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14. ABSTRACT
Mortality from prostate cancer (PC), an estimated 33,330 deaths in 2020, is associated with development of aggressive and treatment-insensitive metastatic castration-resistant prostate cancer (mCRPC). We will investigate the status and role of Y chromosome (ChrY) genes in regulating drug sensitivity and mCRPC development and progression. Though ChrY loss in men is associated with increased risk of disease and mortality, the role of ChrY genes in regulating PC progression is poorly understood. To investigate the clinical impact of ChrY gene expression, we developed new methodology to analyze mutational variants of ChrY genes in PC patient cohorts, previously unsuccessful due to the high number of repetitive sequences and paralog families. Using a custom reference for each paralog family, our method increased ChrY read depth coverage to be on par with whole-exome sequencing allowing for normal/tumor variant calling. We also generated the first CRISPR/Cas9 library targeting human ChrY to further understand the role of individual ChrY genes in regulating antiandrogen treatment sensitivity and mCRPC development in PC models in vitro and in vivo. This multifaceted approach will potentially identify predictive markers for treatment sensitivity based on ChrY. These markers will allow for development of tailored therapies and serve as targets for drug development.

15. SUBJECT TERMS
Metastatic castration-resistant prostate cancer, Y chromosome, antiandrogen therapy, epigenetics, biomarkers, tumor suppressor, mutations, CRISPR/Cas9 library screening

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MUTATIONAL LANDSCAPE OF THE Y CHROMOSOME AND PROSTATE CANCER

1. INTRODUCTION

Prostate cancer (PC) is the second most common cancer and second leading cause of cancer death among men in the United States with an estimated 33,330 deaths in 2020. PC associated mortality is attributed to the development of metastatic castration-resistant prostate cancer (mCRPC) which is characterized by its aggressiveness and poor response to treatment. Though loss of the Y chromosome (ChrY) in men has been associated with increased risk of disease and mortality, the role of ChrY genes in disease progression is poorly understood (Dumanski et al., 2016; Forsberg, 2017; Noveski et al., 2016). Our team presented the first report of a ChrY gene, *KDM5D*, which regulates tumor growth and docetaxel sensitivity through epigenetic modification of key cell cycle regulators and androgen receptor signaling (Komura et al., 2016; Komura et al., 2018). The study also reported the loss of *KDM5D* to be associated with increased mortality and aggressive disease in patient cohorts suggesting its role as a potential biomarker for mCRPC. Together, these studies highlight the urgency to further explore the role of ChrY genes in PC progression and further determine its mutational landscape to develop therapeutic targets as well as biomarkers and gene expression signatures which will allow physicians to predict drug response in patients and thereby prescribe effective treatment regiments. This multidisciplinary approach will help determine the clinical impact of ChrY genes on PC progression and treatment resistance.

2. KEYWORDS: Prostate cancer, metastatic castration-resistant prostate cancer, Y chromosome, antiandrogen therapy, drug insensitivity, docetaxel, epigenetics, biomarkers, tumor suppressor, precision medicine, mutations, CRISPR/Cas9 library screening

3. ACCOMPLISHMENTS

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

The major goals of the project as outlined in the statement of work (SOW) are:

SPECIFIC AIM 1: To determine the mutational landscape of the Y chromosome (ChrY) in men with prostate cancer in the SU2C/PCF, TCGA, and other cohorts

Major Task 1: Structural analysis of the Y chromosome (ChrY). This goal is 100% complete, in accordance with the SOW (1–36 months, responsible PIs and sites: Schultz, Memorial Sloan Kettering Cancer Center [MSK]; Van Allen, Dana-Farber Cancer Institute [DFCI]).

Major Task 1, Subtask 1: Identify the samples with ChrY loss. This goal is 100% complete, in accordance with the SOW (1–36 months, responsible PIs and sites: Schultz, MSK; Van Allen, DFCI).

Major Task 1, Subtask 2: Quantify the focality of ChrY loss. This goal is 100% complete, in accordance with the SOW (1–36 months, responsible PI and site: Schultz, MSK).

Major Task 1, Subtask 3: Assess mutual exclusivity of ChrY loss with genomic lesions in prostate cancer pathways. This goal is 100% complete, in accordance with the SOW (1–36 months, responsible PI and site: Schultz, MSK).

Major Task 1, Milestones: Define the extent of ChrY loss in metastatic prostate cancer and evaluate the association with clinically actionable signaling pathways. This goal is 100% complete, in accordance with the SOW (At 36 months).

Major Task 2: Determine functional features associated with ChrY mutations. This goal is 100% complete, in accordance with the SOW (1–36 months, responsible PI and site: Schultz, MSK).

Major Task 2, Subtask 1: Identify the putative tumor suppressors that are inactivated on ChrY. This goal is 70% complete, in accordance with the SOW (1–36 months, responsible PI and site: Schultz, MSK).

Major Task 2, Subtask 2: Assess differential AR activity between samples that show ChrY loss and samples without alterations on ChrY. This goal is 50% complete, in accordance with the SOW (1–36 months, responsible PI and site: Schultz, MSK).

Major Task 2, Subtask 3: Correlation of ChrY loss with Gleason score and sample type. This goal is 100% complete, in accordance with the SOW (1–36 months, responsible PI and site: Schultz, MSK).

Major Task 2, Milestones: Define the mutational landscape of ChrY and determine if the LOY is significantly associated with disease risk. This goal is 100% complete, in accordance with the SOW (At 36 months).

SPECIFIC AIM 2: To perform genetic screening by CRISPR to identify ChrY genes that are of importance in the development of castration-resistant prostate cancer (CRPC) or resistance to androgen receptor (AR)–targeted therapies.

Major Task 3: Forward genetic screening of ChrY genes. This goal is 100% complete, in accordance with the SOW (1–12 months, responsible PIs and sites: Kantoff and Schultz, MSK).

Major Task 3, Subtask 1: Establish barcoded cell line model systems. Cell lines used: LNCaP, VCaP, LAPC4, LNCaP-Abl, and RWPE-1 (Kantoff Lab). This goal is 50%

complete, in accordance with the SOW (1–12 months, responsible PI and site: Kantoff, MSK).

Major Task 3, Subtask 2: Construct and optimize ChrY CRISPR/Cas9 library. This goal is 100% complete, in accordance with the SOW (1–12 months, responsible PI and site: Kantoff, MSK).

Major Task 3, Subtask 3: Screening of ChrY CRISPR/Cas9 library. Cell lines used: LNCaP, VCaP, LAPC4, LNCaP-Abl, and RWPE-1 (Kantoff Lab). This goal is 100% complete, in accordance with the SOW (1–24 months, responsible PI and site: Kantoff, MSK).

Major Task 3, Subtask 4: Sequencing analysis of sgRNAs and barcodes to identify target genes. This goal is 100% complete, in accordance with the SOW (1–24 months, responsible PIs and site: Kantoff and Schultz, MSK).

Major Task 3, Milestones: Identify ChrY candidate genes that are of importance for development of CRPC and drug resistance and generate hypothetical models for further functional validations. This goal is 100% complete, in accordance with the SOW (At 24 months).

SPECIFIC AIM 3: To characterize the functional significance of genes involved in resistance in cell culture and animal models. We will confirm the functional importance of KDM5D and UTY in progression to CRPC. The clinical significance of these genes will be corroborated with an evaluation of the ChrY landscape in prostate cancer specimens.

Major Task 4: Perform functional validation of candidate genes including KDM5D and UTY in cell line models. Cell lines used: LNCaP, VCaP, LAPC4, LNCaP-Abl, RWPE-1, LNCaP-104R2, CWR22RV1, DU145, PC3 and corresponding target gene knockdown or over-expression cell lines. This goal is 50% complete, in accordance with the SOW (1–36 months, responsible PIs and sites: Kantoff and Schultz, MSK; Gerke, Moffitt Cancer Center [MCC]).

Major Task 4, Subtask 1: Apply specific gene silencing and/or over-expression (as relevant) in a broader panel of prostate cancer cell lines to assess the impact of the expression of a specific candidate gene on cell growth, invasiveness, and drug sensitivities with or without androgen treatment. This goal is 80% complete, in accordance with the SOW (1–36 months, responsible PI and site: Kantoff, MSK).

Major Task 4, Subtask 2: To identify the pathways/mechanisms that a specific gene involved in leading to the observed phenotypes by RNA-seq, ChIP-seq, or phospo-kinase screening. This goal is 0% complete, in accordance with the SOW (1–36 months, responsible PIs and site: Kantoff and Schultz, MSK).

Major Task 4, Milestones: Determine molecular mechanisms/pathways underpinning the involvement of ChrY genes in prostate cancer progression This goal is 50% complete, in accordance with the SOW (At 36 months).

Major Task 5: Perform functional validation of candidate genes in mouse xenograft model. This goal is 0% complete, in accordance with the SOW (1–36 months, responsible PI and site: Kantoff, MSK).

Major Task 5, Subtask 1: Generate mouse xenografts using stable cell lines with inducible knockdown or overexpression of candidate genes. This goal is 0% complete, in accordance with the SOW (1–36 months, responsible PI and site: Kantoff, MSK).

Major Task 5, Subtask 2: Treat mouse xenografts with drug or vehicle and measure tumors. 10 NOD/SCID IL-2 gamma null mice will be used in each experimental or control arm [10 mice per group X 2 groups = 20 mice per experiment]; the number of experiments will be determined by the number of candidate genes identified in Specific Aim 2. This goal is 0% complete, in accordance with the SOW (1–36 months, responsible PI and site: Kantoff, MSK).

Major Task 5, Milestones: Determine the impact of gain and loss of a specific candidate gene on tumor growth in the absence of androgen or in response to a specific drug treatment in vivo. This goal is 0% complete, in accordance with the SOW (At 36 months).

Major Task 6: Clinical validation of the role of target genes on PC progression within PC cohorts. This goal is 25% complete, in accordance with the SOW (12–36 months, responsible PIs and sites: Kantoff and Schultz, MSK; Gerke, MCC).

Major Task 6, Subtask 1: Assess the clinical impact of KDM5D and UTY expression on prostate cancer outcomes in four different cohorts. This goal is 70% complete, in accordance with the SOW (12–24 months, responsible PIs and sites: Kantoff and Schultz, MSK; Gerke, MCC).

Major Task 6, Subtask 2: Validate the clinical impact of newly identified target genes on prostate cancer outcomes in four different cohorts. This goal is 25% complete, in accordance with the SOW (24–36 months, responsible PIs and sites: Kantoff and Schultz, MSK; Gerke, MCC).

Major Task 6, Milestones: Characterization of ChrY-associated genes that impact prostate cancer clinical outcome in the context of treatment with androgen deprivation therapy or AR-targeted drugs. This goal is 25% complete, in accordance with the SOW (At 36 months).

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

Major progress has been made towards the aims outlined in the original application, following the timeline indicated in the SOW (1-36 months).

SPECIFIC AIM 1: To determine the mutational landscape of the Y chromosome (ChrY) in men with prostate cancer in the SU2C/PCF, TCGA, and other cohorts

Major Activities

The analysis of the landscape of somatic copy number alterations and somatic mutations of the ChrY in prostate cancer were supervised by the Schultz/Van Allen groups at MSK and DFCI, respectively. To circumvent the problem of numerous paralogs on ChrY, we first developed a new method to specifically call mutations in paralogous genes, allowing us to map the landscape of mutations on the ChrY. We then switched our focus from mutation calling to determining copy-number alterations, which was another challenge on ChrY due to the fact that there is only a single copy, and all existing copy number methods were written for chromosomes that exist in two copies. While the methods are now complete, we have yet to run them on a larger cohort. We will submit a 12-month no-cost extension (NCE), with a proposed plan to next run the pipeline on a combined whole-exome sequencing (WES) cohort that contains >1,500 tumor samples from various sources.

Specific Objectives

The specific objectives proposed in the SOW were to 1) identify the samples with ChrY loss 2) quantify the focality of ChrY loss, 3) assess mutual exclusivity of ChrY loss with genomic lesions in prostate cancer pathways, 4) identify the putative tumor suppressors that are inactivated on ChrY, 5) assess differential AR activity between samples that show ChrY loss and samples without alterations on ChrY, 6) correlate ChrY alterations with Gleason score and sample type, and 7) define the mutational landscape of ChrY and determine if loss of ChrY is significantly associated with disease risk.

Significant Results or Key Outcomes

FacetsY, an extension of the allele-specfic copy number analysis tool FACETS (Fraction and Allele-Specific Copy Number Estimates from Tumor Sequencing) was used to investigate ChrY loss in prostate cancer samples (Shen et al., 2016). In order to methodologically justify the utilization of FacetsY, we compared the segmentation profiles of 333 primary prostate cancer samples from The Cancer Genome Atlas (TCGA) that were obtained by FacetsY and Affymetrix Genome-Wide Human 6.0 Array data. Breakpoint locations as well as signal intensities obtained from both methods were highly similar (**Figure 1A**). Moreover, chromosome arm alteration (defined as >80% of respective chromosome arm length with an absolute segmentation mean (value >0.2) frequencies showed a high concordance (**Figure 1B**). While previous reports on MSK patients with prostate cancer exclusively relied on MSK-IMPACT panel sequencing data, we now have extended our analysis to include whole-exome recapture sequencing data on 238 prostate cancer samples. Whole-exome sequencing (WES) provides a more continuous and granular view on the ChrY, as most of the protein coding genes are

covered, and hence, a comprehensive deduction of the integrity of the ChrY is possible (**Figure 1B**).

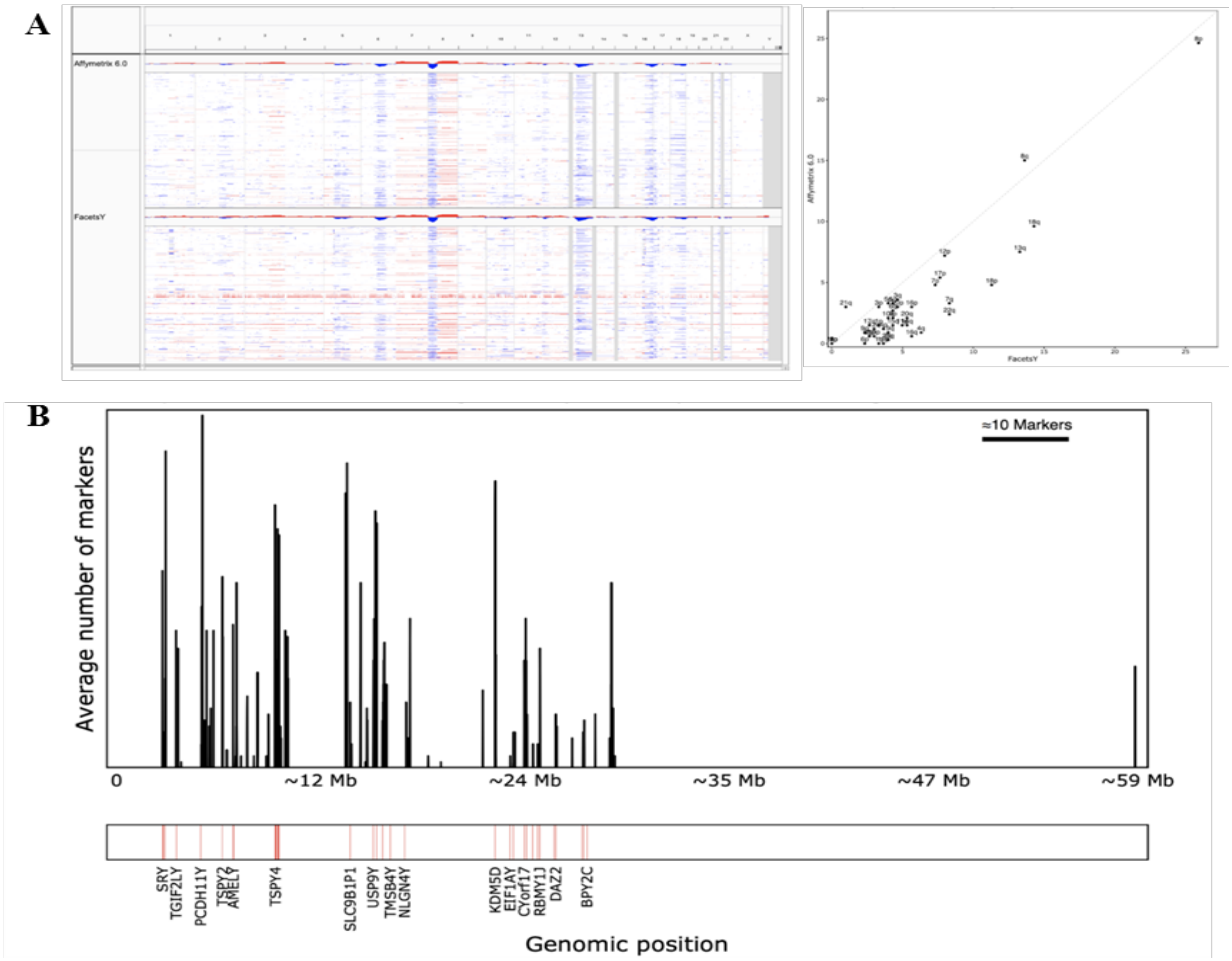


Figure 1. Copy number analysis of Y chromosome. A) Comparison of continuous segmentation profiles obtained by Affymetrix Genome-Wide Human 6.0 Array and FacetsY on prostate cancer samples from TCGA (n = 333). FacetsY is a reliable tool for copy number profiling and deciphers the overall SCNA profile in TCGA-PRAD samples. The right panel shows the relationship between chromosome arm alteration frequencies called in either Affymetrix Genome-Wide Human 6.0 Array (y-axis) or FacetsY (x-axis). FacetsY was run on 333 WES TCGA-PRAD samples (default parameters) and recovers chromosome arm alteration frequencies, similar to those called by Affymetrix 6.0. **B)** WES data provide sufficient information for the assessment of Y chromosome status in individual patients with prostate cancer. Peaks along the x-axis depict the average number of markers in given bins (1 bin = 1 Kb), which were retained for segmentation analysis using FacetsY. Red bars in the bottom annotation lane highlight protein coding genes where putative losses can directly be assessed, as the marker density is adequate for predictions. PRAD, prostate adenocarcinoma; SCNA, somatic copy number aberration; TCGA, The Cancer Genome Atlas; WES, whole-exome sequencing.

Integer copy number calls obtained from FacetsY were used to determine the presence of complete chromosome loss. We defined a sample to have complete chromosome loss if a chromosome had greater than 50% of loss (defined as total copy number [TCN] = 0). Due to an integer copy number transformation (using the median copy number log ratio of respective called segments), a direct comparison between the two sequencing approaches was enabled. We obtained a strong linear relationship ($r = 0.834$; $p < 2.2 \times 10^{-16}$) on a per-sample

basis, suggesting that the MSK-IMPACT sequencing platform provides sufficient information to accurately call ChrY losses (**Figure 2A**). In addition, we investigated genomic correlates with ChrY loss. A total of 133 patients with available WES data and clinical annotations were included (one sample selected per patient), out of which 23% of patients (n = 30) had ChrY loss and 77% of patients (n = 103) had an intact ChrY (**Figure 2B**). While fraction genome altered (FGA) along with chr16q and 13q alterations portend to a greater likelihood of ChrY loss, none of the mentioned variables showed significance (**Figure 2C**).

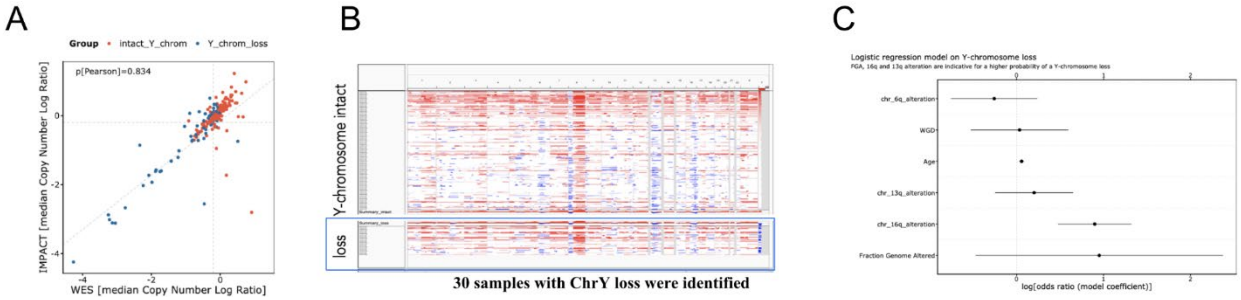


Figure 2. Targeted sequencing provides sufficient information to call Y chromosome (ChrY) loss. A) MSK-IMPACT (Memorial Sloan Kettering-Integrated Mutation Profiling of Actionable Cancer Targets) panel sequencing provides reliable data for ChrY copy number calling. FacetsY was used in a cohort of 238 patients with prostate cancer whose MSK-IMPACT and WES data were both available. Binary ChrY calls of both sequencing strategies were transferred into a continuous scale and compared against each other. Blue dots represent samples with ChrY loss; whereas, red denotes an intact ChrY. B) Segmentation profiles of the two respective groups. C) A logistic regression model suggests that fraction genome alteration (FGA) and specific chromosome arm alteration may be indicative for a ChrY loss in prostate cancer.

To explore the association of known clinical features according to ChrY loss, we examined several features such as sample type, tissue localization, Gleason grade, disease extent, and prostate-specific antigen (PSA) (**Figure 3A**). We did not observe any significant association with any of the above clinical features. In addition, we also performed a genomic analysis to compare the intact ChrY group with the ChrY loss group, which demonstrated that ChrY loss was associated with a slightly higher FGA ($p = 0.05$); however, it was not associated with tumor mutation burden (TMB) (**Figure 3B**).

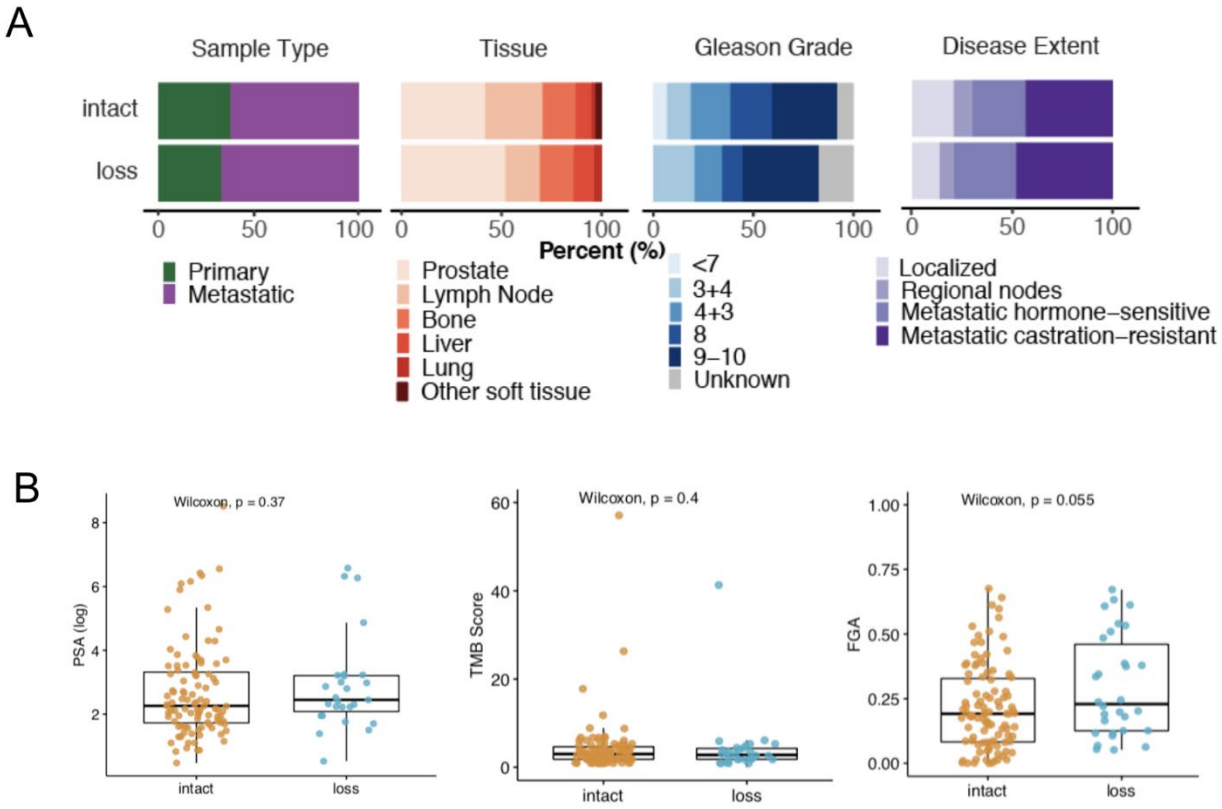


Figure 3. Clinical and pathological associations with Y loss. A) Comparison of categorical clinical features, such as sample type, tissue localization, Gleason grade, and disease extent, from patients in the intact Y chromosome (ChrY) versus ChrY loss groups. No significant differences were found between the groups. B) Comparison of continuous clinical variables, such as prostate-specific antigen (PSA), tumor mutation burden (TMB), and fraction genome alteration (FGA) in the intact ChrY versus loss of ChrY groups. No significant differences were found between the groups, but there was an association of FGA with the ChrY loss group (p = 0.055).

To evaluate the differences in prevalence of genomic alterations by ChrY loss status, we selected for gene alterations annotated as oncogenic or likely oncogenic function based on the curated precision oncology database OncoKB, data version 2.8 (September 17, 2020). We found that samples in the ChrY loss group had a slightly higher frequency of *RB1*, *ZFHX3*, *CCNE1*, *CHEK2*, and *FLT3* alterations (p <.05), however, these association were not significant after correcting for multiple testing **(Figure 4A**). We also analyzed the prevalence of 11 oncogenic signaling pathways (cell cycle, Myc, Hippo, Notch, PI3K, TGF-Beta, WNT, p53, Nrf2, RTK-RAS, and epigenetic pathways (Sanchez-Vega et al., 2018)). We did not observe any significant difference based on pathway level alterations between the two groups (**Figure 4B**).

We interrogated the association between chrY loss status and overall survival (OS) of patients with prostate cancer. For OS, follow-up started at the time of sequencing and ended with patient death, with censorship occurring at the last patient contact. Patients with chrY loss had a shorter median OS (36.4 months) than those with intact ChrY (44.3 months). This difference was significant in an unadjusted model (hazard ratio [HR]: 1.9, 95% CI, 1-3.5; p =

0.04). However, when we accounted for a known prognostic factor (such as FGA), the OS trend remained the same, but was no longer statistically significant. (HR: 1.3, 95% CI, 0.69-2.5; $p = 0.49$) (**Figure 4C**). All analyses were performed using R version 3.5.2 (www.R-project.org).

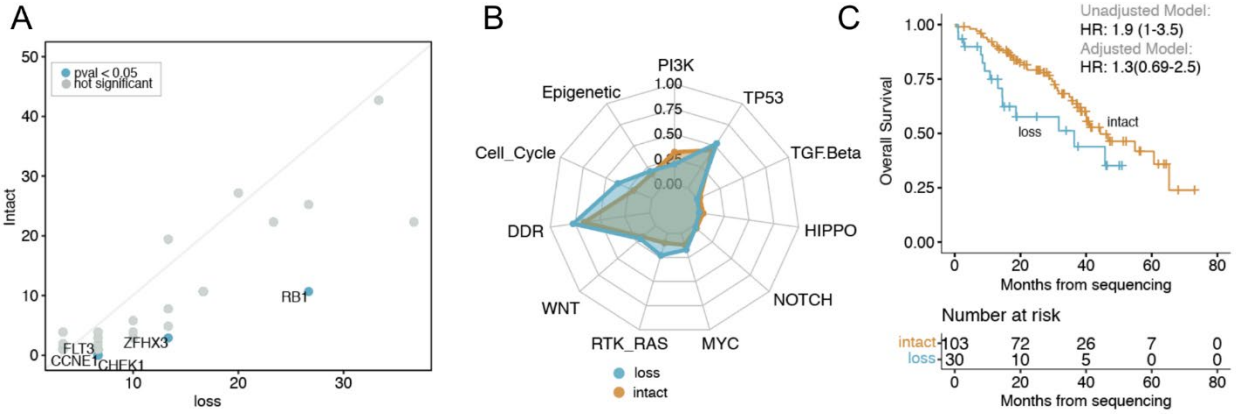


Figure 4. Genomic and overall survival analyses of samples with ChrY loss in an MSK-IMPACT WES recapture cohort. A) Gene enrichment analysis between intact ChrY versus ChrY loss groups. Samples with ChrY loss were more frequently observed with alterations in *RB1*, *ZFHX3*, *CCNE1*, *CHEK2*, and *FLT3* than those with intact ChrY (not significant when corrected for multiple testing). B) Oncogenic pathway level alteration comparison between intact ChrY versus ChrY loss groups using curated pathways templates. No significant pathway level enrichment between the two groups were observed. C) Kaplan-Meier overall survival curve of patients harboring chrY loss versus those with intact ChrY. Kaplan-Meier curve showing unadjusted and adjusted hazard ratios (HRs), with 95% confidence intervals estimated using Cox proportional hazard models. ChrY, Y chromosome; WES, whole-exome sequencing.

Other Achievements

Nothing to Report

SPECIFIC AIM 2: To perform genetic screening by CRISPR to identify ChrY genes that are of importance in the development of castration-resistant prostate cancer (CRPC) or resistance to androgen receptor (AR)–targeted therapies.

Major Activities

Genetic screening by CRISPR was conducted by the Kantoff group at MSK. Sequencing analysis of sgRNA and barcodes to identify target genes were conducted by the Kantoff and Schultz groups. As proposed in the SOW (1-24 months), we have completed the positive genetic screens for identifying genes regulating enzalutamide sensitivity.

Specific Objectives

The objectives proposed in the SOW were to: 1) establish barcoded cell line model systems; 2) design and construct ChrY CRISPR/Cas9 library; 3) optimize the ChrY CRISPR/Cas9 library in target cell lines; and 4) conduct positive selection screens with the ChrY

CRISPR/Cas9 library to identify genes responsible for mCRPC development and antiandrogen resistance.

Significant Results or Key Outcomes

We have successfully generated the first, to our knowledge, ChrY-targeting CRISPR/Cas9 library. The pooled library is constructed to be used in a lentiviral system allowing high transduction efficiency. The CRISPR library (**Figure 5A**) contains 4 sgRNAs/gene and is targeting 45 protein coding genes, 53 non-coding genes, and 188 pseudogenes. In addition, we have included 150 AASV1 (Adeno-Associated Virus Integration Site 1) controls, 150 negative controls, and 72 positive controls, resulting in a total of 1,519 sgRNAs.

LNCaP, LNCaP-Abl, and RWPE-1 cell lines expressing the ChY CRISPR/Cas9 library were created (**Figure 5B**). LNCaP-Abl and RWPE-1 cell lines were used as controls for enzalutamide-insensitive cell lines. Following enzalutamide treatment, the surviving population was analyzed to identify populations with sgRNA enrichment. Enrichment and depletion at the sgRNA and gene level were determined using the MagecK algorithm (version 0.5.6) (Li et al., 2014).

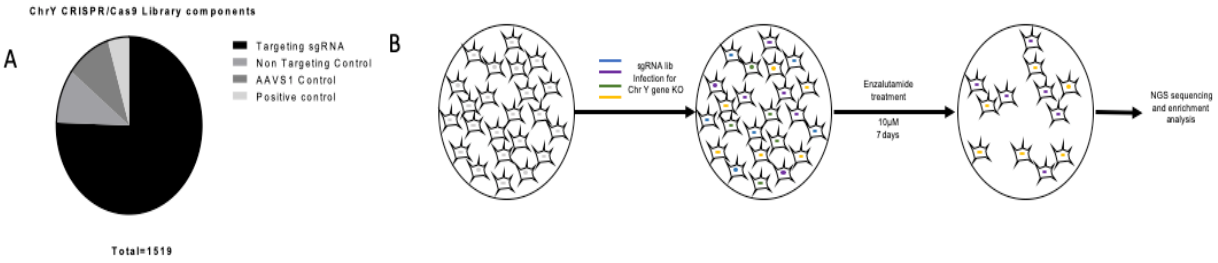


Figure 5. ChrY-targeting CRISPR/Cas9 library generation and experimental design. A) Library composition. B) Protocol for generating cell lines expressing ChrY-targeting CRISPR/Cas9 library. AAVS1, Adeno-Associated Virus Integration Site 1; ChrY, Y chromosome; NGS, next generation sequencing; sgRNA, single guide RNA.

The gene targets obtained were *TMSB4Y*, *DDX3Y*, *TTTY17C*, *TTTY6*, *GPR143P*, and *ZNF736P9Y*. The protein coding gene *TMSB4Y* was excluded from further analysis, as it was not expressed in prostate cancer cell line systems. *TTTY17C* and *TTTY6* genes were excluded from the analysis, as their expression profiles could not be discerned from overall long non-coding RNA (lncRNA) family expression.

The protein coding gene, *DDX3Y*, and pseudogenes, GPR143P and ZNF736P9Y sgRNA, were significantly enriched in enzalutamide-treated samples compared to control samples in castration conditions, indicating their loss conferred a growth advantage under enzalutamide treatment (**Figure 6**).

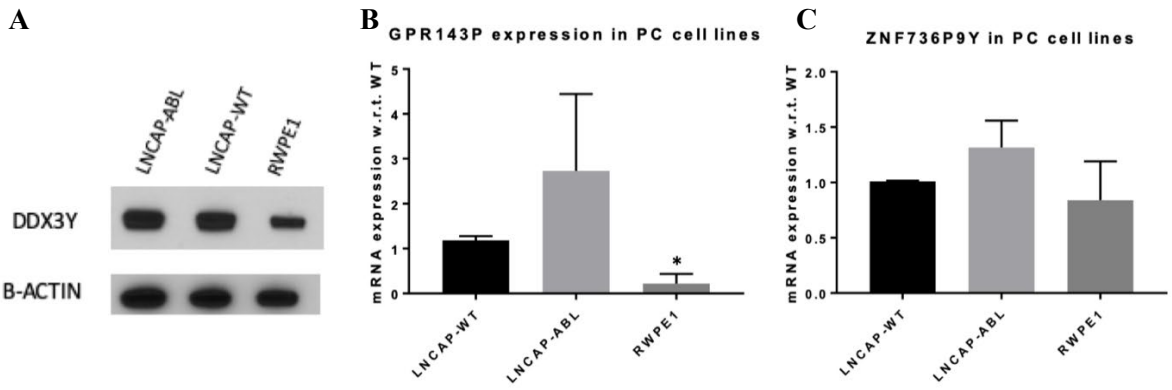


Figure 6. Target gene expression in prostate cancer (PC) cell line models. A) DDX3Y baseline expression in LNCAP-ABL, LNCAP-WT was analyzed using immunoblotting. B) GPR143P expression in prostate cancer cell line models. *GPR143P baseline expression was significantly lower in RWPE-1 cell line model compared to LNCAP-WT cell line model ($P \leq 0.05$). C) ZNF736P9Y expression in prostate cancer cell line models.

Other Achievements

In addition to spectral karyotyping in target cell lines, we also conducted XY FISH (fluorescence in situ hybridization) on patient-derived prostate cancer organoids (Gao et al., 2014). All organoids were derived from metastatic prostate cancer tissue and cultured according to conditions outlined by Gao et al (2014). We screened 13 patient-derived prostate cancer organoid cultures. The FISH probe system marked the X chromosome as well as the euchromatic Yp (short arm) and heterochromatic Yq (long arm) regions of the ChrY with orange, green, and red fluorochromes, respectively (**Figure 7**). Using this system, we were able to identify prevalent ChrY loss in organoids, presenting the first report on the status of ChrY in patient-derived prostate cancer organoids.

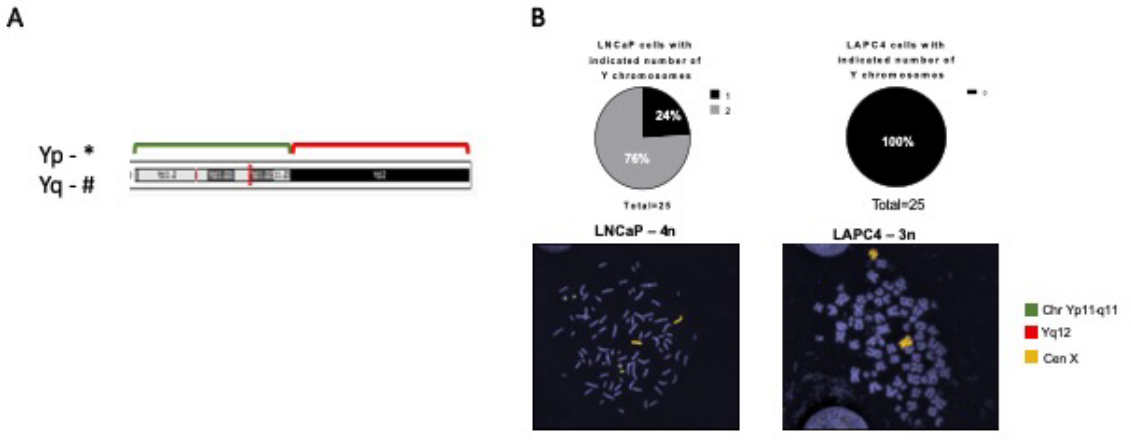


Figure 7. XY paint probe design and validation. A) Chr Y probe design. B) FISH probe validated in prostate cancer cell lines. LNCaP-WT cells had heterogenous ChrY status. LAPC4 cells, which do not have ChrY, showed no signal with the probe. ChrY, Y chromosome; FISH, fluorescence in situ hybridization; WT, wildtype.

Six out of 13 (46.2%) organoid samples were heterogenous in terms of ChrY status. We classified all organoid samples with >40% ChrY or Yq loss as having ChrY or Yq loss, respectively. Using this cutoff to classify chromosomal loss, approximately 30% of the tested organoid samples were classified with loss of the ChrY, indicating a potential role for the anomaly in disease progression. Interestingly, Yq loss was observed in 38.5% of our sample set, occurring at a more frequent rate than total ChrY loss (**Figure 8**).

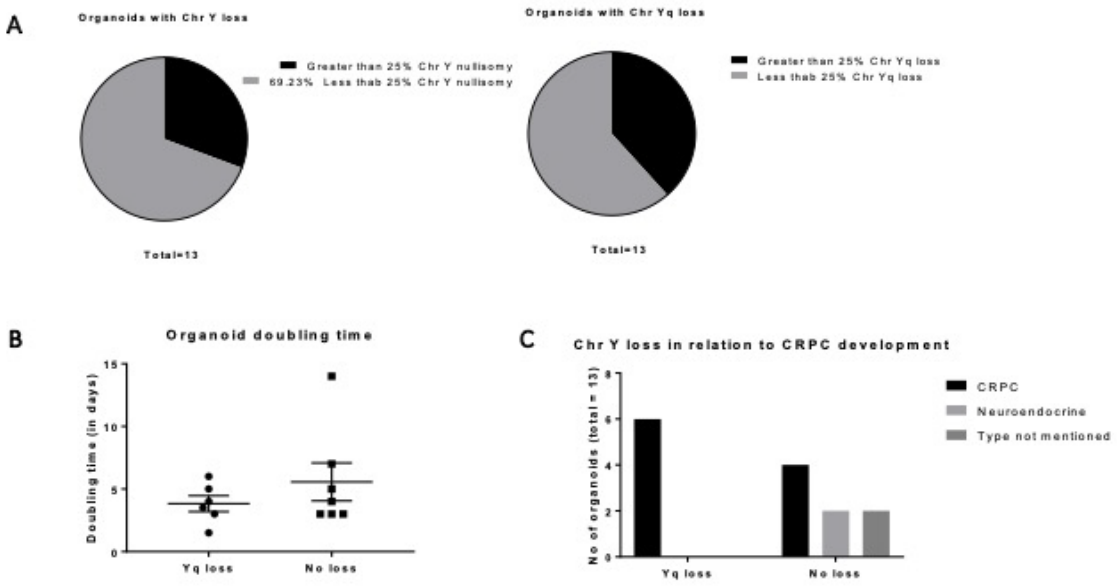
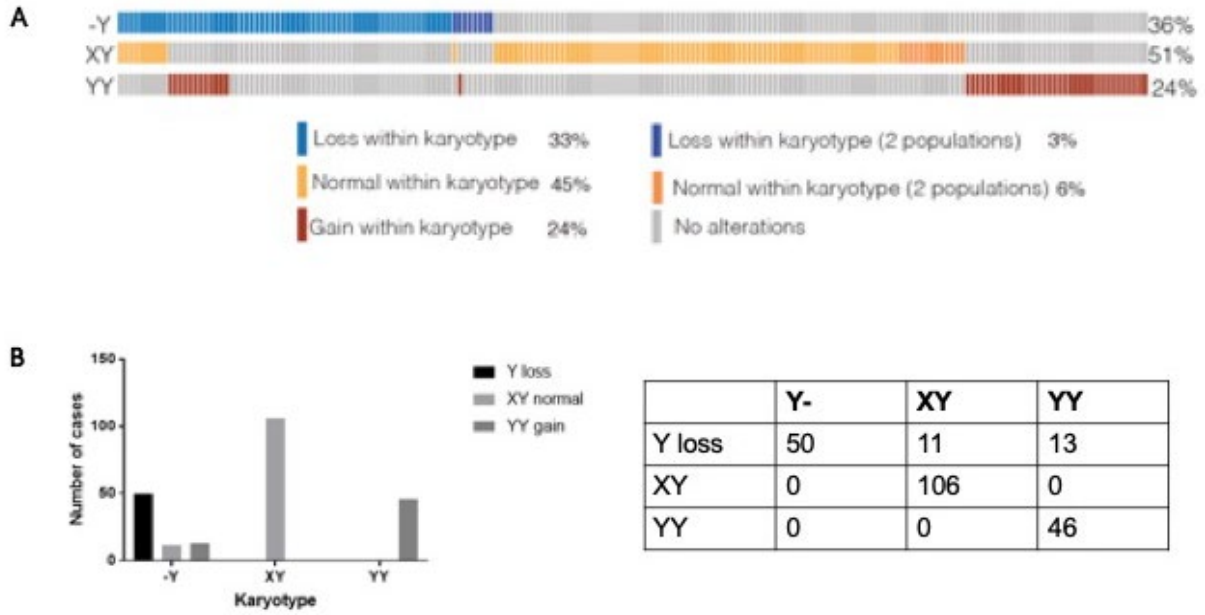


Figure 8. Characteristics of prostate cancer organoids. A) Percentages of prostate cancer organoids with ChrY and Yq loss. B) Doubling times of prostate cancer organoids. C) Breakdown of prostate cancer organoids and cancer tissue type used to derive the organoids.

We also queried Mitelman's database to analyze the status of ChrY loss in primary prostate adenocarcinoma samples. Interestingly, ChrY loss occurred at 36% frequency in primary prostate samples (**Figure 9A**). Additionally, heterogeneity within prostate cancer samples in terms of ChrY status was observed only in samples with ChrY loss and not in patients without loss, indicating that ChrY loss could be an indicator for genomic instability in prostate cancer (**Figure 9B**).



	Y-	XY	YY
Y loss	50	11	13
XY	0	106	0
YY	0	0	46

Figure 9. Y chromosome (ChrY) status. A) ChrY status in primary prostate cancer samples. B) ChrY heterogeneity in primary prostate adenocarcinoma samples. Chi-square test reporting significance.

SPECIFIC AIM 3: To characterize the functional significance of genes involved in resistance in cell culture and animal models. We will confirm the functional importance of *KDM5D* and *UTY* in progression to CRPC. The clinical significance of these genes will be corroborated with an evaluation of the ChrY landscape in prostate cancer specimens.

Major Activities

As per the timeline in the SOW, work on Specific Aim 3 began in month 24, following identification of candidate genes from Specific Aim 2.

Specific Objectives

The specific objectives described in the SOW were to: 1) perform functional validation of candidate genes including KDM5D and UTY in cell lines models; 2) identify pathways/mechanisms that a specific gene is involved in leading to the observed phenotype by RNA-seq, CHIP-seq, or phosphor-kinase screening; 3) generate mouse xenografts using stable cell lines with inducible knockdown or overexpression of candidate genes and treat them with drug or vehicle to measure tumors followed by molecular characterization, and 4) assess the clinical impact of KDM5D, UTY, and candidate gene expression on prostate cancer outcomes in clinical datasets from the following patient cohorts: Stand Up To Cancer/Prostate Cancer Foundation (SUC2/PCF), TCGA, Harvard Prostate Tumor (Health Professionals Follow-Up Study [HPFS] and Physician's Health Study [PHS]) study cohorts.

Significant Results or Key Outcomes

Gene targets *DDX3Y*, *GPR143P*, and *ZNF736P9Y* were selected for functional analysis studies. Small interfering RNA (siRNA)-mediated knockdown in LNCaP cells resulted in ~50% silencing (**Figure 10**). However, this silencing did not result in any change in short-term growth.

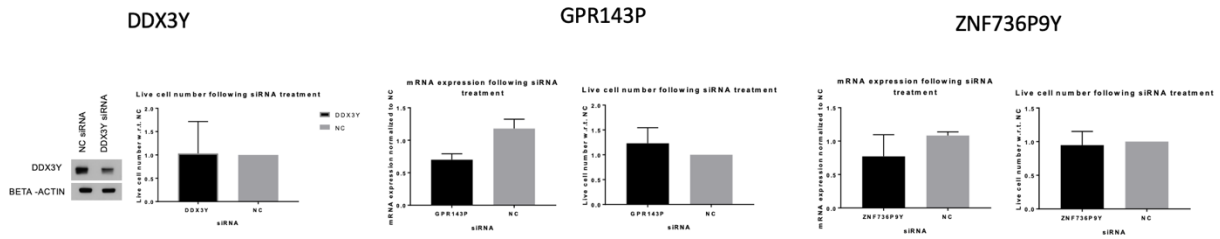


Figure 10. DDX3Y, GPR143P, and ZNF736P9Y were identified from the CRISPR screen. Silencing expression using siRNA did not result in decreased cell growth at 48 hours.

Short hairpin RNA (shRNA)-mediated knockdown of *DDX3Y* was achieved using two constructs, DD6 and DD7. DD6 was used for functional studies, as it resulted in greater knockdown. *DDX3Y* knockdown rescued cells from enzalutamide-mediated growth inhibition when stimulated with dihydrotestosterone (DHT; **Figure 11A**). *DDX3Y* knockdown also resulted in increased basal expression of PSA in LNCaP cells compared to control (**Figure 11B**). DHT stimulation coupled with enzalutamide treatment did not abolish PSA expression in *DDX3Y* knockdown models compared to control, indicating a heightened androgen receptor (AR) response (**Figure 11C**).

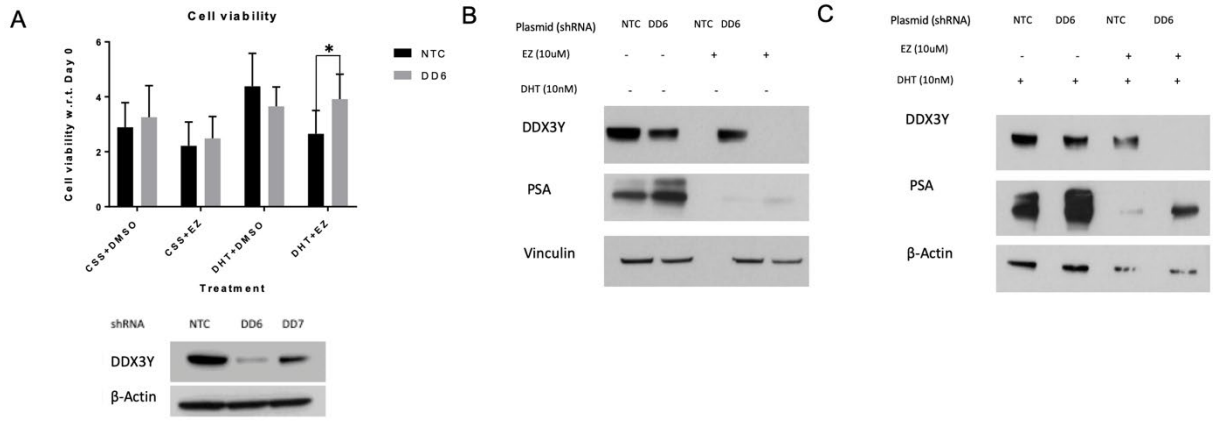


Figure 11. *DDX3Y* knockdown augmented enzalutamide-resistant phenotype in cell line models. A) Knockdown of *DDX3Y* conferred resistance from enzalutamide-mediated growth inhibition. B) DDX3Y knockdown resulted in increased expression of prostate-specific antigen (PSA). C) dihydrotestosterone (DHT) stimulation (10 nM) coupled with enzalutamide (EZ; 10 μM) treatment did not abolish PSA expression in *DDX3Y* knockdown models compared to control, indicating a heightened androgen receptor (AR) response.

Other Achievements

Nothing to Report.

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

  Nothing to Report.

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

  Nothing to Report.

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

  We have submitted requests for a 12-month no-cost extension (NCE, 36-48 months) and a change in PI from Dr. Kantoff (who has left MSK) to Dr. Schultz. As described in these requests, we will continue to conduct the relevant studies as outlined in a revised SOW, with Dr. Schultz and MSK as the responsible PI and site, respectively, for these Collaborative Awards.

  The project is currently underway, in accordance with the timeline outlined in the SOW.

  As outlined in Specific Aim 3, functional validation of candidate genes will be conducted by the Schultz group at MSK. We will perform RNA-seq analysis as part of the functional studies to investigate effect of loss of target genes in cell line models. Clinical validation of candidate genes in patient cohorts will continue to be conducted by the Schultz group at MSK.

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8. Li W, Xu H, Xiao T, et al. MAGeCK enables robust identification of essential genes from genome-scale CRISPR/Cas9 knockout screens. *Genome Biol*. 2014;15(12):554. PMCID: PMC4290824.
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4. IMPACT

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

  Our work presented the first evidence of ChrY genes regulating enzalutamide sensitivity in prostate cancer cell line systems. We also were the first to quantify and evaluate ChrY loss in patient-derived prostate cancer organoids and primary prostate cancer tissue. This work has uncovered novel insights into the role of ChrY loss in prostate cancer and as a potential surrogate marker for chromosomal instability.

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

  Our ChrY-targeting CRISPR/Cas9 library can be utilized to screen for multiple phenotypes in disease models other than prostate cancer. The library is a versatile tool used to investigate the role of ChrY genes in male responses in diseases that affect both sexes. The FISH probes developed for XY paint may also be used to quantify X or Y chromosome status in disease/non-disease models, not limited to cancer. Overall, our developed resources and protocols can be easily adapted to investigate the role of ChrY loss in any disease model.

- **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**

  The initial narrative proposed RWPE-1, LNCaP, LAPC4, LNCaP-Abl, and VCaP as cell line systems. Our efforts to optimize lentiviral transduction protocols in VCaP and LAPC4 cells have resulted in sub-optimal selection. The low transfection efficiency of these cells resulted in models that were unsuitable to conduct shRNA and CRISPR screens. We have therefore excluded VCaP and LAPC4 cells from our analysis. This will not affect the power of our analysis, as cell lines, RWPE-1, LNCaP, and LNCaP-Abl, are sufficient and present enough ChrY heterogeneity for our purposes.

  The target generation and functional analysis were delayed due to COVID-related halts in sequencing analysis. We were therefore unable to conduct studies in xenograft and other *in vivo* models. This does not affect the power of our analysis, as we will continue to pursue these relevant studies this year by requesting a 12-month NCE and PI change from Dr. Kantoff (who will be departing from MSK following the end of this reporting period) to Dr. Schultz (NCE, 36-48 month, responsible PI and site: Schultz, MSK).

- **Actual or anticipated problems or delays and actions or plans to resolve them**

  We experienced a delay of 3 months because of a mandated shutdown of facilities due to the COVID-19 pandemic, related lockdowns, and decreased personnel. This has resulted in a lack of sufficient time to generate and conduct studies in *in vivo* models. However, lack of xenograft studies (activities related to Major Task 5) does not compromise the relevance of our functional analysis, as validation studies are conducted in cell line models and publicly available clinical cohorts. Therefore, we submitted requests for a 12-month NCE and a change in PI from Dr. Kantoff to Dr. Schultz (W81XWH1810199/W81XWH-18-1-0200; Dr. Schultz will be the PI for these Collaborative Awards due to Dr. Kantoff's departure from MSK), allowing for the completion of relevant studies following the end of this award.

  During the 12-month NCE period, we will propose to run our methods on a larger cohort, existing of approximately 1,500 prostate cancer exomes (p-1500 dataset), which will vastly increase our power to identify recurrent events and associations with clinical outcomes. Our initial results from a relatively small set of tumors identified no recurrent event. However, running the method on the P-1500 dataset will significantly increase our power to detect recurrently mutated genes. Once we have the full P-1500 cohort analyzed and have quantified ChrY loss events and mutations, we will be able to analyze it in the context of disease status (is ChrY loss more common in metastatic disease vs primary disease, is there an association with treatment response?) and Gleason grade. The responsible PI and site for completing the relevant studies during the 12-month NCE will be Dr. Schultz at MSK.

- **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.**

    Nothing to Report.

  - **Books or other non-periodical, one-time publications.**
    Nothing to Report.

  - **Other publications, conference papers, and presentations.**

    Nothing to Report.

- **Website(s) or other Internet site(s)**

  Nothing to Report.

- **Technologies or techniques**

Nothing to Report.

- **Inventions, patent applications, and/or licenses**

  Nothing to Report.

- **Other Products**.

  Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

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

  Subhiksha Nandakumar, Bioinformatic Engineer, no change

Name:	Philip Kantoff
Project Role:	Initiating Principal Investigator
Researcher Identifier (e.g. ORCID ID):	0000-0001-7275-0597
Nearest person month worked:	0.6
Contribution to Project:	Dr. Kantoff oversees all aspects of this project. Following the end of this reporting period, Dr. Kantoff will no longer continue as the Initiating Principal Investigator (PI) due to his departure from MSK. Dr. Kantoff will be replaced with Dr. Schultz, who will serve as the PI for these Collaborative Awards.
Funding Support:	NIH, DoD

Name:	Nikolaus Schultz
Project Role:	Collaborating Principal Investigator
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1.2 months
Contribution to Project:	Dr. Schultz oversees all efforts related to this proposal in close collaboration with Drs. Kantoff and Van Allen. Following the end of this reporting period, Dr. Schultz will replace Dr. Kantoff as the PI for these Collaborative Awards due to Dr. Kantoff's departure from MSK.

Funding Support:	NIH, DOD, Cholangiocarcinoma Foundation, Prostate Cancer Foundation, MSK, The Gray Foundation, Functional Genomics Initiative

Name:	Eliezer Van Allen
Project Role:	Site Principal Investigator
Researcher Identifier (e.g. ORCID ID):	vanallen81
Nearest person month worked:	0.36 months
Contribution to Project:	Dr. Van Allen oversees all efforts related to this proposal in close collaboration with Dr. Schultz. Dr. Van Allen will not be continuing as a site PI/key personnel in the following year.
Funding Support:	NIH, DOD, Damon Runyon Cancer Research Foundation, Novartis, Prostate Cancer Foundation, Bristol Myers Squibb, Movember Foundation, Brown Performance Group, Leidos Biomedical Research, Inc, Starr Consortium

Name:	Travis Gerke
Project Role:	Site Principal Investigator
Researcher Identifier (e.g. ORCID ID):	0000-0002-9500-8907
Nearest person month worked:	0
Contribution to Project:	Dr. Gerke is the Moffitt Cancer Center (MCC) Principal Investigator (site location 1). In collaboration with Drs. Kantoff and Schultz, he was responsible for the analysis of gene expression data from the HPFS and the PHS cohorts and broadly assisting with the epidemiologic and statistical interpretation of findings. Effective as of 5/16/2020, this subaward was terminated (revised subaward period of performance: 6/15/2018 – 5/16/2020; effective date of amendment number 3: 6/15/19); Dr. Gerke did not contribute to the project during this period and will not be continuing as a site PI/key personnel in the following year.
Funding Support:	H. Lee Moffitt Cancer Center and Research Institute, Inc.

Name:	Sai Harisha Rajanala
Project Role:	Research Fellow
Researcher Identifier (e.g. ORCID ID):	0000-0002-7096-3756
Nearest person month worked:	3
Contribution to Project:	Dr. Rajanala has replaced Yuki Yoshikawa, MD. She will conduct genetics screens to identify regulators of antiandrogen therapy sensitivity using generated CRISPR/Cas9 screens. Dr. Rajanala will also conduct functional validation of candidate genes following positive genetic screens.
Funding Support:	Institutional

Name:	Rahim Hirani
Project Role:	Research Technician
Researcher Identifier (e.g. ORCID ID):	0000-0002-9304-9916
Nearest person month worked:	6
Contribution to Project:	Mr. Hirani has replaced Mohammad Atiq, MD. He will assist Dr. Rajanala with the functional validation of candidate genes.
Funding Support:	DOD

Name:	Bastien Nguyen
Project Role:	Research Fellow
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	4
Contribution to Project:	Dr. Nguyen will collaborate with Dr. Van Allen's (DFCI) laboratory on sequence analysis.
Funding Support:	DOD

Name:	Anisha Luthra
Project Role:	Bioinformatics Software Engineer
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	3
Contribution to Project:	Ms. Luthra will assist Dr. Schultz on bioinformatic software engineering.
Funding Support:	DOD

Name:	Sabrina Camp
Project Role:	Computational Biologist
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	2
Contribution to Project:	Ms. Camp has replaced Dr. Eric Kofman as the Computational Biologist assisting Dr. Van Allen. Ms. Camp's focus is on the analysis, method development, and application pertaining to identifying genomic features that correlate with mutational signature analysis of prostate cancers.
Funding Support:	DOD

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

SCHULTZ, NIKOLAUS

New Grants Since Last Submission

Title: P01 CA228696, The Impact of DNA Damage Repair Abnormalities in Prostate Cancer (Genomics Core)
Role: Co-Investigator
Sponsoring Agency: NIH/NCI **Effort:** 0.6 calendar
Level of Funding: **Dates:** 9/1/2019–8/31/2025 **Agency Contact (Email):** Roger Gross (rogers.gross@nih.gov)
Goals/Aims: Aberrations in genes that help repair damaged DNA are seen in 20-25% of men with metastatic castration resistant prostate cancer, the lethal form of prostate cancer. We have assembled a multidisciplinary team to increase our understanding of the role the spectrum of DNA repair aberrations play in early prostate cancer.
Overlap: No overlap

Title: The cBioPortal for Cancer Genomics for Cholangiocarcinoma Research
Role: Principal Investigator
Sponsoring Agency: Cholangiocarcinoma Foundation **Effort**: 0.00 calendar
Level of Funding: **Dates**: 07/01/2020–06/30/2022 **Agency Contact (Email)**: Alli Ward (info@cholangiocarcinoma.org)
Goals/Aims: Aim 1. Collect additional cholangiocarcinoma studies for cBioPortal. Aim 2. New cBioPortal features. Aim 3. Community Outreach
Overlap: None

Title: 19CHAL04, A Genomics-Guided Clinical Interpretation and Translational Discovery Engine for Prostate Cancer

Role: Principal Investigator **Effort**: 0.60 calendar
Level of Funding: **Dates**: 10/11/2019–10/10/2021 **Sponsoring Agency**: Prostate Cancer Foundation (formerly CaP CURE)
Agency Contact (Email): Howard R. Soule (info@pcf.org)
Goals/Aims: Develop a prostate cancer patient registry that will capture clinical data (which includes treatments and outcomes), pathology and imaging data, and data on tumor mutations, from over 10,000 patients spanning all stages of disease.
Overlap: None

Title: GC238999, Implementation of a Liquid Biopsy to Enrich Responses to Immunotherapy in Patients with Advanced Liver Cancer.
Role: Computational Biologist
Sponsoring Agency: MSKCC Society **Effort**: 0.60 calendar
Level of Funding: **Dates**: 09/01/2019 – 08/31/2021 **Agency Contact (Email)**: Jaclyn Regan (reganj@mskcc.org)
Goals/Aims: Aim 1: To prospectively determine the mutational status from tumor derived cfDNA of patients with advanced liver cancer prior to receiving standard of care immunotherapy by targeted next generation sequencing using MSK-ACCESS. Aim 2: To prospectively compare the proportion of patients with tumor shrinkage (objective response rate) to immunotherapy between WNT altered and non-altered groups. Aim 3: To establish a prospectively annotated tumor and peripheral blood bank from all liver cancer
Overlap: None

Title: GC260871, RCA-related Cancer Research Consortium
Role: Principal Investigator
Sponsoring Agency: The Gray Foundation **Effort**: 0.00 calendar
Level of Funding: **Dates**: 09/01/2020 – 08/30/2022 **Agency Contact (Email)**: Amy Truong, amy.truong@sagebionetworks.org
Goals/Aims: MSK will perform all work related to the proposed BRCA Pre-Cancer Atlas instance of the cBioPortal for Cancer Genomics. Specifically, MSK will build, maintain and run automated pipelines that transfer data from Synapse into cBioPortal, maintain all user logins, and will develop new cBioPortal features as they become relevant for the project. MSK will also provide in user support, documentation, and outrace for the entire BRCA Pre-Cancer Atlas.
Overlap: None

Grants Terminated Since Last Submission

Title: Identification and characterization of novel mutations in non-unique regions of cancer genes
Role: Principal Investigator
Sponsoring Agency: Functional Genomics Initiative **Effort**: 0.00 calendar **Level of Funding**: **Dates**: 12/20/2019 – 12/19/2020 **Agency Contact (Email)**: John H.H. Petrini (petrinij@mskcc.org)
Goals/Aims: Current mutation calling methods ignore regions of genes that are not unique in the genome. These are often small regions duplicated in other genes or

pseudogenes, but as is the case with members of the histone gene family, can affect entire genes. Our hypothesis is that these regions can be altered by functional mutations in cancer that have so far gone undetected.
Overlap: None

Title: 5 R01 CA182503-05, Defining Response and Resistance to PI3K and AR inhibition in Prostate Cancer
Role: Co-Investigator
Sponsoring Agency: NIH/ NCI **Level of Funding**: **Effort**: 0.60 calendar **Dates**: 09/12/2014–08/31/2020 NCE
Goals/Aims: Through this proposed work, are optimally positioned to directly inform and improve the development of clinical trials evaluating PI3K and AR inhibitors in prostate cancer, and develop future clinical trials evaluating novel therapies targeting mechanisms of resistance.
Overlap: None

Title: GC238999, Implementation of a Liquid Biopsy to Enrich Responses to Immunotherapy in Patients with Advanced Liver Cancer
Role: Computational Biologist
Sponsoring Agency: MSKCC Society **Effort**: 0.60 calendar
Level of Funding: **Dates**: 09/01/2019 – 08/31/2020 **Agency Contact (Email)**: Megan Mitchell (mitchem1@mskcc.org)
Goals/Aims: Aim 1: To prospectively determine the mutational status from tumor derived cfDNA of patients with advanced liver cancer prior to receiving standard of care immunotherapy by targeted next generation sequencing using MSK-ACCESS. Aim 2: To prospectively compare the proportion of patients with tumor shrinkage (objective response rate) to immunotherapy between WNT altered and non-altered groups. Aim 3: To establish a prospectively annotated tumor and peripheral blood bank from all liver cancer.
Overlap: None

- **What other organizations were involved as partners?**

1. **Organization Name:** Dana-Farber Cancer Institute
2. **Location of Organization:** Boston, MA
3. **Partner's contribution to the project**
   a. **Financial support:** None
   b. **In-kind support**: None
   c. **Facilities**: MSK and MCC staff will use the facilities resources at their respective institutions for this project. Dr. Van Allen and his team will use the facilities and resources available to them at DFCI.

d. **Collaboration**: Dr. Van Allen oversees all efforts related to this proposal in close collaboration with Dr. Schultz. Personnel from both the Schultz and Van Allen groups will collaborate on sequencing analysis for the project.
e. **Personnel exchanges***:* None
f. **Other: None**

8. SPECIAL REPORTING REQUIREMENTS

- **COLLABORATIVE AWARDS**

  Drs. Kantoff and Schultz are submitting duplicative reports with tasks clearly marked with the responsible PI and research site.

- **QUAD CHARTS**

  Not applicable.