OXPHOS remodeling in high-grade prostate cancer involves mtDNA mutations and increased succinate oxidation

Bernd Schöpf1, Hansi Weissensteiner1, Georg Schäfer2, Federica Fazzini1, Pornpimol Charoentong3, Andreas Naschberger1, Bernhard Rupp1, Liane Fendt1, Valesca Bukur4, Irina Giese4, Patrick Sorn4, Ana Carolina Sant’Anna-Silva5, Javier Iglesias-Gonzalez6, Ugur Sahin4, Florian Kronenberg1, Erich Gnaiger5,6 & Helmut Klocker7

Rewiring of energy metabolism and adaptation of mitochondria are considered to impact on prostate cancer development and progression. Here, we report on mitochondrial respiration, DNA mutations and gene expression in paired benign/malignant human prostate tissue samples. Results reveal reduced respiratory capacities with NADH-pathway substrates glutamate and malate in malignant tissue and a significant metabolic shift towards higher succinate oxidation, particularly in high-grade tumors. The load of potentially deleterious mitochondrial-DNA mutations is higher in tumors and associated with unfavorable risk factors. High levels of potentially deleterious mutations in mitochondrial Complex I-encoding genes are associated with a 70% reduction in NADH-pathway capacity and compensation by increased succinate-pathway capacity. Structural analyses of these mutations reveal amino acid alterations leading to potentially deleterious effects on Complex I, supporting a causal relationship. A metagene signature extracted from the transcriptome of tumor samples exhibiting a severe mitochondrial phenotype enables identification of tumors with shorter survival times.
Prostate cancer (PCa) is the most prevalent male non-cutaneous cancer type in Western countries, accounting for an estimated 10% of all cancer related deaths in Europe. Recent studies suggest a multifactorial etiology encompassing an accumulation of genetic and epigenetic aberrations. Although primary PCas has been extensively studied only few genomic aberrations including PTEN deletion, TMPRSS2-ERG fusions, and CDKN1B deletion but no driver mutations have been found. Among other carcinogenic alterations, adaptations in metabolism and energy turnover might contribute to PCa formation and progression.

Alterations in mitochondrial (mt) metabolism including oxidative phosphorylation (OXPHOS) are a hallmark of cancer. Mitochondria play an important role during tumorigenesis by orchestrating cellular energy transformation, apoptosis, and reactive oxygen species (ROS) signaling. The bulk of cellular ATP is produced in the mitochondria by the stepwise oxidation of substrates via the tricarboxylic acid (TCA) cycle. Electrons are fueled into the mt-electron transfer system (ETS), catalyzed by NADH and succinate-linked dehydrogenases in the mt-matrix and mt-inner membrane. Translocation of protons generates an electrochemical potential difference across the mt-inner membrane, which is used by ATP synthase. While the majority of ETS machinery proteins are encoded by nuclear DNA (nDNA), 13 ETS subunits are encoded by mtDNA, a small circular genome. mtDNA mutations have been linked to PCa formation and progression. Compared to nDNA, mtDNA exhibits a higher mutation rate caused by increased exposure to ETS-derived ROS and less efficient DNA repair. Random segregation and subpopulation replication of mtDNA variants lead to “heteroplasmy” (HP), the presence of different populations of mtDNA variants in a mitochondrion, cell or tissue. mtDNA mutations are frequently found in localized PCa; however, their functional consequences remain elusive.

Only limited data on TCA cycle and OXPHOS activity in primary PCa tissue is available and little is known about specific malignant PCa metabolism. Understanding the impact of mtDNA mutations might help to characterize metabolic adaptations exploited to drive PCa formation and progression. In this study, we aim to unravel the interplay between mtDNA mutations, the expression and function of mitochondrial enzymes and the metabolic PCa phenotype to identify specific mitochondrial signatures guiding towards new approaches for therapeutic intervention. Our results reveal a shift towards higher oxidation of succinate, which is associated with deleterious mutations in mitochondrial Complex I genes and a rewired expression of mitochondrial metabolic enzymes in primary prostate cancer.

Results
Shift to succinate-driven OXPHOS in prostate tumors. Paired benign and malignant prostate tissue samples were isolated after radical prostatectomy from fifty PCa patients. Benign samples were taken distant from the tumor to minimize field effects (Fig. 1a). Each biopsy was split for histopathological diagnostics (Fig. 1b) and high-resolution respirometry (HRR). See Table 1 for patient and tumor characteristics. OXPHOS was analyzed simultaneously in paired benign/malignant tissue biopsies of each prostate specimen by sequentially assessing respiratory coupling and capacities of single and combined mt-electron transfer (ET) pathways (Fig. 2a–c, Supplementary Tables 1–2). A short-term treatment with H2O2 was included to simulate oxidative stress.

Benign tissues showed a significantly higher NADH-pathway OXPHOS capacity (N; electron entry to the Q junction via CI) supported by glutamate&malate, GMI2 and pyruvate&glutamate&malate, N (PGM2), respectively, compared to PCa tissue \( p = 2 \times 10^{-5} \) and \( p = 8 \times 10^{-6} \), two-sided paired-samples t-test, Fig. 2d). However, upon further addition of succinate (S), no differences in the OXPHOS (NSp) and ET (NSr) respiratory capacities were observed, indicating compensation of the N-pathway deficiency by increased convergent electron transfer through the succinate-pathway (electron entry to the Q junction via CII). Full restoration of NSp and NSr capacities in tumor tissue was driven largely by succinate and to a smaller extent by pyruvate. Glutamate&malate-driven OXPHOS by addition of ADP triggered an increase of O2 flux of 2.4 pmol s\(^{-1}\) mg\(^{-1}\) in tumor compared to 4.5 pmol s\(^{-1}\) mg\(^{-1}\) in benign tissue samples (Fig. 2e). Addition of pyruvate and succinate, respectively, elicited

![Fig. 1 Sample workflow and tissue sample confirmation.](image-url)

- **a** From each of 50 radical prostatectomy specimens a tumor and a non-malignant benign tissue punch needle biopsy was extracted by an experienced uropathologist from contralateral sites of the specimens (blue/red circles). While a small portion of the extracted tissue cores was fixed and used for histological stains (pink arrow) and confirmation of tissue identity, the rest was used immediately for high-resolution respirometry (HRR, blue arrow), subsequent NGS mtDNA profiling (orange arrow) and mtDNA copy number determination (gray arrow). From 16 cases of this cohort, frozen tissue samples directly adjacent to the biopsy cores (green area) were isolated by macrodissection followed by RNA extraction for gene expression profiling via total RNA-NGS (green arrow). Representative hematoxylin and eosin staining (H&E) and p63 (non-malignant tissue marker, brown)/AMACR (malignant cell marker, red) double-immunostaining (p63/AMACR) of fixed and paraffin-embedded benign and malignant prostate tissue samples extracted for HRR. One representative of 50 cases is shown. Scale bars indicate 2000 μm (H&E, P63/AMACRA stains) and 1000 μm (P63/AMACR higher magnification), respectively.
Table 1 Patient and tissue sample characteristics.

| Characteristic                        | Patient [a] | Tissue sample wet mass (mg) |
|---------------------------------------|-------------|-----------------------------|
| Age [a]                                | 62.7 ± 8.0 (39.9–73.4) | 6.49 ± 1.48 (4.0–9.0)     |
| PSA (ng mL⁻¹)                          | 12.7 ± 27.2 (2.0–181.6) | 6.51 ± 1.46 (4.0–9.0)     |
| PSA (%)                                | 14.3 ± 5.8 (3.6–28.1)   |                             |
| Prostate weight (g)                    | 44 ± 14 (20–79)         |                             |
| Pathological stage, N (%)              | 25 (50.0)               |                             |
| pT2 (localized)                       | 21 (42.0)               |                             |
| pT3 (extracapsular extension)          | 8 (8.0)                |                             |
| pT4 (invasion into adjacent structures)| 4 (8.0)                |                             |
| Tumor histology PCA tumor samples, N (%)| 11 (22.0)               |                             |
| Gleason score 6 (patterns 3 + 3)       | 11 (22.0)               |                             |
| Gleason score 7 (patterns 3 + 4)       | 9 (18.0)                |                             |
| Gleason score 7 (patterns 4 + 3)       | 21 (42.0)               |                             |
| Gleason score 8 (patterns 5 + 3)       | 1 (2.0)                 |                             |
| Gleason score 9 (patterns 4 + 5)       | 6 (12.0)                |                             |
| Gleason score 10 (patterns 5 + 5)      | 2 (4.0)                 |                             |
| Tissue sample wet mass (mg)            | 4.69 ± 1.48 (4.0–9.0)   |                             |

Paired malignant and non-malignant tissue biopsy samples were extracted from the rectal prostatectomy specimens by an experienced uropathologist. Age, total serum prostate specific antigen (PSA) and percentage of free PSA (fPSA) at the time of tumor diagnosis. Tissue biopsy wet mass was measured in high-resolution respirometry (HRR) was determined prior to the transfer of permeabilized tissue samples into the O2k chambers. Tumor histology characteristics of radical prostatectomy specimens (Gleason scores of tissue samples and pathological stages) were determined using routine histopathological procedures. Data represent mean ± SD, median (age), range or quantity and percentage, respectively.

significantly higher increases of O₂ flux in malignant compared to benign samples (Np, minus GMPₚ, NSp, minus NP) and thus recovered full respiratory capacity in tumors (Fig. 2e). Finally, CI inhibition by rotenone confirmed significantly higher N-pathway capacity of benign compared to tumor tissue (NSGₚ minus SGₓ, Fig. 2e). Oxidative stress resulted in a reduction of O₂ flux in both tissue types, however, significantly more in the tumor tissue (1.7 vs. 1.1 pmol s⁻¹ mg⁻¹, p = 0.004, two-sided paired-samples t-test, Fig. 2e, GMPₚ minus GMPₚ, pre).

Differences in OXPHOS capacities were mainly driven by high-grade tumors (Gleason score >7), Fig. 2f, g). Whereas relative respiration (FRC) of low-grade tumors (Gleason score ≤7) was similar to benign tissue, it was significantly different in the high-grade tumors (Fig. 2f). A direct comparison of specific O₂ fluxes confirmed a significant reduction of N-pathway respiration (Fig. 2g—effects of ADP and rotenone) and increase of pyruvate and especially succinate respiration (Fig. 2g—effects of pyruvate and succinate) in the high-grade tumors. Altogether, HRR analysis of paired benign/cancer tissue samples uncovered a substrate shift from glutamate&malate to succinate and to a lesser extent pyruvate in tumors, particularly in the high-grade tumors, and higher vulnerability of tumors by oxidative stress.

Several control experiments were performed. Split tissue samples (N = 6) analyzed with or without the oxidative stress step indicated a consistent difference through all post-treatment states (Supplementary Fig. 1a). O₂ flux differences were reduced only for the oxidative stress step (Supplementary Fig. 1b) and analysis of N- and NS-pathway capacities without prior H₂O₂ treatment confirmed the differences between benign and tumor samples (Supplementary Fig. 1c–d). Although the extent of inhibition of O₂ flux by oxidative stress was higher in tumor tissue, it remained consistent through all substrate/coupling states within the benign and the malignant samples compared to untreated samples (Supplementary Fig. 1e). Finally, measurement of the single enzymatic step of CIV indicated no selective inhibition by H₂O₂ (Supplementary Fig. 1f, compare to Supplementary Fig. 1e–BE). Comparison of benign/benign paired samples confirmed that the observed respiratory differences between benign and malignant samples were not caused by tissue heterogeneity or sampling bias. Twenty paired benign/benign samples subjected to the same analysis protocol as benign/malignant samples showed no differences (Supplementary Fig. 2a–c). There were also no differences between zonal origins of benign samples (medial or posterior peripheral zone or transitional zone) (Supplementary Fig. 2c–e).

Increased mtDNA mutation load in primary human PCa tissue. We sequenced the mtDNA genome of all tissue samples recovered from HRR analysis and assessed a potential link of respiratory patterns and HPs in ET-protein or tRNA genes. Average mtDNA sequence coverage was >10,000 fold (Supplementary Fig. 3). Mutations with HP levels of higher 2% were considered for further analysis. Details of all 147 HPs found across the sample pool, including their presence and pathological context listed in the MitImpact 2.9 database¹⁵, are presented in Supplementary Data 1. Private mutations, defined as HPs found only in one tissue type, were more frequent in the malignant samples (Fig. 3a). In benign samples 33 private HPs were found compared to 84 in the malignant tissues. Significantly more HPs were located in the mtDNA coding region of cancer tissue (73 vs 23, odds ratio=2.7, p = 0.007, Fisher’s exact test), whereas HP frequency in the non-coding control region was similar in benign and cancer tissues (10 vs 12) reflecting an accumulation of HPs in the coding mt-genome of the tumors (Fig. 3b). Additionally a higher frequency of non-synonymous HPs in all ET-protein-coding genes (MT-ND1-6 = CI, MT-CO1-3 = CIII, MT-CYB = CIII and MT-ATP6-8 = F₀F₁-ATPase) was detected in the malignant tissue samples (38 vs 10) while the number of mt-tRNA mutations (MT-T = tRNA) was the same (2 vs 2, Fig. 3c). Thirty five HP sites in the malignant samples showed an allele frequency (AF) >10% compared to only 11 in the benign samples, while 15 of the HPs detected in the malignant samples showed an AF >50% compared to only 5 in the benign samples (Fig. 3d). Consequently, overall HP levels were significantly higher in malignant compared to benign tissue samples (p = 0.03, Wilcoxon rank-sum test, Fig. 3e). Almost half (46%) of the tumor samples harbored ≥2 HPs while 60% of the benign samples harbored no HP (Fig. 3f). The number of HPs per mt-gene was correlated to gene size as shown for MT-ND genes (Pearson’s r = 0.93, p = 0.004, two-tailed t-test, Fig. 3g), suggesting no cancer-specific mutational “hotspots” in primary PCa.

Of all non-synonymous mutations in protein coding genes, only two variants (T15719C and T10551C) have been previously detected in human cancers and only two variants were associated with a specific clinical phenotype according to MitoMap, maternally inherited diabetes-deafness syndrome (MIDD; G3421A) and developmental delay, seizure and hypotonia (G4142A) (Supplementary Data 1). Only the T10551C mutation (S28P in ND4L protein), exhibited a high HP level (58%) while the allele frequencies of all other variants was below 20% in our samples. Of the four mutations in mt-tRNA (MT-T) genes (Supplementary Data 1, Supplementary Fig. 4a), one variant (G15995A, MT-TP, tRNAPro) has been previously detected in a cystic fibrosis patient¹⁶ and was classified as “likely pathogenic” by Mitotip (https://www.mitomap.org/cgi-bin/mitotip). This mutation leads to a G>A substitution in a highly conserved region of the anticodon-stem, resulting in a base-pair mismatch with likely effects on RNA folding and stability (Supplementary Fig. 4b). However, the frequency of this tRNA variant (15%) most certainly does not impact mt-function¹⁷.

To evaluate the functional relevance of the mtDNA variants we determined the MutPred Pathogenicity score¹⁸,¹⁹ for all
non-synonymous HPs (Fig. 3h). While only 14% of HPs of benign samples exhibited a high MutPred score (>0.75), half of the HPs of malignant samples fell into this category (Fig. 3i).

Non-synonymous mtDNA mutations in high-grade tumors. To evaluate a correlation of clinical parameters and mtDNA mutation frequency a logistic regression analysis was performed (Supplementary Table 3). Overall mtDNA mutation load correlated significantly with increasing patient age \( (p = 0.04, \text{likelihood-ratio test}) \), in line with recent reports\(^{9,20,21} \) and with lower free to total PSA ratios (IPSA%, \( p = 0.05, \text{likelihood-ratio test} \), Supplementary Table 3). A low IPSA% value is a prognostic indicator of poor prognosis\(^{22} \). While a correlation trend was detected for the pathological tumor (pT) stage \( (p = 0.09, \text{likelihood-ratio test}) \), we found no significant association with histological tumor (Gleason) grade or total serum PSA, contrary to previous findings\(^{23} \). When looking only at non-synonymous mtDNA mutations, however, their frequency correlated with high-grade staging (Gleason > 7, \( p = 0.02, \text{likelihood-ratio test} \)) and again with lower IPSA% \( (p = 0.05, \text{likelihood-ratio test}) \). Thus, non-synonymous HPs with a potential impact on ET-function tended to accumulate in younger patients with unfavorable clinical characteristics.

Deleterious mtDNA mutation load and OXPHOS capacity. While increasing numbers of potentially deleterious mtDNA mutations in cancer samples are reported\(^{9-12,21,23} \), their functional significance is rarely evaluated. We correlated the frequency and type of mtDNA mutations with functional data for each sample. Non-synonymous mutations in genes relevant for

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**Fig. 2 High-resolution respirometry of prostate tissue samples.** 

**a** Coupling/pathway control diagram showing the sequential steps in the substrate-uncoupler-inhibitor titration (SUIT) protocol with different coupling states. See Supplementary Tables 1-2 for HRR protocol details. 

**b** Representative HRR traces with permeabilized tissue. Red line (left Y-axis): wet mass-specific \( \text{O}_2 \) flux (oxygen consumption) \( (\text{pmol g}^{-1} \text{min}^{-1}) \). Blue line (right Y-axis): \( \text{O}_2 \) concentration \( \left[ \text{O}_2 \right] \) (µM). 

**c** Schematic representation of mitochondrial electron transfer from NADH-linked substrates through Complexes I, II, and IV (N-pathway) and from succinate through CII, CIII, and CIV (S-pathway). 

**d** A Schematic representation of respiratory capacity in benign (blue, \( N = 50 \)) versus malignant (red, \( N = 50 \)) tissue samples: OXPHOS-capacity \( (\text{GM}_p, N_p \text{ and } N_{NS_p}) \) and ET-capacity \( (N_{S_p} \text{ and } S_p) \). 

**e** Effects of substrates GM, pyruvate, succinate, oxidative stress, uncoupler FCCP, and CI inhibitor rotenone on \( \text{O}_2 \) flux in benign (blue, \( N = 50 \)) and malignant (red, \( N = 50 \)) tissue samples. 

**f** Normalized respiratory capacities of high-grade tumor (Gleason > 7, dark red, \( N = 10 \)) and low-grade tumor (Gleason ≤ 7, light red, \( N = 40 \)) compared to benign samples (blue, \( N = 50 \)). 

**g** Effects of substrates, oxidative stress, uncoupler, and CI inhibitor on \( \text{O}_2 \) flux in high-grade (light red, \( N = 40 \)) and high-grade (dark red, \( N = 10 \)) tissue samples. Data in (d-g) are presented as mean values ± SD. Statistical differences were tested using two-tailed paired-samples test \( (\text{d-e}) \), one-way ANOVA followed by Tukey’s HSD \( (\text{f}) \) or Wilcoxon rank-sum test \( (\text{g}) \), respectively. Correction for multiple testing was performed using the Bonferroni-Holm procedure. Source data are provided as a Source Data file.
Fig. 3 mtDNA heteroplasmies in benign and malignant tissue samples. a Levels and locations of HPs in benign (blue, N = 50) and malignant (red, N = 50) samples across indicated loci of the mtDNA. The regions shown include the non-coding control region (CR) and the 13 protein coding genes, marked by colored boxes. ND, CO and ATP refer to genes coding for subunits of Complex I (CI); NADH:ubiquinone oxidoreductase), Complex IV (CIV; ferrocytochrome c:oxygen oxidoreductase) and ATP synthase, respectively, whereas CYB encodes a subunit of Complex III (CIII; ubiquinol:ferrocytochrome c oxidoreductase). b Total cumulative count of private mutations, located in either the non-coding D-loop or coding areas of the mt-genome in benign (blue, N = 50) and malignant tissue samples. Differences were tested for significance using Fisher’s exact test. c Incidence of synonymous (Syn) vs non-synonymous (Non-syn) HPs located in protein coding sequences (FOF1-ATP synthase – CV; tRNA genes = tRNA) found in either benign (blue, N = 50) or malignant (red, N = 50) tissue samples. d Detailed proportions of samples harboring variants with defined HP levels (<10%, 10–20%, 20–50% and >50%) in the benign (blue, N = 50) and malignant (red, N = 50) samples. e Boxplot of HP levels comprising all heteroplasmies in benign and malignant tissue samples. Data are presented as boxplots indicating median, 25–75th percentile (box) and median ± 1.5IQR (whiskers), minimum and maximum values (dots). Differences in mean values were tested for significance using Wilcoxon rank-sum test. f Numbers of HPs found per sample in benign (blue circle, N = 50) and the malignant (red circle, N = 50) samples. g Correlation of gene size in kbp to HP count as detected in the mt-ND genes of malignant samples (N = 50). Linear correlation was established using Pearson’s correlation coefficient (r) while correlation was tested for significance using two-tailed t-test. h MutPred Pathogenicity Scores of all non-synonymous HPs in benign (blue, N = 50) and malignant samples (red, N = 50) identified across the mtDNA. The size of the spots indicates the likely functional effects. i Proportion of benign (blue, N = 50) and malignant (red, N = 50) samples carrying HPs with defined MutPred Pathogenicity scores reflecting their likely functional effect. Source data are provided as a Source Data file.

the assembly or function of the OXPHOS machinery (MT-ND, MT-CO and MT-CYB genes) were considered as potentially deleterious. Samples carrying such mutations showed a significant decrease of relative GM-pathway capacity in both benign and malignant samples (p = 0.005 and 0.004, respectively, Wilcoxon rank-sum test, Fig. 4a). In contrast, mutations in the D-Loop exerted no effects (Fig. 4b). GM-pathway capacity was reduced only in samples carrying non-synonymous mutations in CI genes (MT-ND1-6) but not in those with CIII or FoF1-ATPase (COI-III, CYB and ATP6-8) gene mutations (p = 0.0004; one-way ANOVA followed by Tukey’s HSD test, Fig. 4c).

Even moderate levels of deleterious mtDNA mutations in CI genes can lead to significant functional effects24,25. To evaluate the impact of increasing HP levels on CI-function in detail, we divided malignant samples into subgroups exhibiting a HP level of either 30–60% or >60%. Higher HP levels were associated with a more pronounced reductions in respiratory N-pathway capacity (CI-dependent) and correspondingly with a higher proportion of S-pathway capacity to retain full aerobic ATP production (Fig. 4d). In the samples exhibiting the highest HP loads (>60%, N = 4), N-pathway capacity with glutamate&malate was reduced to 16% of total NS-OXPHOS capacity. Comparison of the respiratory capacities in samples harboring high levels of non-synonymous HPs in either CI or CI genes only (Fig. 4f) showed similar N-pathway capacity while significantly higher S-pathway capacity was observed in the group harboring CI mutations.
with different degrees of N-pathway inhibition is shown for the benign (blue) or malignant (red) samples carrying either no or only synonymous HPs (N\(_{\text{BE}}\) = 36 and N\(_{\text{CA}}\) = 23) versus samples carrying non-synonymous HPs (N\(_{\text{BE}}\) = 14 and N\(_{\text{CA}}\) = 27) in the coding regions of the mt-genome. Values present mean ± SD. Differences in mean values were tested for significance using Wilcoxon rank-sum test. b Comparison of relative GM-OXPHOS capacity in samples without mutations (N\(_{\text{BE}}\) = 38 and N\(_{\text{CA}}\) = 37) versus samples with mutations (+) (N\(_{\text{BE}}\) = 12 and N\(_{\text{CA}}\) = 13) within the non-coding (control) region of the mt-genome. Values represent mean ± SD. c Impact of location of non-synonymous HPs on relative GM-OXPHOS capacities. Tumor tissue samples were categorized according to no HPs (–) (orange; N = 24), and HPs located in genes encoding proteins of CIII-CV (CO, ATP, CYB; green; N = 10) or in genes encoding proteins of CI (ND1-NDS; blue; N = 20). d-e Relative GM-OXPHOS (GM\(_{\text{p}}$/NS\(_{\text{p}}\)) and relative S-ET (S\(_{\text{p}}$/NS\(_{\text{p}}\)) respiratory capacities in malignant tissue samples harboring non-synonymous HPs in CI-coding mt-genes. Tumors were grouped into samples carrying no non-synonymous HPs (–); N = 23), samples with variant levels of 30–60% (30–60%; N = 6) and samples with variant levels >60% (>60%; N = 4). Differences in mean values were tested for significance using one-way ANOVA followed by Tukey’s HSD test. f N-pathway (blue) and S-pathway (orange) respiratory capacities in malignant samples harboring high-level (>60%) non-synonymous HPs in either CI-coding genes (N = 4) or CI-coding genes (N = 4). Differences of in mean values were tested for significance using Wilcoxon rank-sum test. Values presented in c-f represent mean values and individual data points. g S-pathway OXPHOS capacity upregulation by partial inhibition of N-pathway oxidative flux in benign (RWPE1, N = 3; EPI56T, N = 3) and malignant (PC3, N = 6; LNCaP, N = 4; DuCaP, N = 3, N represents number of biologically independent experiments) prostate cell lines. Relative S-pathway OXPHOS capacity (normalized to total respiratory capacity, NS\(_{\text{p}}\)) with different degrees of N-pathway inhibition is shown for the five cell lines. Values represent mean ± SD. Source data are provided as a Source Data file.

\(p = 0.002\), Wilcoxon rank-sum test) indicating that a defect in CIV will affect total respiration, while defects in CI can be sufficiently compensated.

To address whether reduction of N-pathway capacity triggers a respiratory pathway shift we subjected three malignant and two non-malignant prostate cell lines to HRR analysis and applied low concentrations of rotenone to partially inhibit NADH-linked respiration. In all cell lines partial inhibition ranging from 40 to 90% elicited a compensatory N→S-pathway shift in the OXPHOS state despite unchanged S-pathway capacity (Fig. 4g). Compensation ranged from 50 to more than 100% in the different cell lines. These results indicate mobilization of an S-pathway OXPHOS capacity reserve upon reduction of N-pathway oxidative flux in order to keep total OXPHOS capacity high.

A structural basis for the effect of CI mutations. To characterize the molecular and biochemical mechanisms by which the CI mutations could affect respiration, an in-silico assessment of the potential structural changes in CI was performed, considering mutations exhibiting critical HP levels of ≥30% (Fig. 5a, b). We analyzed the structural impact of these mutations on the basis of the CI subunit in the recently published cryo-EM structure of the human respiratory supercomplex SC I\(_2\)II\(_3\)III\(_2\)IV\(_2\).

To illustrate potential structural consequences of mutations, two examples exhibiting a very high (>80%) variant level and severe alteration of the HRR profile were examined in detail: the variant T11991C, recently also detected in a PCA tissue sample (COSM1132242, [https://cancer.sanger.ac.uk/cosmic/mutation/overview?id=1132242]) leads to substitution of a large hydrophobic, aromatic amino acid by a small, polar amino acid (F411S)
interrupting a hydrophobic interaction network based on Pi-stacking between conserved helices in the ND4 domain of CI (Fig. 5c). The variant A13495G in the ND5 gene leads to the loss of a polar residue within the loop of the discontinued helix 12 in the central axis of CI membrane domain (T387A). This part of the structure was annotated as flexible region that might play an important role initiating local conformational changes necessary to position corresponding key residues in the central axis (e.g. K392) during proton pumping. A hydrogen bond is lost due to the T387A mutation in this important region (Fig. 5d). HRR analysis of these variant tumor samples revealed very low N-pathway capacity (PGM; pyruvate & glutamate & malate) <10% of combined NS-driven respiration compared to the corresponding benign sample (Fig. 5e, f). N-pathway capacities of the malignant biopsies harboring these variants were decreased by >70% (Fig. 5g, h), indicating a causal role.

**Increased mtDNA content and mt-mass in high-grade tumors.** Having found significant differences in mt-bioenergetics and mtDNA mutations between benign and malignant prostate tissue we examined whether mtDNA copy number (mt-CN) or mt-mass might be altered. Duplex mtDNA/nDNA PCR revealed a median mt-CN load of 310 per nuclear genome (range: 172–698) in the benign and 303 (range: 143–645) in the malignant tissue samples, indicating no meaningful difference (Fig. 6a). While it has been proposed that mt-CN constitutes a reliable marker for mt-CN ratios had significantly higher mt-CNs compared to samples carrying no HPs (p = 0.03; Wilcoxon rank-sum test, Fig. 6c). mt-CN ratios
in the high-grade (Gleason score >7) were significantly increased compared to low-grade (Gleason score ≤7) tumors (p = 0.002; Wilcoxon rank-sum test, Fig. 6d). This suggests an increase of mtDNA load with higher malignancy and potentially deleterious mtDNA mutations.

To assess differences in mt-mass 24 cases showing varying N- pathway respiratory capacities were analyzed by immunohistochemistry. Consecutive tissue sections were immunostained with antibodies directed to the mt-mass marker porin (VDAC1, voltage-dependent anion channel 1), the CI marker NADH dehydrogenase (ubiquinone) iron-sulfur protein 4 (NDUFS4), or the CII marker succinate dehydrogenase complex subunit A (SDHA). Compared to the corresponding benign tissues all immunomarkers were significantly increased in the tumors (Fig. 6e–g, Supplementary Fig. 5). In tumors harboring high HP level non-synonymous mutations in CI genes the CI/CII marker ratios were significantly decreased whereas mt-mass did not differ (Fig. 6h, Supplementary Fig. 6a–h).

Transcriptome mirrors altered OXPHOS capacity. Quantitative mRNA expression analysis via NGS was performed for 16 representative paired tumor/benign samples selected to mirror the HRR properties of the whole sample cohort (Supplementary Table 4, Fig. 7a, compare to Fig. 2g). We analyzed 1158 nuclear genes related to mt-function and metabolism based on MitoCarta 2.0.\(^1\) Comparison of expression of these genes between benign and malignant samples identified 512 differentially expressed genes (false discovery rate, FDR <0.01, Fig. 7b). A Pathway Over-Representation Analysis using the InnateDB Pathway online tool\(^{31}\) allocated differentially expressed genes to amino acid and carbohydrate metabolism, pyruvate metabolism, and the OXPHOS, TCA-driven pyruvate degradation and succinate pathways. Expression of key genes related to OXPHOS, TCA-driven pyruvate degradation and succinate provision, and of enzymes involved in ROS detoxification were upregulated in the malignant samples (Fig. 7d). Mt-pyruvate carrier 2 (MPC2) and subunits of pyruvate dehydrogenase (PDHX, PDK1, and PDP2) drive mitochondrial allocation of...
pyruvate and conversion into lactate or acetyl-CoA (Ac-CoA). Citrate synthase (CS) and subunits of mt-isocitrate dehydrogenase (IDH3) promote conversion of Ac-CoA to citrate and ultimately TCA-driven oxidation of pyruvate into α-ketoglutarate. Mt-(SLC25A10) and cytoplasm membrane (SLC13A3) dicarboxylate transporters, two succinate dehydrogenase subunits (SDHB, SDHC) along with subunits of key TCA-enzymes (succinyl-CoA synthetase SULC2, fumarat hydratase FH) and enzymes involved in the conversion of various amino acids into succinyl-CoA (BCAT2, BCKDHA, IVD, MCCCI, MCCC2, MUT, and PCCB) support a more efficient provision of succinate for respiration in tumors. Increased expression of pyrroline-5-carboxylate reductase 1 (PYCR1) enables recycling of proline using NADPH and sustained feeding of electrons into CII by oxidation of proline by PRODH. In a breast cancer model, this PRODH-PYCR1 cycle was shown to enhance invasion and metastasizing properties of tumor cells. Furthermore, the high expression of genes related to ROS degradation such as superoxide-dismutase (SOD1) and peroxiredoxin 1-3 (PRDX1, PRDX2, PRDX3), indicates a higher ROS exposure and consequently increased necessity to detoxify oxygen radicals in the tumors.

Genes of enzymes related to glutamine-driven ATP generation via the TCA-cycle (glutaminase, GLS2; glutamate-pyruvate transaminase, GPT2) and proline dehydrogenase (PRODH) involved in the metabolization of proline to glutamate were downregulated in tumors. This is in line with the lower GM-pathway capacity in malignant compared to benign tissue. Genes
involved in regulation of the pyruvate dehydrogenase supercomplex (PDH), including pyruvate dehydrogenase kinases (PDK2, PDK3, and PDK4), were expressed higher in benign tissue suggesting a more stringent regulation of pyruvate catabolism. Taken together, the pathway alterations suggest a consistent pattern distinguishing benign and cancer tissue (Fig. 7c).

Shorter survival with a severe mitochondrial phenotype. We performed survival analysis using publicly available PCA cohort data annotated with progression-free or overall survival data to evaluate the prognostic value of the specific expression pattern associated with a decrease in N-pathway respiratory capacity. We grouped our cancer cases with expression profiles into two equal cohorts according to their GM-OXPHOS capacity, a low GMₚ ("severe") and a high GMₚ ("mild") mt-respiratory phenotype group. The relative GM-respiratory capacity of the severe respiratory phenotype tumors was about half compared to the mild respiratory phenotype tumors (\( p = 0.002 \), Wilcoxon rank-sum test, Fig. 8a). The severe phenotype group featured a significantly higher incidence of non-synonymous mtDNA mutations, particularly in CI genes (odds ratio=10.4, \( p = 0.04 \); Fisher’s exact test, Fig. 8b). Of note, five severe but only one mild respiratory phenotype tumors harbored a non-synonymous CI gene mutation. The variant level in this single mild phenotype tumor was only 3.8% compared to 5.4–60.2% in the severe phenotype tumors (Supplementary Table 4). Another distinctive property of the two groups was a significantly higher mt-CN load in the severe phenotype tumors (\( p = 0.02 \), Wilcoxon rank-sum test, Fig. 8c).

Comparison of the transcriptomes of the severe and mild respiratory phenotype tumors revealed upregulation of branched-chain amino acids and fatty acid degradation, pyruvate metabolism, oxidative phosphorylation and TCA cycle pathways in the severe respiratory phenotype tumors. In contrast the pathways for metabolism of other amino acids (glutamine, alanine asparagine, phenylalanine, arginine, proline) were higher expressed in the mild respiratory phenotype tumors (Supplementary Fig. 7). We used the transcriptome profiles to extract a metagene set of 11 genes strongly correlated to the severe respiratory phenotype tumors, to test if these two distinct phenotypes were associated with different disease outcomes (\( r > 0.4, p < 0.05 \), multiple t-test followed by Benjamini–Hochberg correction for multiple testing, Table 2). We then dichotomized samples of the cancer genome atlas (TCGA-PRAD) cohort, \( N = 497 \) into two cohorts according to their metagene set expression scores using an optimum cut-off value. The high score tumor group exhibited a statistically significantly shorter disease-free survival probability (Fig. 8d; hazard ratio=0.53, confidence interval=0.32–0.89, \( p = 0.001 \), stratified log-rank test). This result was confirmed with four PCA gene array expression cohorts.
Table 2 Metagene signature of severe respiratory phenotype tumors.

| Severe respiratory phenotype metagenes | Gene ID | Gene name         |
|--------------------------------------|---------|-------------------|
|                                      | ACADL   | Acyl-CoA dehydrogenase long chain |
|                                      | ALDH7A1 | Aldehyde dehydrogenase 7 family member A1 |
|                                      | AUH     | AU RNA binding methylglutaconyl-CoA hydratase |
|                                      | BPHL    | Biphenyl hydrolase like |
|                                      | CHDH    | Choline dehydrogenase |
|                                      | CRL51   | Cardiolipin synthase 1 |
|                                      | FECH    | Ferrochelatase |
|                                      | LDHD    | Lactate dehydrogenase D |
|                                      | MAOA    | Monoamine oxidase A |
|                                      | MPEP    | Mitochondrial intermediate intermediate peptidase |
|                                      | NUDT8   | Nudix hydrolase 8 |

Significantly overexpressed genes exhibiting a significant correlation (r > 0.4, p < 0.05, multiple test followed by Benjamini–Hochberg correction for multiple testing) were extracted from the “severe” phenotype samples and classified as representative “metagenes” for this phenotype. 

(N = 570) (Fig. 8e, Supplementary Table 5). Together, these results indicate worse outcome associated with metabolically remodeled PCa.

Discussion

The specific metabolism of prostatic epithelial cells is characterized by secretion of citrate into the prostatic fluid. Exploiting glutamate and other amino acids as anaerobic fuel substrates and hence as major sources for ATP production allows sparing of pyruvate and glutamate and other amino acids as anaplerotic fuel substrates. The malignant tissue showed an increased utilization of mainly sucrose and N-pathway contribution. Upregulation of succinate-triggered ATP production in tumors despite CI protein mutations affecting CI activity by increasing CI/CII marker ratio by 3D modeling, (4) increased mtDNA content and (5) increased mt-mass marker and reduced CI/CII maker ratio. The mt-ICP marker pattern confirms upregulation of CII as an important mechanism to enable sustained OXPHOS capacity for ATP production in tumors despite CI protein mutations affecting CI and N-pathway contribution. Upregulation of succinate-triggered OXPHOS respiration upon inhibition of N-pathway oxidative flux in prostate cells indicates that the S-pathway is in general not working at its maximum capacity. This allows to recruit further S-pathway capacity to keep OXPHOS high when oxidative flow from CI to the Q junction is compromised, e.g., by CI gene mutations.

Importantly, our results show that primary human PCa tissue is highly capable of aerobic ATP production. Despite decreased N-pathway capacity, the cancer cells reorganize OXPHOS capacities and metabolism to compensate this loss. A high respiratory capacity is crucial for high malignant and invasive potential. In contrast to tumors, human skeletal muscle and fibroblasts heavily rely on N-pathway capacity and a similar compensatory mechanism is not known for mt-myopathies. Ultimately, our results suggest that the immediate net effect of deleterious mtDNA mutations is compensated and does not hamper cellular ATP homeostasis in primary human PCa. Nevertheless, the association with shorter survival and in vitro and in vivo data indicate that deleterious MT-ND variants increase the tumorigenic potential. This points to a significance of such mutations beyond the immediate effect on cellular energy supply.

Non-synonymous mtDNA mutations are associated with specific changes in the expression of OXPHOS-related proteins. In our study, pathways linked to glutamate metabolism were decreased, those related to pyruvate and succinate utilization increased in tumor tissue, thus mirroring the respiratory patterns and suggesting a specific significance of such mutations to rewrite metabolism. The mechanistic link between mutations and altered enzyme expression levels remains elusive at this stage, however, our results provide a first view on altered expression patterns that control rate is half of Hopkins et al. (0.1 vs. 0.2) (Supplementary Fig. 8). It is important to consider the different matched control tissues (paired benign vs. blood tissue) as mtDNA mutations vary across organs, even within one individual.

Our study highlights non-synonymous, potentially deleterious mutations in mtDNA-encoded genes of the mitochondrial machinery as the crucial alterations impacting on mt-function in primary PCa. Non-synonymous mutations in mt-CI protein genes turned out to be closely linked to respiratory and metabolic differences, whereas mutations in genes encoding proteins of other mt-complexes seem to be better tolerated. This warrants exploration of mtDNA CI HPs as diagnostic indicators of PCa progression risk.

mtDNA heteroplasmy levels up to 80% may exhibit little impact on energy production. Although this observation is well supported by deleterious mtDNA mutations affecting CIV, ATP synthase or tRNAs, like the A3243G tRNALeu mutation causing MELAS syndrome or the A8344G tRNAIle mutation causing MERRF syndrome, CI gene mutations lead to symptomatic mt-myopathies at much lower thresholds. LHON or Leigh’s syndrome patients consistently show ~50% decrease in N-pathway capacity at variant levels of 20–50%.

These findings are in agreement with our study with heteroplasmy levels of 30–60% exhibiting a significant N–S-pathway shift. Strong support for a causal link between potentially deleterious mtDNA mutations and the respiratory alterations comes from: (1) strong N–S-capacity phenotype in samples harboring CI gene mutations, (2) clustering of these mutations to the central axis of CI, a highly-charged channel involved in redox coupling and proton translocation, (3) significant structural impact by mutations indicated by 3D modeling, (4) increased mtDNA content and (5) increased mt-mass marker and reduced CI/CII maker ratio by IHC in tumors with high mutation load in mt-CI genes. The mt-ICP marker pattern confirms upregulation of CII as an important mechanism to enable sustained OXPHOS capacity for ATP production in tumors despite CI protein mutations affecting CI and N-pathway contribution. Upregulation of succinate-triggered OXPHOS respiration upon inhibition of N-pathway oxidative flux in prostate cells indicates that the S-pathway is in general not working at its maximum capacity. This allows to recruit further S-capacity to keep OXPHOS high when oxidative flow from CI to the Q junction is compromised, e.g., by CI gene mutations.
can guide future studies of tumorigenesis and progression-supporting metabolic pathways.

Metabolic alterations and high load of non-synonymous mtDNA mutations are accompanied by an increase in mtDNA content and mt-density, which is a characteristic of more aggressive PCa.23,30 Confiming this hypothesis, a set of highly correlated metagenes extracted from the gene expression profile of PCa samples exhibiting a severe respiratory phenotype was able to predict significantly shorter survival. Taken together, the severe N→S-shifted respiratory phenotype provides an additional prognostic indicator for aggressive PCa.

Our study has limitations: (1) functional changes observed by HRR analysis were detected using artificially high concentrations of substrates and oxygen, which may lead to an overestimation of the effects. However, tissues are composed of a mixture of malignant and non-malignant cells (e.g., the stroma), resulting in a "dilution" of the malignant phenotype. (2) HRR analysis involves multiple sequential enzymatic steps and further experiments exploring isolated TCA cycle proteins and/or ETS subunits are needed to validate their single contribution in more detail. On the other hand, our method allows a realistic evaluation of the complex interplay of the various enzymatic steps of respiration. (3) We focused on evaluation of functional and genomic data, while gene expression was analyzed at mRNA level and protein levels by IHC for mt-markers only. It is widely accepted that the combination of HRR and mtDNA sequence analysis provides the most powerful tool to study the effect of (potentially) deleterious mtDNA variants on TCA cycle and OXPHOS function.31-53 The IHC expression data of mt-markers and a recent immunohistochemical study revealed a significantly elevated expression of CI1-protein subunits in primary PCa tissue.54 In line with that, Grupp et al.55 reported increased mitochondrial mass associated with PCa progression. (4) More comprehensive functional characterization of the potentially deleterious mtDNA variants described in our study are necessary to evaluate their impact on the N-pathway function and cellular ATP homeostasis. Patient follow-up is warranted to estimate the effects on long-term disease outcome.

In conclusion, this is the first study analyzing strain-specific OXPHOS capacities in native primary human prostate tissue. It links remodeling of OXPHOS in PCs to potentially damaging mtDNA mutations and differential expression of mt-genes. Our findings suggest that decreased N-pathway capacity associated with potentially deleterious, high-level mtDNA heteroplasmy in mt-CI genes, higher mtDNA load and increased mt-mass are distinct characteristics of high-grade tumors, highlighting the diagnostic and prognostic potential of metabolic rewiring. Analysis of the CI molecular structure suggested a structural basis for the effects of functionally relevant mutations. Furthermore, we provide evidence that both, potentially deleterious mtDNA mutations and an aberrant gene expression account for these alterations in a subgroup of PCa samples. We defined a severe respiratory phenotype PCa subtype characterized by a significant N→S-pathway shift and show that the distinct expression signature of this subgroup is associated with worse disease prognosis in large and independent PCa cohorts. This signature could thus help to identify patients at a higher risk, complementing the classical tumor grading and risk assessment system. Our results warrant exploring therapeutic strategies to target alterations of metabolism in PCa and particularly suggest succinate-linked respiration as a therapeutic target.

**Materials and methods**

**Prostate cancer subjects.** Fifty prostate cancer patients were recruited for the study and radical prostatectomy was the first-line treatment in all cases (Table 1). All patients underwent surgery at the University Hospital for Urology of the Medical University of Innsbruck and received standard care radical prostatectomy. Immediately after surgery, prostate specimens were transported to the Department of Pathology where small tissue samples were excised for the study. After formalin fixation, sections were obtained from all patients. Tissue samples were used for diagnostic purposes. Following fixation, the tissue samples from at least 1 cm of tissue were used to prepare diagnostic histological sections. A sufficient amount of tissue was collected for further experiments. We focused on evaluation of functional and genomic data, involving multiple sequential enzymatic steps and further experiments exploring isolated TCA cycle proteins and/or ETS subunits are needed to validate their single contribution in more detail. On the other hand, our method allows a realistic evaluation of the complex interplay of the various enzymatic steps of respiration. (3) We focused on evaluation of functional and genomic data, while gene expression was analyzed at mRNA level and protein levels by IHC for mt-markers only. It is widely accepted that the combination of HRR and mtDNA sequence analysis provides the most powerful tool to study the effect of (potentially) deleterious mtDNA variants on TCA cycle and OXPHOS function.31-53 The IHC expression data of mt-markers and a recent immunohistochemical study revealed a significantly elevated expression of CI1-protein subunits in primary PCa tissue.54 In line with that, Grupp et al.55 reported increased mitochondrial mass associated with PCa progression. (4) More comprehensive functional characterization of the potentially deleterious mtDNA variants described in our study are necessary to evaluate their impact on the N-pathway function and cellular ATP homeostasis. Patient follow-up is warranted to estimate the effects on long-term disease outcome.

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corresponding OXPHOS capacity, ADP (2.5 mM) was added at saturating concentrations. This was followed by incubation with H2O2 (500 µM) for 15 min in the closed chamber to induce oxidative stress and assess the effect of damage mediated by elevated cellular ROS levels. The excess H2O2 was then removed by addition of catalase (280 U/mL) and the chambers were briefly opened to regain initial O2 concentrations. After titration of pyruvate (P, 5 mM), succinate (S, 10 mM) was added to fuel electrons via CII, thus reconstituting TCA cycle function and inducing convergent NADH- and succinate-linked (NS) electron flow through CII and CIII into the O2-junction. Subsequently stepwise titration of uncoupler carbonyl cyanide p-trifluoromethoxyphenyl hydradrazine (FCCP, 0.5 µM) enabled the determination of maximal non-coupled NS-mediated ET-respiratory capacity. Inhibition of CI by rotenone (Rot, 0.5 µM) revealed S-pathway ET-respiratory capacity. Residual oxygen consumption (ROX) was measured after additional inhibition of CII and CIII by malonate (Mna, 5 mM) and antimycin A (Ama, 2.5 µM), respectively.

In control experiments assessing the effect of simulated oxidative stress by H2O2 exposure, tissue samples were split, one half was analyzed using the H2O2 treatment step, while the other half was incubated with H2O2 as a control. In addition, a series of paired BE/CA tissue samples was measured using a protocol omitting the H2O2 treatment. The impact of oxidative stress treatment on the single enzymatic step of Complex IV was assessed in split BE tissue samples using a protocol including rotenone (Rot, MIT, Mg], Mg and Ascorbate was added followed by N,N,N′,N′-Tetramethyl-L-4-phenylendiamine (TMPD). Chemical background oxygen consumption was measured after inhibition of CIV with sodium azide and was used for correction (Supplementary Tables 1–2).

Five measurements in cell lines, cells were harvested by trypanosmization, counted and re-suspended in MRS5 buffer. Measurements were performed with 0.5–1 Mill cells per mL in the O2k chamber and the SUT protocol used for tissue samples with an additional step of plasma membrane permeabilization by titration of digitonin was applied (SUT-014, [http://www.mitoft.org/index.php/SUT-014]). To simulate injury of CI, rotenone was added in stepwise titration of 1 nM until 0.5+1 Mill cells per mL in the O2k chamber and the SUIT protocol used for tissue samples with an additional step of plasma membrane permeabilization by titration of digitonin was applied (SUT-014, [http://www.mitoft.org/index.php/SUT-014]). To simulate injury of CI, rotenone was added in stepwise titration of 1 nM until partial inhibition of N-pathway capacity in OXPHOS. In control experiments vehicle (ethanol) was added.

Next-generation sequencing of the entire mitochondrial genome. After the HRR experiments tissue biopsies were recovered, washed twice with pre-chilled PBS and stored at –20 °C until isolation of DNA. Total genomic DNA was extracted using the EZI DNA Tissue Kit (Qagen, Hilden, Germany) following the manufacturer’s instructions. The whole mt-genome was amplified using the primers 5′-ACTAGGCTTGCTGGTGGTTG-3′ (forward) and 5′-GGCGGATCTTACGGTCTTGG-3′ (forward). PCR reactions were performed with 8.5kb PCR fragments using the primers 5′-CGCCTGTTT-3′ and 5′-AAATCTTACCC-3′ (fragment A), 5′-AAATCTTACCC-3′ and 5′-GGCGGATCTTACGGTCTTGG-3′ (fragment B, for- ward) and 5′-GCAATTCTTACGGTCTTGG-3′ (fragment B, reverse). After PCR, all amplicons were quality checked, after purification on a MoBio Qiagen AxyPrep DNA Amplicon Kit and amplified using the DNE-930 dsDNA Reagent Kit (both Advanced Analytical, Ames, IA). For generation of barcoded 200 bp insert Ion Torrent NGS libraries, 1 µg of DNA of a 1:1 mixture of both mtDNA fragments A and B was enzymatically fragmented using the NEBNext dsDNA Fragmentase Kit (New England Biolabs). DNA of a 1:1 mixture of both mtDNA fragments A and B was enzymatically fragmented using the NEBNext End Repair Module (New England Biolabs) and ligated with IonXpress adapters (Life Technologies, Waltham, MA) using T4 DNA Ligase and Bst DNA Polymerase (New England Biolabs). The ligation products were size selected on an E-Gel SizeSelect Agarose Gel, and the average size of each fragment was determined on a Fragment Analyzer while the concentration was determined on a Nanodrop instrument. Purification of fragmented DNA was assessed on Fragment Analyzer while the concentration was determined on a Nanodrop instrument. Purification of fragmented DNA was assessed on Fragment Analyzer while the concentration was determined on a Nanodrop instrument. Purification of fragmented DNA was assessed on Fragment Analyzer while the concentration was determined on a Nanodrop instrument. Purification of fragmented DNA was assessed on Fragment Analyzer while the concentration was determined on a Nanodrop instrument. Purification of fragmented DNA was assessed on Fragment Analyzer while the concentration was determined on a Nanodrop instrument. Purification of fragmented DNA was assessed on Fragment Analyzer while the concentration was determined on a Nanodrop instrument. Purification of fragmented DNA was assessed on Fragment Analyzer while the concentration was determined on a Nanodrop instrument. Purification of fragmented DNA was assessed on Fragment Analyzer while the concentration was determined on a Nanodrop instrument. Purification of fragmented DNA was assessed on Fragment Analyzer while the concentration was determined on a Nanodrop instrument. Purification of fragmented DNA was assessed on Fragment Analyzer while the concentration was determined on a Nanodrop instrument. Purification of fragmented DNA was assessed on Fragment Analyzer while the concentration was determined on a Nanodrop instrument. Purification of fragmented DNA was assessed on Fragment Analyzer while the concentration was determined on a Nanodrop instrument. Purification of fragmented DNA was assessed on Fragment Analyzer while the concentration was determined on a Nanodrop instrument. Purification of fragmented DNA was assessed on Fragment Analyzer while the concentration was determined on a Nanodrop instrument. Purification of fragmented DNA was assessed on Fragment Analyzer while the concentration was determined on a Nanodrop instrument. Purification of fragmented DNA was assessed on Fragment Analyzer while the concentration was determined on a Nanodrop instrument. Purification of fragmented DNA was assessed on Fragment Analyzer while the concentration was determined on a Nanodrop instrument. Purification of fragmented DNA was assessed on Fragment Analyzer while the concentration was determined on a Nanodrop instrument. Purification of fragmented DNA was assessed on Fragment Analyzer while the concentration was determined on a Nanodrop instrument.

Immunohistochemistry for mt-markers. IHC staining was performed using the antibodies anti-VADDCI (D73D12, Cell Signaling Technologies, Leiden, The Netherlands; 1:500), anti-SHDA (D697M, Cell Signaling Technologies; 1:400) and anti-Ndufs4 (EP7832, Abcam, Cambridge, UK; 1:200)[45]. Immunostaining was evaluated using a modified “quickscore” procedure considering staining intensity and the percentage of stained cells with scores ranging from 0 to 3 (0 = absent, 1 = weak, 2 = intermediate, 3 = strong). In cases with two recognizable tumor foci or tumor patterns, both foci were scored and mean scores taken for statistical analysis.

Global transcriptome next-generation sequencing. Global gene expression analysis was performed by RNA-NGS-sequencing. RNA was isolated from frozen tissue blocks from areas surrounding the tissue samples extracted for HRR/mtDNA sequencing (Fig. 1a). Libraries were prepared using the Illumina TrueSeq stranded mRNA LT Sample Preparation Kit and pooled-end sequencing was performed on an Illumina HiSeq 2500 instrument (Illumina, San Diego, CA) according to the manufacturer’s instruction. The mean relative GM-pathway capacity for all malignant samples in this study was 0.27 ± 0.10 as determined by HRR. Therefore, a GM-pathway contribution of ≤27% was considered as “low” whereas a relative GM-pathway contribution of >27% was considered as “high” GM-pathway capacity.

RNA expression analyses. As a first step before raw sequence data analyses, the FastQC tool was applied as quality control on the sequencing reads from all datasets. The sequencing reads were aligned to the human genome (GRC38/hg38 assembly) using STAR and processed with Salmon[46]. Gene-level read counts were determined using the Python package Salmon[47]. To simulate the default union-counting mode[46]. The differential gene expression analysis of tumor and non-malignant benign samples was calculated using the DESeq2 package in R[70].
Clustering and visualization were done with the software Genesis. A collection of 1158 human mt-genes for expression analysis was retrieved from MitoCarta2.0 database (https://www.hb institute.org/files/shared/metabolism/mitocarta/human.mitochart2.0.html).

**Metabolic pathway assignment.** For pathway analysis and annotation the InnateDB Pathway Analysis online tool was used (http://www.innatedb.ca/redirrect.do?go=batchPw). Overexpressed mt-genes as identified by the RNA-seq analysis were used to perform a Pathway Over-Representation Analysis. The KEGG DAVID analysis list restricted to the proteins and enzymes listed in the MitoCarta list was used to group the differentially expressed mt-related genes into respective pathways. To test for significant differences in gene expression between benign/malignant and mild/severe phenotype samples, a pairwise comparison between groups for each gene listed in the MitoCarta catalog was performed based on the FPKM values. To control for type-I error accumulation, \( p \)-values were corrected by the Benjamini–Hochberg procedure.

**Severe respiratory phenotype phenotype metagene signature.** For the identification of a set of differentially expressed genes representative for the severe (low GM) mitochondrial respiratory phenotype ("metagenes"), we selected only significantly correlated genes with an average correlation \( r>0.4 \) (\( p<0.05 \), multiple \( t \)-test followed by Benjamini–Hochberg correction for multiple testing). We thus tried to avoid that their correlation might be due to chance while preserving a reasonable number of genes for the severe phenotype metagene set. Clinical variables including disease-free survival (time to biochemical tumor recurrence) and the corresponding survival RNA-seq expression profile of prostate cancer patients diagnosed in the Cancer Genome Atlas (TCGA) database were downloaded directly from the TCGA Data Portal (https://cancergenome.nih.gov/). For the confirmatory meta-analysis, available clinical data and microarray expression profiles from four additional prostate cancer cohort studies were downloaded from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/): GSE16560 (Human 6k Transcriptionally Informative Gene Panel for DASL; \( n = 281 \)) GSE40272 (SMD Print_1529 Homo sapiens; \( n = 84 \)) GSE70768 (Illumina HumanHT-12 V4.0 expression beadchip; \( n = 111 \)) and GSE70769 (Illumina HumanHT-12 V4.0 expression beadchip; \( n = 94 \)). The survival information (overall survival OR, disease-free survival DFS or time to biochemical recurrence BCR, respectively) was extracted from the clinical XML files in the complete clinical sets.

**Statistical analyses.** All statistical analyses were performed using IBM SPSS or R software version 2.0 and numerical data are presented as mean ± SD unless otherwise stated. Normality tests were performed using the Shapiro–Wilk Test followed by a Q-Q Plot and histogram examination. Accordingly, either Student’s paired-samples \( t \)-test or Wilcoxon signed rank test was used for group comparisons whereas Wilcoxon rank-sum test and student's \( t \)-test was used to compare variables among unpaired samples. If multiple paired \( t \)-tests were performed on the same sample set, results were corrected according to the Bonferroni-Holm method. For multiple comparisons, one-way analysis of variance (ANOVA) followed by Tukey’s honest significant difference (HSD) test was used. Linear regression models followed by one-way ANOVA were used to test for relationships between independent variables. A multivariate logistic regression analysis was performed to evaluate the relationship between several independent variables (patient age, free PSA ratio and tumour stage) and the presence or absence of heteromielies in the dependent variable. Spearman’s rank correlation coefficients were calculated to evaluate the presence of significant correlations among non-parametric variables and to test for multilinear dependency among single independent variables used in the logistic regression model. The significant differential expression of genes was determined using a false discovery rate (FDR) below 0.01 as determined by the Benjamini–Hochberg procedure to control for type-I errors after multiple testing. To test associations between categorical variables either Pearson’s Chi-squared test or Fisher’s exact test was used. Survival times were defined using the latest information. For survival analysis, the patients were dichotomized based on the expression levels of the low-GM metagens. The optimal cut-points were searched within the inner 80% selection interval and chosen based on a minimal corrected \( p \)-value and based on a maximum Harrell’s C indices. Survival probability curves were calculated via the Kaplan-Meier method while differences in disease outcome were analyzed using Hazard ratios and stratified log-rank test. Univariate and multivariate Cox regression analysis was performed using age, Gleason score, TNM staging and total serum PSA values as binary covariates, for all cohorts mentioned above. For statistical analysis, IBM SPSS or R software version 2.0 including the survival package were used. \( p \leq 0.05 \) was considered not statistically significant.

**Data availability**

Data that support the findings of this study are available within the article and its supplementary files or from the authors upon reasonable request. Supplementary Information provides Supplementary Tables and Figures, Supplementary Data 1 provides a list of mtDNA Heteromelies, the Source Data File provides datasets underlying Figs. 2–8 and Supplementary Fig. 1–4 and 6–8. The Supplementary Software iSee Package for 3D visualization of Complex 1 mutations is provided for download or online access at [https://github.com/genepli-mt-c1]. DNA and RNA sequence datasets have been deposited in EGA (European Genome-Phenome Archive [www.ega-archive.org]) under the following accession numbers: EGAD00001005931 (RNAseq data set, [https://ega-archive.org/datasets/EGAD00001005931]) and EGAD00001005946 (mtDNA data set). Prostate cancer gene expression datasets with survival information were accessed at the Cancer Genome Atlas (TCGA) Data Portal ([https://cancergenome.nih.gov/]; TCGA-PRAD and the Gene Expression Omnibus portal ([https://www.ncbi.nlm.nih.gov/geo/]): GSE16560, GSE40272, GSE70768 and GSE70769. Protein structure data were accessed at the protein Data Bank (PDB), [https://www.rcsb.org/](https://www.rcsb.org/): 4HE8, 4HEA, 5XTD ([https://www.rcsb.org/structure/5XTD], 5XTC).

**Code availability**
The iSee package can be accessed from GitHub using the following link https://github.com/genepli-mt-c1

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H.K., E.G., and B.S. conceived and designed the research; B.S., G.S., H.W., A.N., L.F., V.B., U.G., E.G., F.K., A.N., and H.K. planned experiments; B.S., G.S., A.N., F.F., A.C.S.A.S. and J.I.G. performed experiments; V.B., I.G., P.S., and U.S. performed RNA-seq; A.N. and B.R. performed structural analyses; all authors analyzed and/or interpreted data; B.S., H.W., F.F., C.P., A.N., E.G. and H.K. wrote the manuscript; H.K. organized and supervised the study. All authors reviewed and approved the manuscript for publication.

Competing interests
E.G. is founder and CEO, and J.I.G. is employee of Oroboros Instruments, Innsbruck, Austria. All other authors declare no conflict of interests.

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Correspondence and requests for materials should be addressed to H.K.

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