of AR-Vs in TFR+- vs EpCAM+-CTCs with AR-V7 and AR-v567es detected in 21% and 18% of TFR+-CTCs vs 0% of EpCAM+-CTCs, respectively. AR-FL expression frequency was similar between the two CTC immunophenotypes **(Table 2)**

Table 2. AR-V transcript prevalence in single TFR+-CTCs vs. single EPCAM+-CTCs.

Single CTCs from 3 patients

AR-FL		AR-V7		AR-v567es	
TFR+	EpCAM+	TfR+	EpCAM+	TfR+	EpCAM+
(8/34)	(5/21)	(7/34)	(0/21)	(6/34)	(0/21)
24%	24%	21%	0%	18%	0%

Single CTCs were isolated from 3 patients with mCRPC and processed for quantification of each AR transcript by ddPCR.

We also isolated and assessed the expression of AR-FL, AR-V7 and AR-v567es **in 159 and 51 single CTCs from mCRPC and NEPC patient samples**, respectively. 53 single mCRPC-CTCs and 17 single NEPC-CTCs were analyzed for each transcript alone. (**Table 3**) The analysis identified significant enrichment for AR-v567es in patients with neuroendocrine PC (NEPC) in 10/17 CTCs (59%) compared to mCRPC in 6/53 CTCs (11%), indicating a previously unrecognized role of AR-v567es in lineage plasticity, which warrants further mechanistic interrogation. Enrichment of AR-v567es expression in NEPC was further confirmed in NEPC organoids. We observed no significant difference in the AR-FL and AR-V7 prevalence between the two single CTC subpopulations from mCRPC and NEPC patients.

AR-FL+		AR-V7+		AR-v567es+	
CRPC	NEPC	CRPC	NEPC	CRPC	NEPC
14/53	2/17	7/53	0/17	6/53	10/17
26%	12%	13%	0%	11%	59%
n.s.		n.s.		p < 0.005	

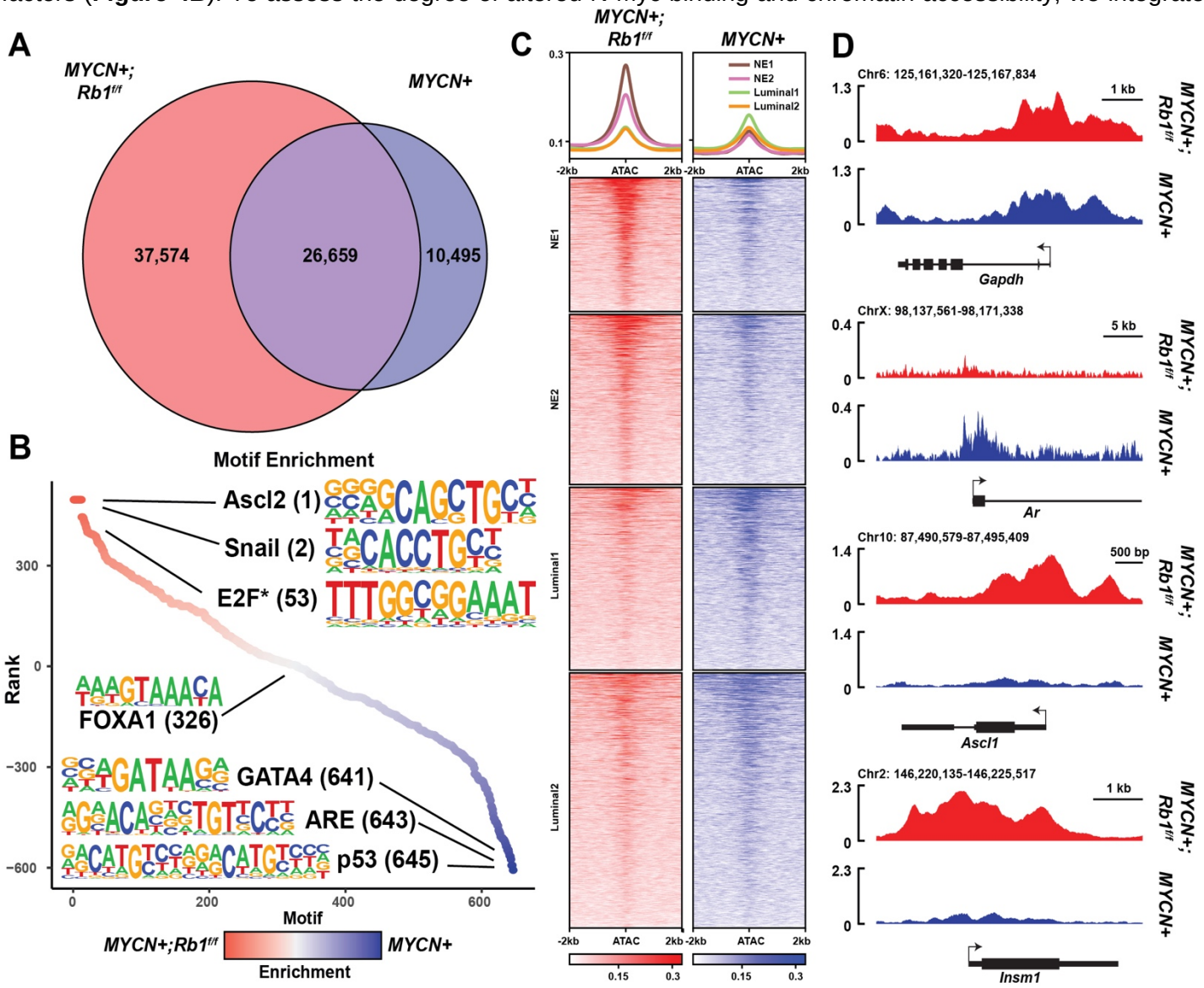
Table 3. AR transcript expression in single CTCs from patients with mCRPC vs. NEPC. *Prevalence of each transcript across single CTCs from patients with mCRPC vs. NEPC. Fisher Exact test, CRPC vs. NEPC, n.s.; not significant*

Specific Aim 3. Explore the functional relationship between TfR and Myc in patient-derived tumor and animal organoids and their impact on NEPC lineage plasticity.

In our preliminary data presented above (**Figure 2**), we identified enrichment for RB-loss pathway in CTCs from patients with mCRPC who did not respond to ARSI treatment and who also displayed enrichment in TFRC-pathways. To start exploring the functional relationship between TfR and Myc we took advantage of already established GEMs with MYCN overexpression in combination with loss of Rb1, to recapitulate the gene expression clinical associations between RB-loss, treatment resistance and disease progression, presented in Aim 1.

Based on single-cell based sequencing data from the GEMs, we observed that the overexpression of *MYCN* in combination with the loss of *Rb1* resulted in the rapid formation of aggressive, metastatic tumors with neuroendocrine-like features. Thus, we hypothesized that the loss of *Rb1* would synergize with *MYCN* overexpression to reshape the N-Myc cistrome and drive tumor progression. To address this, we performed N-Myc chromatin immunoprecipitation sequencing (ChIP-seq) on multiple, independent tumors collected from *MYCN*+ or *MYCN+;Rb1*$^{f/f}$ mice. Consistent with its role as a transcription factor, N-Myc binding was highly enriched near the transcription start site (TSS) of genes in both genotypes. We found 36,789 N-Myc peaks in *MYCN*+ tumors. Surprisingly, following the loss of *Rb1*, the N-Myc cistrome was dramatically expanded to 62,171 binding sites with nearly 60% of these sites corresponding to new binding sites not seen in *MYCN*+ mice alone

(**Figure 4A**). Additionally, GSEA of the *MYCN*+ and *MYCN+;Rb1*$^{f/f}$ unique peaks revealed distinct sets of genes bound by N-Myc between the conditions. To determine the transcriptional co-factors which may contribute to this differentially remodeled cistrome, we performed motif enrichment analysis between N-Myc peaks unique to the *MYCN*+ and *MYCN+;Rb1*$^{f/f}$ mice. While the most differentially enriched motifs in *MYCN*+ tumors consisted of TP53 and ARE motifs, *MYCN+;Rb1*$^{f/f}$ tumors were enriched for ASCL and SNAIL family motifs (**Figure 4B**). More strikingly, a high degree of enrichment was observed for a motif consistent with the E2F family of transcription factors (**Figure 4B**). To assess the degree of altered N-Myc binding and chromatin accessibility, we integrated



the scATAC-seq and the ChIP-seq data and restricted the analysis to regions of differential accessibility between subpopulations. In the presence of wild-type levels of *Rb1*, N-Myc was bound at regions of accessible chromatin associated with luminal populations (**Figure 4C**). In the context of *Rb1* loss, N-Myc binding was reduced in the luminal-specific regions and instead was dramatically enriched in regions which were accessible only in the neuroendocrine populations (**Figure 4C**). Specifically, we found that N-Myc binding was reduced at the *Ar* locus and redirected to neuroendocrine-associated genes, such as *Ascl1* and *Insm1*, in *MYCN+;Rb1*$^{f/f}$ tumors (**Figure 4D**). Importantly, the loss of *Rb1* appeared to remodel the N-Myc cistrome in a very specific manner, as binding at the *Gapdh* locus (a known N-Myc target gene) remained consistent between *MYCN*+ and *MYCN+;Rb1*$^{f/f}$ tumors (**Figure 4D**). Together, these data suggest that the loss of *Rb1* synergizes with N-Myc overexpression to redirect the N-Myc cistrome to new genomic loci to regulate gene expression, possibly with the help of ASCL- and E2F-family members.

Figure 4. The N-Myc cistrome is altered upon Rb1 loss. A. Venn diagram showing the overlap of N-Myc binding by ChIP-seq from *MYCN+;Rb1*$^{f/f}$ (n=3) and *MYCN*+ (n=2) tumors (peak cutoff: q < 0.0001). **B.** Differential transcription factor motif enrichment between N-Myc binding sites in *MYCN+;Rb1*$^{f/f}$ and *MYCN*+ tumors. **C.** Heatmaps of N-Myc binding in

MYCN+;Rb1$^{f/f}$ and *MYCN+* tumors at regions of accessible chromatin associated with neuroendocrine and luminal subpopulations by scATAC-seq in Figure 5C. **D.** N-Myc binding at indicated loci in *MYCN+;Rb1$^{f/f}$* (n=3) and *MYCN+* (n=2) tumors.

- **What opportunities for training and professional development has the project provided?**

Drs Giannakakou, Beltran, Tagawa and Rickman are fully committed to furthering the training and professional development of the postdoctoral fellows and students affiliated to this project. Due to the unexpected COVID-19 crisis we had limited opportunities to present our preliminary data. Parts of the data were presented at the scientific conferences and internal research progress meetings mentioned in the following section.

- **How were the results disseminated to communities of interest?**

Prostate Cancer Foundation, Carlsbad, CA (Annual Scientific Retreat, October 2019)

Annual Scientific Retreat

Belfer Basic Research Working Group, New York, NY (February 2020)

Internal research in progress meeting in the Meyer Cancer Center at Weill Cornell Medicine.

Multi-Institutional Prostate Cancer Program Retreat, Ft. Lauderdale, FL (March 2020)

Annual meeting of institutions with an NCI-funded P50 SPORE grant in prostate cancer research.

- **What do you plan to do during the next reporting period to accomplish the goals?**

In the next reporting period, we plan to continue our investigations in all 3 Aims. In **Specific Aim 1**, we will continue molecular profiling of CTCs enriched from patients with mCRPC and NEPC using RNA-Seq. We will isolate pools of TfR+ and EpcAM+ CTCs and perform differential gene expression and pathway analyses to identify genes/pathways that are significantly associated with clinical outcomes. We will correlate differentially expressed pathways with response to ARSI treatment, progression-free survival and overall survival. In addition, serum Chromogranin and NSA will be assessed for each NEPC patient/time point and correlated with CTC characteristics. In parallel with CTC RNA-Sequencing, a matching PBMC sample will be submitted for RNA-Seq as a germline control. We will use our established bioinformatics analysis pipeline which includes GSEA and pathway analyses. In **Specific Aim 2** we will expand the number of patients with mCRPC or NEPC and perform and single CTC analyses to determine the heterogeneity and clinical impact of AR-V expression using our established AR-V ddPCR assay. Along these lines we have a manuscript in revision, describing the analytical specificity, sensitivity and clinical performance of this assay. In **Specific Aim 3** we will determine if C-Myc and N-Myc are bone fide regulators of TFRC expression and will also determine the impact of TFRC depletion on the landscape of N-Myc binding, N-Myc target genes and associated epigenomic alterations during the transformation from CRPC to NEPC phenotype.

4. IMPACT

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

  Nothing to Report

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

  Nothing to Report

- **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:

Nothing to Report

6. PRODUCTS:

Nothing to Report