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Increased mitochondrial function may render some cancers vulnerable to mitochondrial inhibitors. Since mitochondrial function is regulated partly by mitochondrial DNA copy number (mtDNAcn), accurate measurements of mtDNAcn could help reveal which cancers are driven by increased mitochondrial function and may be candidates for mitochondrial inhibition. However, prior studies have employed bulk macrodissections that fail to account for cell type-specific or tumor cell heterogeneity in mtDNAcn. These studies have often produced unclear results, particularly in prostate cancer. Herein, we developed a multiplex in situ method to spatially quantify cell type specific mtDNAcn. We show that mtDNAcn is increased in luminal cells of high-grade prostatic intraepithelial neoplasia (HGPIN), is increased in prostatic adenocarcinomas (PCa), and is further elevated in metastatic castration-resistant prostate cancer. Increased PCa mtDNAcn was validated by two orthogonal methods and is accompanied by increases in mtRNAs and enzymatic activity. Mechanistically, MYC inhibition in prostate cancer cells decreases mtDNA replication and expression of several mtDNA replication genes, and MYC activation in the mouse prostate leads to increased mtDNA levels in the neoplastic prostate cells. Our in situ approach also revealed elevated mtDNAcn in precancerous lesions of the pancreas and colon/rectum, demonstrating generalization across cancer types using clinical tissue samples.

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MYC-driven increases in mitochondrial DNA copy number occur early and persist throughout prostatic cancer progression

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Conflict of interest: ESA is a paid consultant/advisor to Janssen, Astellas, Sanofi, Dendreon, Pfizer, Amgen, Lilly, Bayer, AstraZeneca, Bristol-Myers Squibb, Clovis, and Merck; he has received research funding to his institution from Janssen, Johnson & Johnson, Sanofi, Dendreon, Genentech, Novartis, Tokai, Bristol Myers-Squibb, AstraZeneca, Clovis, and Merck; and he is the co-inventor of an AR-V7 biomarker technology (Patent # CA2959336A) that has been licensed to Qiagen. SRD has received research funding to his institution from Astellas. AMD is a paid consultant/advisor to Merck and Cepheid and has received research funding from Janssen and Myriad for unrelated work. SY receives research funding to his institution from Bristol-Myers Squibb and Celgene, Janssen, and Cepheid for unrelated work and has served as a consultant for Cepheid. He owns founder’s equity in Brahm Astra Therapeutics and Digital Harmonic. MCM is a paid consultant to Clovis and Exelixis.
Abstract

Increased mitochondrial function may render some cancers vulnerable to mitochondrial inhibitors. Since mitochondrial function is regulated partly by mitochondrial DNA copy number (mtDNAcn), accurate measurements of mtDNAcn could help reveal which cancers are driven by increased mitochondrial function and may be candidates for mitochondrial inhibition. However, prior studies have employed bulk macrodissections that fail to account for cell type-specific or tumor cell heterogeneity in mtDNAcn. These studies have often produced unclear results, particularly in prostate cancer. Herein, we developed a multiplex in situ method to spatially quantify cell type specific mtDNAcn. We show that mtDNAcn is increased in luminal cells of high-grade prostatic intraepithelial neoplasia (HGPIN), is increased in prostatic adenocarcinomas (PCa), and is further elevated in metastatic castration-resistant prostate cancer. Increased PCa mtDNAcn was validated by two orthogonal methods and is accompanied by increases in mtRNAs and enzymatic activity. Mechanistically, MYC inhibition in prostate cancer cells decreases mtDNA replication and expression of several mtDNA replication genes, and MYC activation in the mouse prostate leads to increased mtDNA levels in the neoplastic prostate cells. Our in situ approach also revealed elevated mtDNAcn in precancerous lesions of the pancreas and colon/rectum, demonstrating generalization across cancer types using clinical tissue samples.
Introduction

Warburg observed in the 1920s that cancer cells take up excess glucose that is metabolized mostly to lactate, even in the presence of oxygen. He postulated that such aerobic glycolysis (the "Warburg effect") was universal in cancer, essential for cancer cell proliferation and caused by disrupted mitochondrial respiration (1). The concept of mitochondrial dysfunction as oncogenic was later supported by studies showing that a number of cancer types contain somatic point mutations and/or deletions in mitochondrial DNA (mtDNA) (2–5). In addition, inactivating mutations in nuclear genes whose protein products function in mitochondria occur in several hereditary cancer syndromes, including those caused by mutations in fumarate hydratase (FH) and succinate dehydrogenase (SDH) (6–9). In the prostate, somatic mutations in mtDNA have been proposed as potential cancer drivers (10–18). Furthermore, a 3.4 kb deletion in mtDNA has been reported in prostate cancer, and these and other mtDNA deletions accumulate in the aging prostate (16, 17, 19). Nevertheless, the functional significance of somatic mitochondrial DNA alterations as oncogenic drivers in cancer, including prostate cancer, remains to be fully clarified.

The phenomenon of aerobic glycolysis, which is the use of glycolysis rather than oxidative phosphorylation as a source of energy in cells even in aerobic conditions, was long proposed as a unique property of cancer cells indicative of mitochondrial dysfunction in cancer (20, 21). However, recent evidence has shown that such aerobic glycolysis is not cancer-specific and commonly occurs in non-neoplastic proliferating cells as part of a metabolic program during cell growth and division (1, 20–26). While a complete picture of precisely why dividing cells undergo aerobic glycolysis is still emerging, the metabolism of glucose to lactate helps maintain high levels of glycolytic intermediates and/or NAD⁺ to support anabolic reactions needed for cell growth and replication (1, 21, 22, 24, 27). Furthermore, multiple lines of evidence suggest that cancer and other non-neoplastic proliferating cells have increased mitochondrial activity. Several metabolites generated by the TCA cycle are used as intermediates for macromolecular synthesis in proliferating cells. Also, except for a relatively rare subset of cancer types (e.g., oncocytomas of the kidney, renal tumors associated with inactivation of FH or genes encoding the succinate dehydrogenase complex), respiration is not generally markedly impaired in cancer (21, 25, 28), and a functioning electron transport chain is required for proliferation of most normal and cancer cells (21, 29–
Taken together, it is now apparent that mitochondria display increased activity in most cancers; albeit, their metabolic activities are reprogrammed to facilitate cell proliferation and continuous self-renewal (1, 2, 21, 22, 24–28, 33).

Mitochondrial function is regulated by mitochondrial biogenesis, which is controlled, at least partially, by mtDNA copy number (mtDNAcn) (34, 35). Alterations in mtDNAcn have been reported in cancer (35). For example, increased mtDNA copy number has been described in acute myelogenous leukemia (AML) (36, 37), endometrial carcinoma (38), esophageal squamous cancer (39), pancreatic cancer (5), and colorectal cancer (40). Further, treatment with an inhibitor of mtDNA replication (41) or of a mitochondrial complex 1 inhibitor (42) in AML has shown promise in preclinical studies. Notably, increased mtDNA content was mechanistically linked to microsatellite-stable colorectal cancer cell proliferation and metastasis (40). However, in other cancers, such as gastric, breast, hepatocellular, non-small cell lung and renal carcinomas, decreased mtDNA levels, as compared to their normal tissue counterparts, have been reported (35). Thus, there is no general pattern regarding mtDNAcn changes in tumor-normal comparisons in common adult cancer types.

In terms of mtDNAcn changes in prostate cancer, there are contradictory results (16, 43–45). Mizumachi et al. examined single laser microdissected cells from a small number (N=9) of prostate cancers and reported increased mtDNA content in some cancers, decreased amounts in others, and a general increase in cell-to-cell variability in cancer cells compared to matched benign tissues (43). Koochekpour et al. reported lower levels of mtDNA in human prostate cancer compared to normal prostate tissues and suggested that reduced levels of mtDNA can drive aggressive prostate cancer (44). Reznik et al. analyzed mtDNAcn variation across multiple cancers relative to matched non-neoplastic tissues using next generation sequencing (NGS) data from The Cancer Genome Atlas (TCGA), finding that prostate cancer had non-statistically significantly increased bulk tumor mtDNA levels compared to benign prostate tissues (45). Schöpf et al. reported that there was no significant difference in mtDNAcn between benign and malignant prostate tissues, although mtDNAcn was increased in higher grade cancers (18). Higuchi et al. showed that androgen-independent C4-2B cells had lower levels of mtDNA than androgen-sensitive LNCaP cells and that depletion of mtDNA in LNCaP cells resulted in the development of androgen independence (46). Consequently, whether there are increased or decreased levels of mtDNA in prostate cancer, and whether mtDNA levels are associated with disease aggressiveness and
castration resistance remains unclear. To improve our understanding of the role of mtDNAcn alterations in prostate cancer, it is important to resolve these inconsistent findings.

A concern with most prior studies of mtDNAcn alterations in cancer is that they usually employ bulk macrodissection of tumor and matched benign tissues, which is followed by cellular disruption and DNA isolation. These bulk mtDNA measurements could not take into account heterogeneity within or between tumor nodules or the diversity of cell types (47). We have shown, using an in situ hybridization approach, differing mtDNA levels in normal tissue compartments and cell types in most organs (48). Therefore, the use of bulk macrodissections may be ill-suited for tumor/normal comparisons by not accurately representing differences among relevant neoplastic and non-neoplastic cell types or regional heterogeneity in mtDNA levels of neoplastic cells (43). To understand better the potential biological role of mtDNAcn changes in cancer initiation and progression, it is critical to determine when during the carcinogenesis process the alterations occur (49). Since cancer precursor lesions are often difficult to obtain from fresh tissues, and are often microscopic in size, most studies of mtDNAcn alterations on such precursors require either laser capture microdissection (LCM) or an in situ approach (49).

Here, we report the enhancement of our in situ approach to mtDNA quantification (48) by combining it with iterative sequential immunohistochemistry in a multiplex assay, allowing for cell type and compartment specific mtDNA measurements. This multiplex assay revealed generally increased mtDNAcn in pre-neoplastic lesions and prostate cancer. Because we and others have linked MYC overexpression to prostate cancer development and progression (50–52), and because MYC has been shown to induce mitochondrial biogenesis (53), we also investigated the role of MYC in regulating mtDNA copy number in human and murine prostate cancer cells. These studies revealed that MYC increases mtDNAcn through stimulating mtDNA replication, apparently by increasing expression of mtDNA replication factors, including the helicase TWINKLE.

Results

*Basal cells show higher mtDNAcn than luminal cells in benign prostate glands*
Using chromogenic in situ hybridization (CISH), we previously observed that mtDNA signals were visually higher in prostate basal cells compared with normal luminal cells (48). To quantitatively assess mtDNAcn in tissues, we combined mtDNA CISH with multiplex immunohistochemistry (IHC) using two protein markers, keratin 8 (CK8) and a combination of keratins 5+14 (CK903). Anti-CK8 labels both basal and luminal prostate epithelial cells, and, in normal (e.g. non-atrophic) epithelium, anti-keratins 5+14 labels only prostate basal cells. We employed an iterative chromogenic approach (54) in which we first performed in situ hybridization for mtDNA followed by whole slide scanning and sequential IHC for each of the keratin antibodies (Fig. 1A). We used radical prostatectomy specimens to compare regions containing normal appearing epithelium, HGPIN and adenocarcinoma. Fig. 1A shows the cellular compartmental segmentations from a representative case containing adjacent normal appearing epithelium and invasive adenocarcinoma. Quantitative image analysis demonstrated that basal cells contained an approximately 5-fold higher mtDNA percent signal area on average than normal appearing luminal cells (Fig. 1B). To address whether the increased mtDNAcn in basal cells compared with luminal cells might be a response to nearby cancer, we studied prostate samples from young adult male organ donors without cancer, and the results were highly similar to those obtained using the cancer patient samples (Supplemental Figure 1).

**Increased mtDNAcn in high grade prostatic intraepithelial neoplasia (HGPIN)**

HGPIN is the likely precursor to most human prostate cancers (55, 56), yet mtDNAcn levels have not been studied in these lesions. We sought to compare neoplastic epithelial cells in HGPIN to matched benign normal appearing epithelium. By visual inspection, luminal cells in HGPIN showed increased mtDNA signals as compared with normal luminal cells (Fig. 2A-B). By image analysis, HGPIN showed a consistent increase in the median percent mtDNA signal area of approximately 3-fold compared to the normal prostate glands (Fig. 2F). This increase was found despite the fact that basal cells were not excluded from the image analysis in the normal regions.

**Increased overall and heterogeneously distributed mtDNAcn in primary prostate cancer**
Using the same specimens, we next examined primary invasive adenocarcinomas. Visually, invasive carcinomas generally showed higher mtDNAcn than matched normal regions. Interestingly, there was considerable regional heterogeneity of mtDNA in situ hybridization signals, both within individual tumor foci (intratumorally) and between foci separated in space (intertumorally) (Fig. 2C-D; Supplemental Fig. 3). Quantitative analysis including the different subregions of tumor foci is shown in Supplemental Fig. 2. As an example of intratumoral heterogeneity in a primary prostatic adenocarcinoma, Sup. Fig. 3 shows striking mtDNAcn heterogeneity within an individual tumor from a prostatectomy case. In addition to being contiguous in space, this tumor showed homogeneous ERG expression, indicative of a TMPRSS2-ERG gene rearrangement (57, 58). While additional analyses would be needed to definitively assess clonality, this homogeneous ERG expression is consistent with the likelihood that this large tumor with heterogeneous mtDNAcn represents a single clonal origin (Supplemental Fig. 3) Taken together, carcinoma lesions had significantly higher levels than normal epithelium, and were similar to HGPIN (Fig. 2F).

As an independent method for comparing mtDNAcn in prostatic tumor-normal pairs, we used whole genome sequencing from laser capture microdissected tissues in which tumor regions and matched benign paired prostatic regions (non-high grade PIN containing) were isolated separately (N=115 prostate cancer cases). In this cohort, which is part of a comprehensive genomic analysis of prostatectomy samples, tumor samples showed increased mtDNAcn compared with matched normal appearing glands (Fig. 3A). Figure 3B shows the increased mtDNA levels in carcinomas occurred across all major Gleason Grade groups. The distribution of cases by grade, pathological stage, race and other demographic features are shown in Supplemental Table 2. While there was no clear increase in mtDNAcn by grade, all of the cancers with mtDNAcn signals above 1000 were Gleason grade group 2 or higher (Supplemental Table 3).

A prior study has reported that tumor and benign prostate tissues contain differing levels of mtDNAcn in Black compared to White patients (44). When comparing mtDNAcn in tumor samples between White and Black men (self-identified race) using our NGS approach, there were highly similar mtDNAcn levels between the two, with no statistical difference (Supplemental Fig. 4A).
Aliquots of the same DNA samples used for WGS were subjected to quantitative PCR, as described previously (48). This showed a strong positive correlation between the mtDNAcn obtained using WGS and qPCR (Supplemental Fig. 4B), validating the quantitative nature of our NGS approach. Also, qPCR on a subset of the samples used for WGS also showed an increase in mtDNAcn in matched tumor samples compared with benign (Supplemental Fig. 4C). Thus, three independent/orthogonal methods used in this study all show increased mtDNAcn in tumors versus normal prostatic tissues. We observed a weak positive correlation between age and mtDNAcn in the tumor samples, although not in the benign ones (Supplemental Fig. 4D) (15). When examining the results by self-reported race, the same trends were present in both Black and White patient samples, although this correlation between age and mtDNAcn in tumor tissue reached statistical significance only in the samples from White patients (data not shown).

**Metastatic castration-resistant prostate cancer (CRPC) showed higher mtDNAcn with less heterogeneity than the primary prostate cancer**

We performed the same iterative CISH-IHC and image analysis workflow using tissues from men with known metastatic castration-resistant prostate cancer (mCRPC) (N=23 patients) using pretreatment biopsies of patients in two different clinical trials (51, 59). Visually, these metastases demonstrated high mtDNA in situ hybridization signals compared with the adjacent non-neoplastic tissues (when present), with some cases showing higher mtDNA signals than hepatocytes in liver, which are known for high mtDNA content (Fig. 2E) (48). By image analysis, we observed high mtDNAcn in most of these mCRPC biopsies, which appeared higher as a group when compared to the primary tumors (Fig. 2F).

**Functional relevance of increased mtDNAcn in prostate cancer**

To address the functional significance of mtDNA alterations in prostate lesions, we first sought to determine whether the increased mtDNAcn was accompanied by corresponding increases in mtRNA(s). We used a multiplex fluorescent in situ hybridization assay to perform simultaneous hybridizations for mtDNA along with mtRNAs from three different mtDNA-encoded genes. In the normal appearing prostate regions, we found generally increased signals in the basal versus luminal compartments, and in carcinoma tissues there were increased levels for all 3 of these mtRNAs (Fig. 4A-B). Furthermore, there was a correlation between each of
these 3 mtRNA species and mtDNA (MT-CO1 sense) (Fig. 4C). In fact, the mtDNA levels correlate with the levels of both MT-rRNA and MT-mRNAs that it encodes. These observations suggest that mtRNA levels may be regulated, at least in part, by mtDNA levels. In addition, we performed enzyme histochemistry for cytochrome c oxidase (COX) and succinate dehydrogenase (SDH) on frozen sections from a number of cases (N=12 tissue blocks from 11 patients). In general, there were visually higher levels of both enzyme activities in basal cells compared to luminal cells (Sup Fig. 5). In prostate carcinomas, there were visually apparent increases as well as heterogeneity that were similar in pattern to the mtDNA in situ signals from adjacent tissue sections, providing further support for the hypothesis that the increased mtDNAcn is functional with respect to production of the encoded genes. Interestingly, the lesions with high mtDNAcn, mtRNA expression and mitochondrial enzymatic activities also showed high MYC protein expression, suggesting a mechanistic link between them (Fig. 4D).

**MYC as a Driver of mtDNAcn in Clinical Prostate Cancer**

To begin to assess the molecular mechanism for increased mtDNA levels in HGPIN and PCa, we focused on MYC, since MYC has been shown to regulate mitochondrial biogenesis and mtDNA levels (53, 60). MYC is also a key driver of prostate cancer, whose overexpression starts very early in the disease process in HGPIN (56, 61, 62), continues in primary carcinomas and is also highly expressed in mCRPC (52, 61–63). In human prostate cancer cell lines, we have shown that forced overexpression of MYC results in increased mtDNA (48). Despite these findings, since some MYC-driven processes are context and cell type-specific, including its effects on mitochondrial related gene expression (53, 60), it is important to investigate whether MYC could be a driver of mtDNAcn changes in normal prostatic epithelial cells in vivo in the context of intact tissues. Therefore, we examined mice with targeted MYC overexpression in the prostate in which MYC is known to drive the development of PIN lesions (Hi-MYC mice) (64–66). We focused on the anterior prostate lobe of Hi-MYC, since, despite being driven by AR signaling, MYC expression is quite heterogeneous and it often contains regions of MYC-positive and MYC-negative epithelium within the same lobe. We observed that in regions showing increased MYC mRNA and protein levels, there were increased levels of mouse mtDNA that correspond to the regions of MYC overexpression and accompanying morphological changes diagnostic of PIN, including marked nucleolar enlargement (4 of 4 regions in 3 mice). Fig. 5 shows a representative case in a Hi-MYC mouse.
Decreased mtDNAcn in response to high dose testosterone treatment in mCRPC and correlation with MYC

Prior work has shown that the cyclical treatment of men with mCRPC with supraphysiological levels of testosterone (SPA, or supraphysiological androgen), referred to as Bipolar Androgen Therapy (BAT), produces tumor regression and clinical benefit in ~30-40% of patients (51, 67–71). Many of the samples from men with mCRPC shown in Fig. 2 were from the pretreatment time point of a recent trial, referred to as COMBAT-CRPC (51) (clinical trials.gov NCT03554317) where patients received a pre-treatment biopsy of metastatic site, as well as a biopsy after 3 cycles of SPA (cycle 4, day 1 or C4D1). Interestingly, most patients in the trial who responded to SPA also showed a marked reduction in MYC protein expression, by quantitative IHC, as well as MYC mRNA by RNAseq. This was accompanied by reductions in Ki67 protein as well as a number of proliferation associated mRNAs (51, 72). We performed the mtDNAcn CISH-IHC assay on all available mCRPC patient samples that had matched post-treatment biopsies from COMBAT-CRPC. Fig. 6 shows that there was a decrease in mtDNAcn that correlated with decreases in MYC protein in most cases. These findings provide evidence that, in late stage mCRPC, changes in mtDNAcn after drug treatment correlate with changes in MYC levels, further indicating that MYC may be a key regulator of mtDNAcn in prostatic adenocarcinomas.

mtDNA replication as a potential downstream target of MYC in prostate cancer.

To address whether MYC can drive mtDNA replication in prostate cancer cells (PC3), we inhibited MYC activity with a novel small molecule inhibitor MYCi975 (73, 74), followed by metabolic labeling with EdU for incorporation into newly synthesized DNA. Using an anti-5'-bromodeoxyuridine (BrdU) antibody that also binds to EdU (75) (Fig. 7A), we visualized the EdU signals by IHC. In untreated cells, we observed abundant punctate dot-like structures in the cytoplasm, consistent with mitochondrial localization. As a control for antibody specificity, these IHC signals were absent in cells not treated with EdU. In support of the hypothesis that these signals reflect mtDNA replication, the EdU-IHC signals were greatly reduced in cells treated with the mtDNA polymerase (POLG) inhibitor, ddC. Many cells also showed strong nuclear signals for EdU, indicating nuclear S phase labeling (Fig. 7A). After inhibition of MYC by MYCi975, there were reduced cytoplasmic signals, consistent with a decrease in mtDNA replication. There was also a decrease in nuclear DNA signals after MYC inhibition,
which is expected since loss of MYC will also block nuclear DNA synthesis in prostate cancer cells (76). As a second approach, we performed qPCR for mtDNA and nuclear DNA (nDNA) after immunoprecipitation of EdU containing DNA. Using this assay, we also observed decreased EdU incorporation into mitochondrial genes when MYC levels were reduced (Fig. 7B). Taken together, these results support the hypothesis that MYC activity controls mtDNA synthesis in prostate cancer cells.

**mtDNA Replication Factors in Prostate Cancer**

Genes required for mtDNA replication, which are all encoded by nuclear DNA, include POLG (the main DNA polymerase), POLG2 (a processivity subunit), TWNK (the helicase referred to as Twinkle), SSBP1 (the mtDNA specific single stranded binding protein), TFAM (encoding mitochondrial transcription factor A required for replication and transcription), LIG3 (the DNA ligase), PRIMPOL (the DNA primase-polymerase), TOP1MT (encoding the mitochondrial topoisomerase) and TOP3A (a mitochondrial isoform encoding topoisomerase required to deconcatenate mtDNA circles). Germline mutations in the genes that encode these proteins lead to mtDNA depletion syndromes (77). A number of these genes have been previously shown to be MYC targets in other cell types (53, 78). To examine the potential regulation of these genes in prostate cancer by MYC, we interrogated mRNA levels of these genes after siRNA knockdown of MYC in 3 prostate cancer cell lines (76). Most of the mtDNA replication genes were reduced after MYC knockdown (Sup. Fig. 6A). Publicly available ChIP-seq showed that MYC binding occurs within the promoter regions of multiple mtDNA replisome genes in a prostate cancer cell line; RNA-seq data showed their transcripts were reduced correspondingly (74) (Sup. Fig. 6B). Further, we examined the RNAseq data mentioned above from the same samples that were subjected to laser capture microdissection and whole genome sequencing. Fig. 8 shows increased mRNA in primary prostate cancer lesions compared to matched benign for a number of genes encoding these mtDNA replication factors, especially Twinkle (TWNK/C10ORF2). Further, a number of these factors, including Twinkle, correlate with MYC expression in these primary tumors (Fig. 8C). When we examined RNAseq data from the COMBAT CRPC trial discussed above (51), a similar correlation with MYC and TWNK was present (Sup Fig. 6C). This correlation between MYC and TWNK mRNA levels was also found in other cancer types in publically available TCGA data sets, including those from human breast carcinoma, pancreatic adenocarcinoma and colorectal carcinoma (Sup Fig. 7). These findings suggest that a number of the necessary components of mtDNA replication machinery are
positively regulated by MYC, are bound in their regulatory regions by MYC protein, are overexpressed in human prostatic cancer, and correlate with MYC levels in primary tumors and in mCRPC. To further explore whether TWNK overexpression relates to MYC driven transcriptional activity more generally, we compared TWNK mRNA levels to the Hallmark MYC Pathways V1 and V2 using GSVA and found a strong positive correlation between these (Fig. 8D).

We also sought to determine whether expression of nuclear genes encoding proteins that localize and function in mitochondria were increased in human prostate cancers. For this, we performed GSEA using the Mitocarta 3.0 mitochondrial pathways and found that many of these were enriched in prostate cancer in our U01 samples, a number of which were considered statistically significant (e.g., mitochondrial_central_dogma.Translation, mitochondrial_central_dogma.mtRNA_metabolism, mitochondrial_central_dogma.Protein_homeostasis) (Supplemental Fig. 8).

**Increased mtDNAcn was also seen in other cancer precursor lesions**

To determine the applicability of our in situ approach in other cancer precursor lesions, we investigated mtDNAcn in precancerous lesions from the pancreas and large intestine. We applied the same CISH-IHC workflow on human pancreas tissues with pancreatic intraepithelial neoplasia (PanIN) and benign pancreatic ducts, as well as human colon tissues with tubular adenoma and normal colon mucosa. By visual inspection, PanIN lesions showed higher mtDNA in situ hybridization signals compared to their normal duct counterparts (Figure 9A). By image analysis, we observed approximately 3-fold higher %mtDNA area in PanIN compared to the normal ducts (Figure 9B). In the colonic samples, as recently reported (48), when present, the normal appearing colonic epithelium showed a marked gradation in mtDNAcn with cells located in the stem/proliferative compartments of crypts showing higher mtDNAcn compared to more differentiated cells towards the surface. In 7 of 8 adenomas, by visual inspection the mtDNA signals were more similar to, or apparently higher than, the levels observed in the normal appearing stem cell compartment (Figure 9C). These strong mtDNA signals occurred even towards the surface of the adenomatous epithelium in a manner reminiscent of topographical
infidelity of proliferation markers and other proteins in colorectal adenomas, including MYC protein expression (Figure 9D) (79, 80).
Discussion

To overcome the limitations inherent in most mtDNAcn measurements in tumor/normal pairs, we developed a multiplex chromogenic in situ hybridization and IHC approach to quantify mtDNAcn in specific cell populations. This assay uncovered consistent increases in mtDNAcn in high grade PIN. In addition, we found increases and marked intratumoral heterogeneity in mtDNAcn in most primary prostatic adenocarcinomas and further increases in advanced mCRPC. The increased mtDNAcn in primary prostate cancer was independently verified using whole genome sequencing (WGS) of DNA isolated after LCM, as well as by quantitative real-time PCR. Since three separate complementary approaches produced highly similar results, our findings should resolve previous contradictory publications regarding differences in mtDNAcn in prostatic cancers as compared with matched benign normal appearing prostate epithelium. The increased mtDNAcn in prostate cancer cells associated with increases in mtRNAs and mitochondrial electron transport chain activity, observed by in situ enzymatic activity, suggests increased mitochondrial function. Mechanistically, increased mtDNAcn in mouse prostatic luminal epithelial cells in vivo is driven by forced overexpression of MYC. Additionally, reductions in mtDNAcn correlate with reductions in MYC that are observed in mCRPC clinical samples after treatment with supraphysiological testosterone (51). Since MYC is known to be consistently overexpressed in human PIN (compared with normal prostate luminal cells) and most primary and metastatic castration resistant prostatic adenocarcinomas (51, 52, 56, 63), these findings implicate MYC overexpression as causing mtDNAcn increases in these lesions. We also report that a key mechanism by which MYC drives increased mtDNAcn is through increased mtDNA replication, potentially via positive regulation of mtDNA replication factors, including the helicase, TWINKLE. Finally, our in situ approach also identified increases in mtDNAcn in neoplastic precursor lesions in the colon (adenomas) and pancreas (PanINs), demonstrating the broad applicability of this method.

Comparisons with other prostate cancer studies

Our findings are consistent with the findings of Mizumachi et al. (43) Our in situ method and their single cell LCM approach both measure mtDNAcn with single cell resolution. Our findings also generally agree with a more recent study using whole genome sequencing data from TCGA samples that showed a trend for increased mtDNAcn in prostate cancer versus benign matched tissues (45). Although Reznik et al. reported that there was
no correlation between mtDNAcn and mtRNA expression in prostate cancer using sequencing methods (81), we observed that at least for the 3 mtRNAs, their spatial patterns and expression levels highly correlate with those of mtDNAcn. The present findings differ with those of Schöpf et al., (18) who found no overall increase in mtDNAcn in cancer vs. normal in the prostate, although they did report an increase in mtDNAcn in higher grade tumors as compared with the lower grade ones. By contrast, we found consistently increased mtDNAcn across all grade groups. Nevertheless, in our study, the Gleason grade of all of the cases with the highest levels of mtDNA in primary tumors were greater than Grade Group 1, and we found a further increase in metastatic castration resistant lesions (Fig. 2 and Fig. 3). Schöpf et al. (18) used tissue punch biopsies to isolate tumor and benign tissues, which provides a lower level of tumor purity than our laser capture based method used for our WGS approach, and this could help explain the differences. Our findings differed from those of Koochekpour et al. (44), since we found no difference in Black vs. White men in tumor mtDNAcn, while their study did.

**Basal vs. luminal cells**

In the present study we found that basal cells have approximately 5-fold higher %mtDNA area as compared with normal luminal cells. This indicates a higher mtDNA density in basal cells compared with luminal cells. In comparing mtDNA cellular levels in HGPIN and invasive adenocarcinomas to normal, there was a clear increase overall, even when including normal basal cells in the normal regions, suggesting a higher mtDNA density per unit cellular area (Fig 2). By visual inspection, there was a clear increase in mtDNA in situ signals in luminal cells in PIN and cancer compared with normal luminal cells. Although some prostatic adenocarcinomas appear to possess gene expression signatures, or modules, that resemble basal cells to a degree (82–84), most evidence suggests that the cell of origin for prostate carcinoma is a luminal cell (56, 85–87). When compared specifically to benign normal appearing luminal cells, there was a more striking increase in HGPIN luminal cells (Sup. Fig. 9).

**MYC may drive mtDNA replication**

Prior studies have shown that MYC can regulate mitochondrial biogenesis and mitochondrial DNA synthesis (53, 78). Wang et al., reported that during *Drosophila* oogenesis, *myc* regulated mtDNA replication and COX enzyme activity, while a hypomorphic *myc* allele led to reduced levels of both (78). They further
suggested that such changes may occur through myc’s role as a master transcription factor, since a majority of the genes encoding proteins of the electron transport chain, mtDNA replication and transcription were downregulated in flies with the hypomorphic myc alleles. Our results mirrored theirs as we observed similar trends when examining microarray data after knockdown of MYC in human prostate cancer cell lines, as well as RNAseq results from a clinical trial where responding patients showed reduced MYC was accompanied reduced mRNA levels in genes such as TWNK (Supplemental Fig. 6). Also, a number of mtDNA replication-related genes are upregulated in human prostate cancer and TWNK levels correlate with MYC mRNA (Fig. 8 and Sup. Fig. 6C). Taken together, these findings suggest an essential role of MYC in mtDNA replication and mitochondrial function in both Drosophila normal ovarian tissues and prostate cancer development and progression. Further study is required to assess whether increased mtDNAcn (or de novo mtDNA synthesis) is required for tumor development and progression in MYC-driven cancers.

**Why is mtDNAcn increased?**

We hypothesize that increased mtDNAcn is necessary in the process of carcinogenesis (40, 41). For instance, it is possible that higher mtDNAcn drives mitochondrial biogenesis to increase mitochondrial activity needed for the increase in macromolecular synthesis required for cell growth and replication. That there may be increased mitochondrial biogenesis in prostate cancer is further supported by electron microscopy studies that showed apparently elevated mitochondrial numbers in human prostate cancer samples (88–90) as well as in PIN (referred to as intraductal dysplasia, [(91)]). Since our mCRPC samples generally showed increased mtDNAcn compared to primary prostate cancer, we hypothesize that the cells with high mtDNAcn have a growth advantage and thus may be selected during disease progression. In support of this, we found reduced mtDNAcn in matched samples from patients before and after SPA that correlated with clinical response, Ki67 levels and MYC levels. Studies over several decades have indicated that, compared with normal prostate, prostate cancer cells show increased mitochondrial functions that are important for cell proliferation, and our overall results are concordant with these observations (92–96).

An alternative hypothesis is that, since mtDNA mutations are common in prostate cancer (10, 15, 18,
some of which may be deleterious to mitochondrial functions, increased mtDNAcn may reflect compensatory upregulation of mtDNA levels to help maintain these compromised mitochondrial functions. There is a potential precedent for this in prostate cancer as seen by Schöpf et al., in which they found higher mtDNAcn in cases showing potentially deleterious mtDNA mutations (18). In that study, short term cultures of fresh prostate cancer tissue showed an altered mitochondrial oxidative phosphorylation capacity including a decrease in N-pathway (NADH through Complex I, III, IV) capacity, and an increase in S-pathways (succinate from complex II-IV) capacity, which was accentuated in cases with higher levels of heteroplasmy of potentially deleterious mtDNA mutations in proteins encoding complex I components (18). Furthermore, mtDNA mutations, or nuclear-encoded genes that can alter the TCA cycle flux, can result in increases in "oncometabolites" that can drive epigenetic changes such as DNA CpG methylation that can provide a selective growth advantage to the neoplastic cells (25), despite having somewhat compromised overall mitochondrial function. Thus, in this case, there could also be compensatory increases in mtDNAcn to maintain mitochondrial function.

One additional limitation of our study is that, although we were able to quantify mtDNAcn within specific cell compartments, we did not examine this at the single cell level. Future studies that employ cell membrane markers for combined in situ and IHC analysis, as well as potential future methods that can perform single cell mtDNA sequencing/copy number measurements, could help address the potential mtDNAcn heterogeneity at the single cell level.

Translational Relevance and Future Directions

The discovery of functional mitochondrial reprogramming geared for biomass generation and proliferation in many cancers (1) has nominated pharmacological blockade of mitochondrial oxidative phosphorylation, mtDNA replication, and mitochondrial translation (28, 97) as promising and novel anti-cancer therapeutic avenues. Based upon the present findings, we hypothesize that developing novel drugs to inhibit required mtDNA replication machinery components, including the helicase Twinkle (encoded by TWNK), SSBP1 and mitochondrial topoisomerases, may represent new therapeutic approaches for cancer treatment and/or prevention. This concept is analogous to the recent credentialing of POLMRT, the mtRNA polymerase, as a
novel cancer drug target (98). Related to this, it has been hypothesized that those tumors with high levels of activity of canonical mitochondrial functions, such as TCA cycle and oxidative phosphorylation, may harbor dependencies that could be most susceptible to mitochondrial inhibition therapies (27, 37, 99). Since mtDNAcn regulation is associated with controlling mitochondrial biogenesis and function, accurate measurements of mtDNAcn in tumors and their precursors using our in situ approach may help determine which cancers are most appropriate for mitochondrial therapies. Also, the fact that we observed elevated mtDNAcn in precursor lesions from 3 different tumor types, and others have seen in head and neck cancers (49), suggest that blockade of mitochondrial activities may be an important new approach for precision prevention and cancer interception strategies. Future methods that can measure mtDNA using single cell based sequencing methods may also have an important role here (100), although they would lose critical spatial information that is a key feature in our approach. The method developed in this study is straightforward and should be readily portable to automated in situ assays combined with IHC, when appropriate, that could be implemented routinely in research laboratories and clinical pathology laboratories if additional future studies support clinical utility.

Methods

Mice

Hi-MYC mice (64) on an FVB/N background (n = 3, age = 2-6 months) were conventionally housed according to protocols approved by the Animal Care and Use Committee at the Johns Hopkins University School of Medicine. Animal tissues were harvested and fixed in 10% Neutral buffered formalin for 48 hours.

Chromogenic mtDNA in situ hybridization combined with immunohistochemistry

In situ hybridization for mtDNA for fresh frozen and formalin-fixed paraffin-embedded (FFPE) tissues was performed based on a previous method for human prostates and colons (48). For human pancreas FFPE tissues,
the pretreatment was 15 min steaming in RNAscope® Target Retrieval reagent followed by 10 min incubation in protease plus at 40 °C, and the rest is the same as other tissues.

The mtDNA in situ hybridization signals were developed using 3-amino-9-ethylcarbazole (AEC, Vector Laboratories, SK-4200), counterstained in hematoxylin and blued in Shandon bluing buffer (Thermo Scientific, 6769001). The slides were mounted, coverslipped, scanned, and de-coverslipped. The AEC was de-stained in an alcohol gradient. The slides were then rehydrated in an alcohol gradient and incubated using a HRP blocker (ACD bio) for 15 min at 40 °C before being subjected to sequential immunohistochemistry. This scanning, destaining and blocking process was repeated between all staining cycles.

After in situ hybridization, the slides were incubated with an anti-CK8 antibody (1:1500, Abcam, ab53280) for 45 min, PowerVision Poly-HP anti-Rabbit IgG (Leica, PV6114) for 30 min, and visualized by incubation in AEC for 30 min, followed by counterstaining and bluing. For some slides, an additional CK903 IHC staining cycle was performed after CK8 detection. CK903 (1:50, Enzo, ENZ-C34903) was used as the primary antibody, PowerVision Poly-HP anti-Mouse IgG (Leica, PV6119) as the secondary antibody, and AEC as the chromagen. All primary antibodies were diluted in Antibody Dilution Buffer (Ventana, adb250) unless specified.

The mtDNA in situ hybridization on mouse prostate tissues using Mm-mt-Co1 sense probe was performed as described previously (48). Hs-MYC mRNA in situ hybridization was performed similarly with probes against human MYC (ACD Bio, 311761).

**Multiplex Fluorescent in Situ Hybridization and Immunofluorescence**

Multiplex fluorescent in situ hybridization for mitochondrial RNA(s) expression was performed using HiPlex12 detection kit (ACD Bio, 324105) on fresh frozen tissues. Briefly, fresh frozen prostate tissues were hybridized with RNAscope HiPlex probes against Hs-MT-CO1 sense (mtDNAcn), Hs-MT-CYB, Hs-MT-ATP6 and Hs-MT-RNR1 in two rounds. Whole slides were scanned and fluorophores were cleaved between rounds. After HiPlex, the slides were then incubated in anti-CK8 and anti-CK903 antibodies with the same concentration as above. Secondary antibodies were the same except they were used at 1:125 dilution. Lastly, the slides were developed with Opal fluorescent dye at 1:50 dilution (Akoya Biosciences) in different channels. Catalog numbers
for HiPlex probes were the following: *Hs-MT-CO1* sense (478051), *Hs-MT-CYB* (582771), *Hs-MT-ATP6* (532961) and *Hs-MT-RNR1* (425961), all from ACD Bio.

**Immunohistochemistry (IHC)**

All single-plex 3,3′-Diaminobenzidine (DAB) based chromogenic IHC on tissue samples (FFPE or fresh frozen) was performed on a Ventana Discovery ULTRA system (Roche). Fresh frozen tissues were air dried at room temperature for 15 minutes, fixed overnight in formalin and rinsed off with dH₂O prior to staining. IHC labeling for c-Myc and ERG was performed with primary antibodies against c-MYC (1:600, Abcam, ab32072) and ERG (EPR3864) (Roche, Cat. No 6478450001), respectively. IHC for EdU and MYC on cells grown on chamber slides was performed as described (48). A primary antibody against BrdU (Abcam, ab136650) was validated to be specific for EdU as well, and used at 1:1000. The primary antibody against c-MYC was used at 1:250 dilution (Abcam, ab32072). The secondary antibodies were PowerVision Poly-HRP anti-rabbit or anti-mouse (Leica, PV6114 and PV6119) and developed with DAB.

**Image Analysis**

Whole slides were scanned at x40 (0.25 um per pixel) on a Ventana DP200 slide scanner (Roche) for chromogenic slides, or a TissueFAXS slide scanner for fluorescent slides (TissueGnostics). All the image processing and analysis were performed using HALO version 3.0 (Indica labs). Single-staining whole slide images in the chromogenic multiplex in situ hybridization and immunohistochemistry assays were fused using the Serial Staining Image Fusion protocol provided by HALO (54). Briefly, after scanning, each set of images were registered. Images were color deconvolved in a case-by-case manner based on red, green, blue (RGB) optical density values for each marker in AEC and for hematoxylin. During this process, the registration transforms generated in the previous step were used to align all slides into the same coordinate system. The pseudocolor single stained whole slide images were then fused with the same image resolution as the original images, and then subjected to downstream analyses. Fluorescent scanning images were exported using TissueFAXS viewer software (TissueGnostics) in single channel images and fused similarly in HALO.
A random forest algorithm in the Tissue Classifier Add-on of HALO was used to distinguish epithelium from stroma and empty tissue compartments. The calculated area of each compartment type was based on the CK8 and hematoxylin status for each slide. CK8 was chosen as it is positive for all normal prostatic epithelial cells, prostatic intraepithelial neoplasia (PIN), primary and metastatic prostate cancer. In needle biopsies from metastatic sites in the liver, in which hepatocytes are CK8-positive, the metastatic carcinoma regions were manually outlined. For samples that were also stained with CK903, the classifier was additionally trained to recognize and calculate the area of prostate basal epithelium vs. luminal epithelium. Classifiers were trained for each slide individually. All classifiers had resolution set at 1.04 μm/px and minimum object size at 20 μm². For radical prostatectomy samples, regions of normal prostatic epithelium, HGPIN and prostate cancer were annotated before analysis. To quantify mtDNA in situ hybridization signals, the Area Quantification FL algorithm (v.2.1.2) and above-mentioned classifiers were used in combination, and percentages of mtDNA signal area (%mtDNA area) in each classified compartment (e.g. prostate basal cells, prostate luminal cells, or total prostate epithelium [basal + luminal cells]) were measured and reported. Since we observed that many of the single punctate hybridization signals (dots) appeared to coalesce into larger punctae, instead of counting individual puncta, we quantified the overall area of mtDNA signals as related to the tissue compartments. This %mtDNA area is strongly correlated with mtDNAcn, as defined by the ratio of mtDNA levels to nuclear DNA levels (48). The quantification algorithm of each image was individually optimized using the real-time tuning function based on both the deconvoluted image and the original chromogenic image.

**Cell lines and cell culture**

PC3, LNCaP, CWR22Rv1 prostate cancer cells (ATCC) were cultured in RPMI1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS) at 37°C and 5% CO₂. Cells were maintained on cell culture flasks (Sarstedt) or CC2 chamber slides (Thermo Scientific, 154739). MYCi975 was originally reported by Han et al. (73) and was obtained from AxonMedChem (Axon 3229).

**Western blotting**

Whole cell extracts from cell lines were obtained using RIPA buffer (Thermofisher) containing a protease inhibitor cocktail (Roche) and benzonase (Thermofisher). Protein concentrations were quantified using a BCA
gold assay (Thermofisher). Equal amounts of total protein were prepared in 4X NuPAGE LDS Sample Buffer (NP0007, Invitrogen) and 5% BME, and boiled at 95°C for 5 min. The protein samples were resolved on 4-12% NuPAGE Bis-Tris gel with protein molecular weight standards (PageRuler Prestained Protein Ladder, 10 to 180 kDa, Thermofisher). The gels were transferred onto PVDF membranes using the Trans-Blot Turbo Transfer System (Bio-Rad). Primary antibodies used in this study included: rabbit anti-c-Myc antibody [Y69] (ab32072, Abcam, 1:1000), mouse anti-β-Actin antibody (3700, Cell Signaling, 1:5000). Fluorescent secondary antibodies used in this study were IRDye 680RD Donkey anti-Mouse IgG Secondary Antibody and IRDye 800CW Donkey anti-Rabbit IgG Secondary Antibody (Licor, 1:5000).

EdU-immunoprecipitated DNA and real time quantitative PCR

To measure newly synthesized mtDNA, we developed a method for EdU-immunoprecipitated DNA and real-time qPCR based on a similar approach by Jiang et al. (101). Briefly, cells grown on flasks were treated with MYCi975 at the indicated concentrations for 48-96 hours. Before harvesting, the cells were incubated in cell culture media containing 10 µM EdU for 4 hours or overnight. Cells were divided into two portions, one for total DNA extraction and the other for total protein extraction. Total DNA was extracted using DNeasy blood and tissue kit (Qiagen). Immunoprecipitation of EdU-containing DNA was performed using the iDeal ChIP-seq kit for Transcription Factors (Diagenode) with the following modifications. For each immunoprecipitation (IP) reaction, 30 µL of washed Protein A-coated magnetic beads was combined with 70 µL IP reaction mix containing 6 µL of BSA, 20 µL 5x iC1b buffer, 0.5 µg antibody in ChIP grade water and incubated for 2 hours at 4°C under rotation. Equal amounts of total DNA (5-15 µg) were added to the reaction mix and incubated overnight at 4°C under rotation. The beads were washed and the DNA was eluted and purified by following the manufacturer's protocol. One percent of the INPUT was subjected to the same elution and purification process. DNA (1 µL) from the purified IP and INPUT samples was used for real time quantitative PCR. TaqMan Universal PCR Master Mix II (no UNG) and TaqMan probes (mitochondrial: MT-RNR1, MT-CO1 and nuclear: BGLT3) were used following the manufacturer's protocol (Thermofisher). EdU-containing newly synthesized DNA was calculated and reported by comparing the relative amount of immunoprecipitated DNA to the INPUT DNA for specific mitochondrial and nuclear genes (% of recovery). The anti-BrdU/EdU antibody (Abcam, ab136650) was used,
and Mouse (G3A1) mAb IgG1 (Cell Signaling, 5415) was used as the negative isotype-matched control for the
IP reactions.

**mtDNA copy number and RNAseq in laser capture microdissected prostate tissues**

Fresh frozen tissues were placed in OCT and sections were cut at 7 μm thickness and mounted onto
membrane slides (Leica, 11600288). LCM of regions of interest was performed using Leica LMD 7000
Microscope in which study pathologists were careful to exclude areas with overt inflammatory infiltrates,
carcinoma, and high grade PIN when isolating normal appearing regions. Tissue digestion and DNA/RNA
extraction was performed using Allprep DNA/RNA Kits following the manufacturer’s recommendations (Qiagen,
802804). Sequencing was performed using Illumina HiSeqX and NovaSeq6000 with Paired End 150 bp x 150
bp read configuration. Trim galore v0.6.3 was used to trim the reads. Bwa v0.7.7 (mem) was used to align to the
hg19 and hg38 human genome builds. Piccard-tools v1.119 & GATK v3.6.0 were used to create a recalibrated
bam file. Bedtools v2.27.1 (genomecov) was used to determine the nuclear coverage (i.e., all chromosomes
except mtDNA) and the mitochondrial coverage. mtDNA copy number was computed using the following formula:
mitochondrial coverage/nuclear coverage. RNAseq and gene expression measures on mtDNA replication
related genes were performed as previously described (102). The analysis of the WGS and RNA-seq data here
is limited to just the reported mtDNA copy number assessment and the gene expression measures for known
mtDNA replication genes. Full details and reporting of the WGS and RNA-seq data will be reported as part of
another study.

**Data availability**

Publicly available ChIP-seq and RNA-seq data on cells treated with MYCi975 were obtained from NCBI
GEO accession number GSM5399497 GSM5399571, GSM5399627, and GSM5399631 (74) and visualized
using the Integrated Genomics Viewer (IGV). RNA-seq data on MYC and TWNK in different cancer types from
TCGA PanCancer Atlas was accessed using cBioportal (103, 104). The RNAseq data from laser capture
microdissected prostate tissues described above have been deposited in NCBI’s Gene Expression Omnibus and
are accessible through GEO Series accession number GSE246067.
**Statistics**

Statistical analysis and visualization was performed using Prism GraphPad 9.0 and R version 4.0.2. For box & whiskers plot, unless specified in the figure legend, boxes present medians with interquartile ranges, and whiskers present min to max and all points are shown. Statistical analysis between two groups was performed using Mann-Whitney test for non-paired samples and Wilcoxon test for paired samples. For more than two sample comparisons, Kruskal–Wallis one-way analysis was used.

**Study approval**

*Human studies.* Human prostate tissues (frozen, or formalin fixed and paraffin embedded [FFPE]) were obtained from radical prostatectomies performed at The Johns Hopkins Hospital. Their use was approved by the Johns Hopkins University School of Medicine Institutional Review Board (NA_00087094). **Supplemental Table 1** summarizes the clinical features of the cases used for in situ hybridization study. **Supplemental Table 2** summarizes the clinical features of the patients participating in the whole genome sequencing and RNAseq study. These samples were part of an NIH-U01 funded study that will be reported upon in detail separately and are referred to hereafter as the U01 samples. Organ donor prostates were obtained from the National Disease Research Interchange (NDRI). For each organ donor specimen, the entire prostate was submitted for histology and was histologically confirmed to be free of invasive adenocarcinoma as well as other significant pathology.

*Animal studies.* All animal study protocols were approved by the Animal Care and Use Committee at Johns Hopkins University School of Medicine.
Author contributions

JC and AMD conceptualized and designed the project. JC, QZ, JBV, JLH, LT, BO, TJ, AV, TCL, IK, RW, MM, SRD, KP, SW, RH, EA, AG, SY and AMD acquired, analyzed and interpreted data. JC and AMD wrote the original draft of the manuscript. All authors were responsible for reviewing and editing the manuscript.

Acknowledgements: We thank Hiromi Sesaki for providing the detailed cytochrome c oxidase and succinate dehydrogenase tissue based enzyme activity assay protocol. We thank Sarah Wheelan for helping with WGS and RNAseq analysis. We thank Alan Meeker for helping with fluorescent whole slide scanning. We thank Karen Sfanos, Luke Mummert and Nate Brennen for their help in collecting prostate samples from organ donors. This work is supported by NIH/National Cancer Institute (NCI) Specialized Programs of Research Excellence (SPORE) in Prostate Cancer grant P50CA58236 (AMD), NIH/NCI grant U01 CA196390 (AMD, SY, KP.), NIH/NCI U54 CA274370 (AMD, SY, KP.), U.S. Department of Defense Prostate Cancer Research Program (PCRP) grant W81XWH-18-2-0015 (AMD); The Johns Hopkins Sidney Kimmel Comprehensive Cancer Center Oncology Tissue Services Laboratory is supported by NIH/NCI grant P30 CA006973; The Patrick C. Walsh Prostate Cancer Research Fund at Johns Hopkins (AMD, SY).
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Figures and Figure Legends

Figure 1. Multiplex chromogenic in situ hybridization for mtDNA and IHC in human prostate. (A) Whole slide scans of each staining round were imported into HALO and registered followed by color deconvolution, scan fusions and application of pseudocolors to the resultant channels. mtDNA in situ hybridization signals are shown in white, epithelial cells (both basal and luminal) stained with CK8 in red, basal cells stained with CK903 in green and nuclei in blue (hematoxylin channel). (B) Markup image of classifier result showing basal cells (green, present in normal), luminal cells (red, present in normal and at all stages of prostate cancer tumorigenesis), stroma (blue) and empty space/lumens (gray). (C) Image analysis results showing higher mtDNAcn in normal basal cells compared to normal luminal cells. The center line in the box shows the median %mtDNA area in each group. N = 7 regions from 3 patients for each cell type. Mann-Whitney test, ***P < 0.001. Original magnification, x120.
Figure 2. Chromogenic in situ hybridization for mtDNA from prostate samples at different stages of prostatic tumorigenesis and image analysis results. Tissues were subjected to multiplex CISH-IHC as in Fig. 1, and images from the round of mtDNA in situ hybridization are shown. (A) Normal appearing prostate glands (inset showing higher hybridization signals in normal basal cells), (B) HGPIN, (C) invasive adenocarcinoma with low mtDNA CISH signals, (D) invasive adenocarcinoma with high mtDNA CISH signals, (E) metastatic prostate adenocarcinoma to the liver. (F) Quantification of mtDNA in epithelium presented as %mtDNA area at each stage. Each symbol in the graph is the average of %mtDNA area from multiple regions in a single patient. N = 16, 13, 15 and 23 patients for each lesion type. Kruskal-Wallis test followed by Dunn’s test for nonparametric pairwise multiple comparisons, Normal vs. HGPIN **P = 0.0039, Normal vs. Primary PCa *P = 0.0125, Normal
vs. mCRPC ****P < 0.0001, and Primary PCa vs. mCRPC P = 0.1005. Note that images shown in A-D were from the same patient. Original magnification, x200 (A-E), x400 (inset in A).
Figure 3. Increased mtDNAcn in prostate cancer using whole genome sequencing. (A) Combined dot and box and whisker plot showing increased mtDNA in tumor samples. (B) Increased mtDNAcn was present across all Gleason grade groups (GG; GS 3+3=GG1, GS 3+4=GG2, GS 4+3 = GG3 and 4+4 = GG4; GS9-10=GG5). N = 10, 36, 35 and 33 for GS 6, GS 3+4, GS 4+3 and 4+4, GS 9-10, respectively. Note that GG3 and GG4 are combined due to the relatively low number of GG4 (4+4=8) cases. For benign vs cancer, Wilcoxon test, all cases ****P<0.0001, GS6 P = 0.0645, GS 3+4 **P = 0.0013, GS 4+3 and 4+4 ***P = 0.0005, GS 9-10, **P = 0.0024. Blue dots represent benign samples, and red dots represent cancer samples.
Figure 4. Increased mtDNAcn was accompanied by elevated mtRNA expression as well as MYC protein expression in the prostate. (A) Representative images of simultaneous multiplex detection and visualization of mtDNA and mtRNA(s). (B) Image analysis results of 3 mtRNA(s) expression in cancer regions compared to the adjacent benign tissues. Each symbol represents an analyzed region of interest. For benign vs cancer, Mann-Whitney test, \( MT\text{-ATP6} \ **P = 0.0019, \ MT\text{-RNR1} \ **P = 0.0021, \ MT\text{-CYB} \ ***P = 0.0005. \) (C) Correlations between mtDNAcn and mtRNA expression by image analysis. (D) IHC for MYC from an adjacent frozen section of prostate tissue from the same tissue shown in A. Original magnification, \(~x70.\) Scale bar = 200 \( \mu m \) (A). \( N = 6 \) patients.
Figure 5. Mouse mtDNAcn is increased in prostates of the Hi-MYC mouse model. H&E images (A and D) showed the enlarged nuclei and nucleoli, as well as cytoplasmic hyperchromasia characteristic of the mouse PIN lesions after MYC overexpression. Through chromogenic in situ hybridization, human MYC mRNA (B and E) and mtDNA levels (C and F) were visualized (positive signals are brown). Note the tight spatial correlation with the appearance of cytoplasmic MYC signals, corresponding to human MYC mRNA expression, and increased mtDNA signals. Also note that the MYC probe hybridizes to all nuclei in the transgenic mice as a result of binding to the transgene present in all of the Hi-MYC mouse cells. Original magnification, ×70 (A-C), ×200 (D-F). N = 5 mice.
Figure 6. The decrease in MYC in response to SPA correlates with decreased mtDNAcn in mCRPC. (A) Each pair of bars shows the percentage change from the baseline biopsy in the percentage of MYC positive cells (by IHC staining for MYC protein) among all epithelial tumor cells by image analysis to the percentage change in mtDNA area by image analysis. There was a highly significant correlation (P=0.0021, $R^2=0.63$). (B) Representative images (patient #3) of MYC-IHC and mtDNAcn-ISH for patients at the pretreatment time point and after 3 cycles of BAT. Notice that with both markers, image analysis was only performed in the epithelial compartments. MYC protein and mRNA quantification was reported previously for these cases (51). Original magnification, ~x200. N = 12 patients.
Figure 7. MYC activity controls mitochondrial DNA synthesis. (A) Representative images showing that the Anti-BrdU antibody staining is only present after PC3 cells are pretreated with EdU overnight, demonstrating its specificity for EdU. Cells grown on chamber slides were metabolically labeled with EdU or no EdU and were then fixed and stained by IHC. Note the complete absence of staining in the middle panel in cells without EdU treatment and the marked reduction in cytoplasmic signals after treatment with the mtDNA replication inhibitor, ddC (right most panel). (B-C) When MYC activity and/or levels were decreased using MYCi975, decreased EdU (after 4 hour incubation) incorporation into cytoplasmic or mitochondrial foci was observed by IHC (B) and separately by EdU immunoprecipitation followed by qPCR in PC3 cells (C). n = 2 independent biological replicates in IP. Representative Western blot results confirmed the decrease in MYC after drug treatment (C) in the samples subjected to IP. Original magnification for IHC images, x400 (A), x200 (B).
Figure 8. Genes encoding proteins required for mtDNA replication are upregulated in prostate cancer from human laser capture microdissected samples as determined by RNAseq. (A) Heatmap showing upregulation of transcripts in genes encoding mtDNA replication factors. Top line shows the Gleason grade group and the second line shows the patient race. ERG, AMACR, serve as controls for known upregulated genes in prostate cancer and TP63, KRT5, KRT15 and GSTP1 serve as controls for known downregulated (basal cell...
enriched) genes in prostate cancer. Genes directly involved in mtDNA replication are TFAM, POLG (mtDNA polymerase), POLG2, C10orf2 (TWNK), SSBP1, LIG3, PRIMPOL and TOP3A. (B) TWNK/C10orf2 mRNA was increased in cancer compared with benign. Boxes show medians with interquartile ranges, whiskers: 1.5 * interquartile range. (Wilcoxon test, P<0.0001). (C) Correlation of TWNK/C10orf2 mRNA with MYC mRNA. (D) Correlation of TWNK/C10orf2 with Hallmark MYC pathway scores.
Figure 9. Increased mtDNAcn is commonly found in other cancer precursor lesions. (A) Representative in situ hybridization results of a normal pancreatic duct versus a PanIN. Note that the relatively high mtDNAcn seen does not reflect high levels of proliferation in the normal appearing pancreatic acinar cells but likely reflects the high energetic needs of these cells. (B) Quantification of mtDNAcn in normal ducts and PanINs using image analysis. Each symbol represents a region of the tissue type specified. Mann-Whitney test, ****P < 0.0001. (C-E) Representative images of mtDNA in situ hybridization and MYC IHC in a colon sample containing an adenomatous polyp. Boxed areas in the left-most images in C and E are shown as higher magnification in the middle and right panels. Boxed areas in the middle panel of C are shown in D to illustrate the differential mtDNA levels along the surface (left) versus bases (right) of colonic crypts. Original magnification, x200 (A), x20 (C-D), x40 (middle and right panels in C and E), x400 (D). ****P < 0.0001. N = 11 and 8 for patients for PanIN and colon adenoma, respectively.