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Integrated single-cell and spatial transcriptomic profiling decodes lineage plasticity and immune microenvironment remodeling in prostate cancer progression

Hongyuan Yu^{1†}, Yusong Wang^{2†}, Mingcong He^{3†}, Yiming Chen¹, Jianbin Bi^{1*}, Xuyong Lin^{4*} and Xiaolu Cui^{1*}

Abstract

This study presents a comprehensive single-cell and spatial transcriptomic atlas of prostate cancer progression, integrating 127 single-cell RNA sequencing samples and 9 spatial transcriptomics profiles spanning the disease continuum from healthy prostate to neuroendocrine carcinoma. Our analysis defines four evolutionarily connected malignant epithelial subtypes: luminal-identity (sub1), stress-adaptive luminal (sub2), neuroendocrine (sub3), and a double-negative basal-like state (sub4). We identify FOSL1 as a key driver of lineage plasticity through direct transcriptional regulation of HMGA1, promoting treatment resistance via enhanced proliferation, EMT and stemness. The tumor microenvironment undergoes coordinated reprogramming during progression, with neoadjuvant hormone therapy inducing distinct cellular responses: FOLR2+ and CX3CR1+ TAMs upregulate TGF- β signaling to establish immunosuppressive niches, while CXCL12+ iCAFs and ACTA2+ myCAFs maintain spatial co-localization and facilitate immune cell recruitment. Spatial analyses reveal enhanced chemokine signaling post-therapy, particularly in specific TAM subsets, driving increased but functionally impaired lymphoid infiltration characterized by T-cell exhaustion and regulatory T-cell expansion. This integrated analysis establishes a unified paradigm connecting epithelial plasticity with microenvironmental reprogramming, revealing FOSL1-HMGA1 signaling and macrophage-driven immunosuppression as promising therapeutic targets for advanced prostate cancer.

Keywords Prostate cancer, Single-cell RNA sequencing, Spatial transcriptomics, Lineage plasticity, Tumor microenvironment, Treatment resistance

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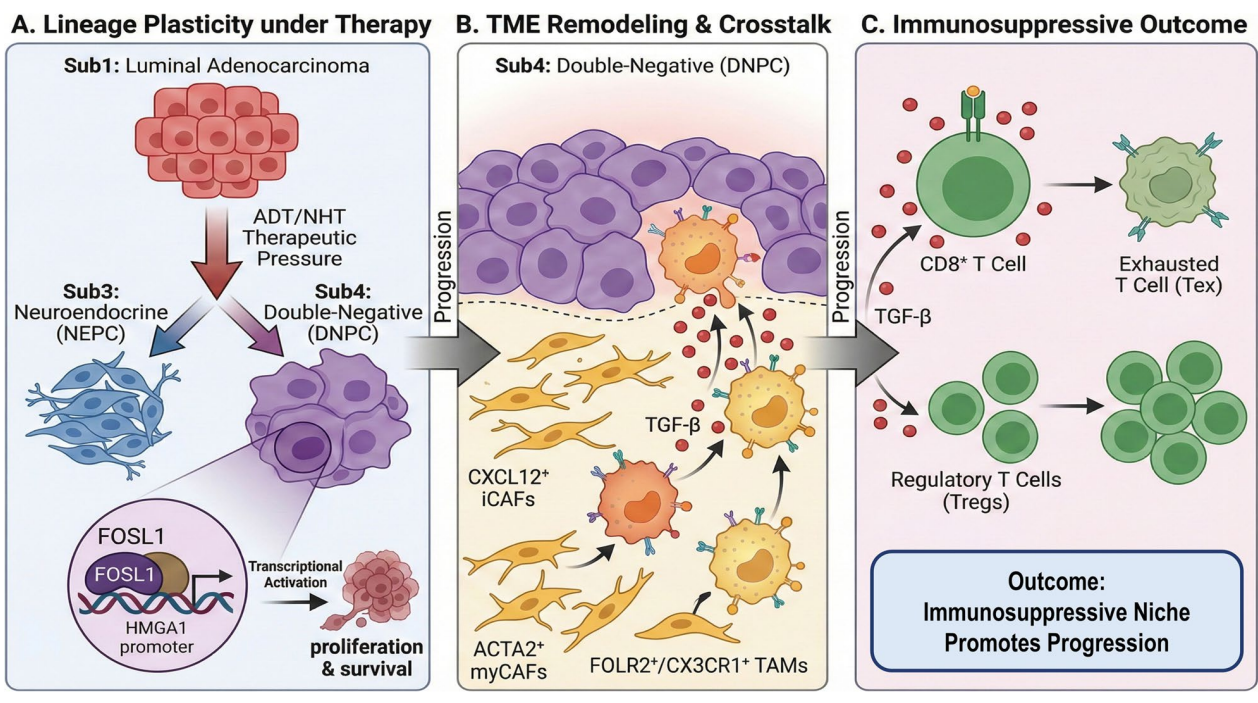
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Graphical Abstract

Single-Cell Atlas of Lineage Plasticity and Immunosuppressive Niche Remodeling in Advanced Prostate Cancer. Based on our study which systematically reveals the mechanisms of lineage plasticity and immune microenvironment remodeling in prostate cancer, we have constructed an integrated progression model (Graphical Abstract). The model proposes that under therapeutic pressure (e.g., ADT/NHT), luminal adenocarcinoma (Subtype 1) can differentiate into either a neuroendocrine phenotype (NEPC, Subtype 3) or a double-negative prostate cancer (DNPC, Subtype 4) through distinct paths. During DNPC progression, the key transcription factor FOSL1 drives tumor cell stemness/EMT maintenance and enhanced proliferation and survival by directly activating HMGA1 transcription (**A**). Concurrently, the tumor microenvironment undergoes significant remodeling, marked by the enrichment of stromal and immune cell subsets such as CXCL12⁺ iCAFs, ACTA2⁺ myCAFs, and FOLR2⁺/CX3CR1⁺ TAMs, forming an active intercellular network (**B**). This remodeling ultimately fosters an immunosuppressive niche, where signals like TGF- β mediate the functional exhaustion of CD8⁺ T cells, thereby promoting tumor immune evasion and disease progression (**C**). Our study elucidates, at single-cell resolution, the cell-intrinsic drivers and microenvironmental synergies underlying prostate cancer malignant evolution, providing a novel theoretical foundation for combination therapeutic strategies targeting lineage plasticity and the immunosuppressive microenvironment



Introduction

Prostate cancer (PCa) continues to pose a substantial global public health challenge, with rising incidence and mortality documented in recent years [1]. Globally, PCa remains the second most common malignancy in men, with recent estimates indicating over 1.5 million new cases and approximately 400,000 deaths annually [2]. Notably, incidence rates have been increasing rapidly in previously low-risk regions including Asia, particularly in China, where the widespread adoption of PSA testing and lifestyle changes have contributed to an annual increase of around 8.1% over the past decade [3]. Despite advancements in treatment that have modestly reduced mortality in high-income countries, PCa still represents a

leading cause of cancer-related death among men worldwide, underscoring its growing epidemiological severity [4]. Therapeutically, novel androgen receptor signaling inhibitors (ARSIs) such as enzalutamide and apalutamide have markedly improved outcomes for patients with advanced disease by significantly delaying progression and enhancing survival [5]. However, the emergence of resistance driven by the pronounced lineage plasticity of prostate cancer often leads to adaptive transformations such as neuroendocrine differentiation, which confers aggressive treatment-resistant characteristics [6]. Consequently, understanding the molecular subtypes and drivers of lineage plasticity through comprehensive genomic and transcriptomic profiling has become essential for

developing more effective precision therapies and overcoming therapeutic resistance [7].

Recent advancements in single-cell RNA sequencing (scRNA-seq) and spatial transcriptomics (ST) have profoundly enhanced our understanding of prostate cancer heterogeneity and lineage plasticity. scRNA-seq studies have delineated the cellular composition and transcriptional states within prostate tumors, revealing continuous evolutionary trajectories from adenocarcinoma to treatment-resistant states, including neuroendocrine and double-negative phenotypes [8, 9]. Complementing these findings, spatial transcriptomics has enabled the mapping of these heterogeneous cell states within their tissue architectural context, uncovering spatially restricted niches that promote malignant progression and therapy resistance [10, 11]. The integration of these technologies—for example, through multi-omics approaches that combine scRNA-seq with ST—has begun to elucidate the cell-cell communication networks and stromal interactions that drive phenotypic plasticity. Studies utilizing such integrated analyses have revealed how specific microenvironmental niches, rich in certain immune or stromal cells, can support the emergence of aggressive subclones [12, 13].

Despite these transformative insights, significant limitations in experimental design and practical application remain. A major constraint is the scarcity of comprehensive spatial transcriptomics studies that encompass the entire disease continuum, from localized prostate cancer through castration-resistant progression to neuroendocrine transformation. Most current spatial studies focus on limited disease stages or sample types, restricting our understanding of spatial dynamics throughout disease evolution. Furthermore, spatial transcriptomics studies often face challenges in sample representativeness, as tumor biopsies may not fully capture intratumor regional variation or rare spatial niches. Issues related to RNA quality—particularly in archived clinical specimens—further restrict the generalizability of current findings [14, 15]. There is an urgent need for multi-institutional efforts to generate comprehensive, well-annotated spatial datasets that encompass the entire disease continuum, thereby enabling the validation of spatial and molecular subtypes across diverse populations and disease stages.

To address these limitations and systematically investigate prostate cancer progression and treatment resistance, we conducted an integrated single-cell and spatial transcriptomic analysis of a well-annotated clinical cohort. Our study includes 127 single-cell RNA sequencing samples spanning healthy prostate tissue, primary prostate cancer, novel hormone therapy (NHT)-treated tumors, castration-resistant prostate cancer, and neuroendocrine prostate cancer. Furthermore, we performed spatial transcriptomics on 8 in-house samples

using CytAssist and incorporated one public dataset (GSE230282), which contains coexisting AR-positive and neuroendocrine regions, thereby covering the complete spectrum from early-stage prostate cancer to castration-resistant prostate cancer (CRPC) and neuroendocrine prostate cancer (NEPC). This multi-modal design enables deep characterization of tumor lineage evolution and microenvironmental dynamics throughout disease progression. A key focus is placed on identifying transcriptional alterations linked to double-negative prostate cancer (DNPC) development. Through these analyses, we aim to provide novel insights into molecular mechanisms driving treatment resistance and to uncover potential therapeutic targets for advanced prostate cancer.

Results

Sample composition and cellular landscape of prostate cancer progression

Our study integrated 127 single-cell RNA sequencing samples from multiple publicly available datasets, including GSE181294 ($n = 39$) [12], GSE137829 ($n = 6$) [11], GSE193337 ($n = 8$) [16], HRA002145 ($n = 4$) [17], GSE206962 ($n = 4$), GSE157703 ($n = 2$) [18], GSE264573 ($n = 12$) [19], HRA004818 ($n = 8$), and GSE274229 ($n = 44$) [20] (Supplementary Table 1). To ensure data quality and comparability, samples with an extremely low cell count or derived from metastatic castration-resistant prostate cancer (CRPC) were excluded. In addition, we incorporated 8 in-house spatial transcriptomic profiles generated via CytAssist, comprising 2 primary prostate cancer samples, 4 NHT-treated specimens, and 2 AR-positive prostate cancer cases with partial neuroendocrine differentiation (Supplementary Table 2). Furthermore, the public spatial dataset GSE230282 [21] was included as a validation cohort due to its co-existence of NEPC and androgen-responsive prostate cancer (ARPC) regions, providing a representative model of tumor heterogeneity. The overall study design and workflow are summarized in Fig. 1A.

After rigorous quality control and batch correction, a total of 555,023 high-quality cells were retained for downstream analysis (Supplementary Table 3). The dataset encompassed 116,651 cells from healthy prostate tissues, 218,725 from primary prostate cancer, 107,337 from ADT-treated tumors, 76,932 from CRPC, and 35,378 from NEPC samples (Supplementary Table 4). Using canonical marker genes, cells were annotated into nine major types: B cells, plasma cells, fibroblasts, endothelial cells, epithelial cells, T & NK cells, mast cells, myeloid cells, and pericytes (Fig. 1B–1D, Supplementary Fig. 1A, Supplementary Table 5).

Proportions of these cell types varied substantially across disease stages (Fig. 1E and F). Epithelial and fibroblast populations were significantly expanded in tumor

tissues compared to healthy controls. Conversely, T and NK cells exhibited a progressive decline throughout cancer advancement. Endothelial cells were notably reduced in CRPC and NEPC. Myeloid cells were enriched in primary prostate cancer but showed a decreasing trend in later stages. Notably, Ratio of Observed to Expected (Ro/e) analysis (Fig. 1G) further highlighted systemic microenvironmental remodeling: immune cells including myeloid, T, and NK cells were consistently under-represented in tumor tissues, suggesting possible immune suppression or exclusion. In NEPC samples, fibroblasts, mast cells, and B cells were aberrantly enriched. Elevated fibroblast abundance may indicate activated stroma and fibrosis—a feature linked to therapy resistance [22]. Increased mast cell infiltration has been associated with neuroendocrine differentiation and poor prognosis [23], while B cell accumulation might reflect ectopic lymphoid structure formation or immune regulatory networks. These shifts suggest a complex interplay within the tumor ecosystem that may drive lineage plasticity and treatment resistance.

Building upon the single-cell data, we performed spatial transcriptomic profiling using the 10X Cytassist platform on 8 additional prostate cancer FFPE specimens. This cohort included 2 primary prostate cancer cases, 4 samples from patients who had undergone neoadjuvant hormone therapy (NHT), and 2 cases pathologically diagnosed with partial neuroendocrine differentiation (exhibiting mixed neuroendocrine and AR-positive prostate carcinoma components, subsequently referred to as NE-differentiation). Immunohistochemical staining for AR in all samples and for neuroendocrine markers (CgA, Syn) in patient 07 and 08 is shown in Supplementary Fig. 1B. All samples passed stringent quality control metrics, including thresholds for unique molecular identifiers (UMIs), gene counts, and mitochondrial gene content (Supplementary Table 6). The distribution of UMI counts per spot, cell type clustering, distribution of cell clusters in each sample, and corresponding H&E staining of the original tissue sections are presented in Supplementary Fig. 1C, 1D, 1E, 1 F, and Supplementary Table 7, respectively.

The cell type annotation was achieved through deconvolution analysis directly applied to the spatial transcriptomics data, which enabled the resolution of cellular heterogeneity without relying on external single-cell references. To quantitatively dissect the tissue architecture and cellular geography, we utilized the spaCET tool to infer cancer cell fractions and major cellular components across the tissue sections. The analysis revealed distinct spatial reorganization associated with disease state and treatment response (Fig. 1H). As expected, the estimated cancer cell fraction was significantly elevated in primary and NE-differentiation samples. In contrast,

NHT-treated specimens exhibited a substantial reduction in malignant epithelial regions, indicative of a favorable therapeutic response. Concomitantly, these treated samples showed a marked expansion of stromal compartments. This stromal expansion, predominantly featuring cancer-associated fibroblasts (CAFs), may signify the establishment of a therapy-resistant niche or a desmoplastic reaction following tumor cell attrition, a phenomenon previously linked to treatment failure and disease progression [13]. Furthermore, we observed a notable increase in lymphoid and myeloid cell infiltration within the NHT-treated tissues. This heightened immune presence could represent either a productive anti-tumor immune response triggered by therapy-induced cell death or, conversely, an immunosuppressive microenvironment facilitating adaptive resistance, as documented in studies of advanced prostate cancer [11, 24]. The spatial co-localization of these immune populations with residual tumor clusters is a subject of ongoing investigation in our cohort.

Molecular characterization of prostate epithelial cell dynamics during disease progression

We performed an in-depth analysis of 182,402 epithelial cells from our integrated dataset to investigate prostate epithelial heterogeneity. Using established marker genes, we categorized these cells into six distinct subtypes: Basal, Club, Hillock, Luminal, lineage-plastic cells (LPC), and neuroendocrine (NE) cells (Fig. 2A, Supplementary Table 8). Representative marker gene expression for each subtype is shown in Fig. 2B. Compositional analysis across disease stages revealed that Luminal cells constituted the predominant population in both primary and CRPC samples (Fig. 2C). Specimens collected after ADT therapy exhibited a more balanced distribution among Basal, Club, and Luminal subtypes. The CRPC cohort contained a detectable proportion of NE cells, while NE cells overwhelmingly dominated the NEPC group.

Application of the Ratio of Observed to Expected (Ro/e) method quantified subtype enrichment across clinical groups, showing significant LPC enrichment in both CRPC and NEPC, with the most pronounced elevation of NE cells specifically in the NEPC cohort (Fig. 2D). We then performed gene signature scoring using established gene sets: an androgen receptor (AR) signature [25], a neuroendocrine (NE) signature [26], a lineage plasticity (LP) signature [27], and the CRPC51 signature associated with treatment resistance [9]. As expected, Luminal cells displayed the highest AR activity, NE cells scored highest for the NE signature, and LPC populations exhibited the strongest enrichment for both LP and CRPC51 signatures (Figs. 2E). Analysis of epithelial cells grouped by clinical stage showed that primary and CRPC samples had the highest AR scores, whereas NEPC

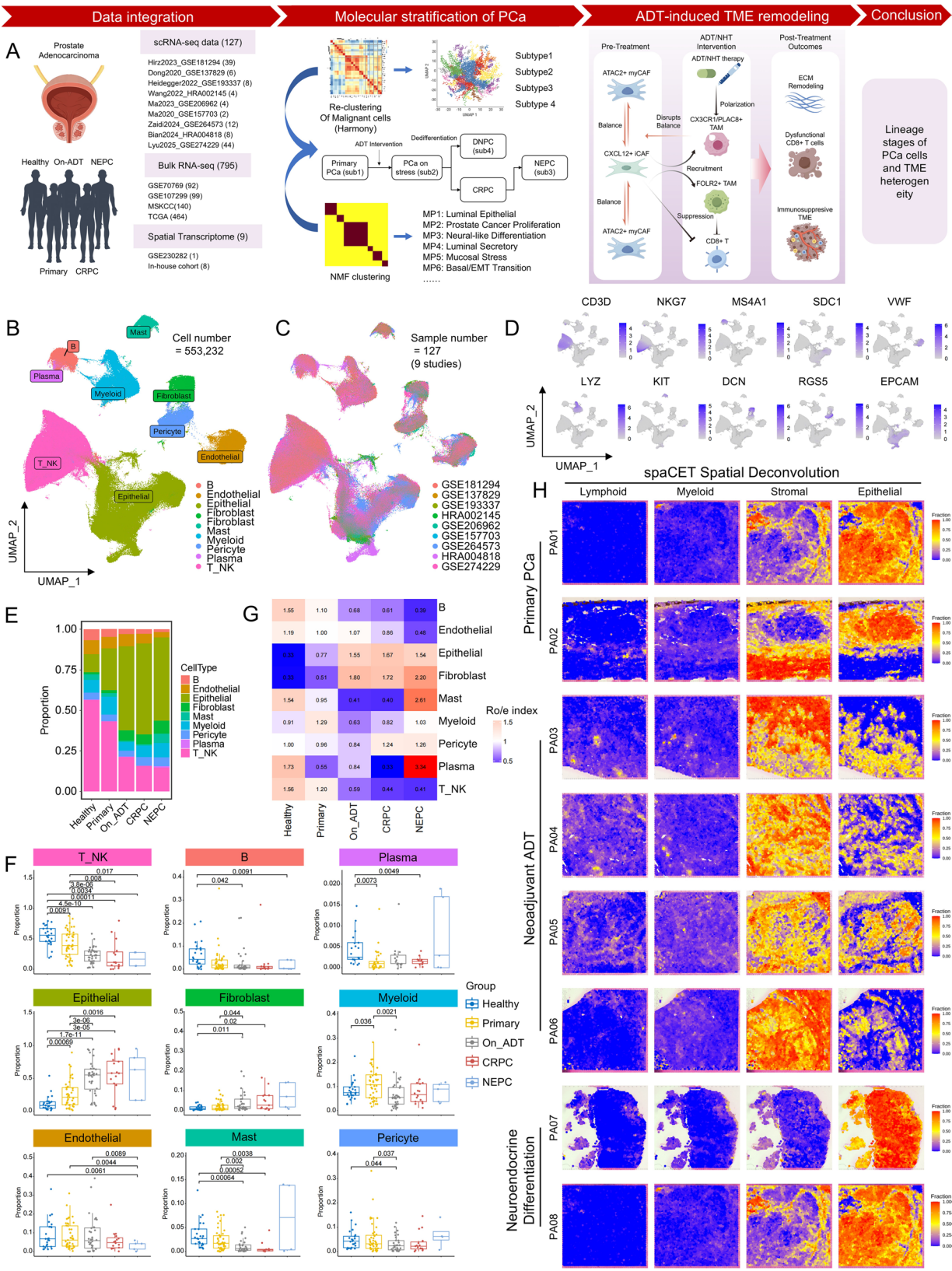


Fig. 1 (See legend on next page.)

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Fig. 1 Integrated single-cell and spatial transcriptomic profiling of prostate cancer progression. **A** Schematic overview of the study design. The workflow encompasses sample enrollment (127 single-cell RNA sequencing samples and 9 spatial transcriptomics profiles), data processing and integration, molecular subtyping identification, and characterization of tumor microenvironment evolution throughout prostate cancer progression. **B** UMAP visualization of 555,023 cells colored by major cell types, with each cell type represented by a distinct color for clear discrimination. **C** UMAP projection showing batch-corrected integration of cells from different sequencing studies, with each study represented by distinct colors, demonstrating successful integration across multiple datasets after Harmony batch correction. **D** UMAP feature plots displaying expression patterns of canonical marker genes used for cell type annotation: EPCAM (epithelial cells), VWF (endothelial cells), DCN (fibroblasts), CD3D (T cells), NKG7 (NK cells), MS4A1 (B cells), SDC1 (plasma cells), LYZ (myeloid cells), KIT (mast cells), and RGS5 (pericytes). Expression levels are represented by color gradient from gray (low expression) to red (high expression). **E** Stacked bar plots illustrating the proportional composition of major cell types across clinical groups: Healthy ($n = 25$), Primary ($n = 45$), NHT-treated ($n = 36$), CRPC ($n = 16$), and NEPC ($n = 5$). Height of each colored segment represents the percentage of each cell type within the respective clinical group. **F** Box plots showing significant differences in cell type proportions between clinical groups. Statistical significance was determined using Kruskal-Wallis test with Dunn's post-hoc correction ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$). Boxes represent interquartile range (IQR), center lines indicate median values, and whiskers extend to $1.5 \times \text{IQR}$. **G** Ratio of Observed to Expected (Ro/e) analysis heatmap depicting enrichment patterns of cell types across clinical groups. The color scale represents the Ro/e index, with red indicating enrichment ($\text{Ro/e} > 1$), blue representing depletion ($\text{Ro/e} < 1$), and white showing neutral distribution ($\text{Ro/e} = 1$). Color intensity corresponds to the magnitude of deviation from expected values, with darker colors indicating stronger enrichment or depletion. **H** Spatial mapping of major cellular compartments across 8 prostate cancer samples analyzed by spaCET. From left to right: P01-P02 (primary prostate cancer), P03-P06 (post-neoadjuvant hormone therapy), P07-P08 (neuroendocrine prostate cancer). Each panel shows spatial distribution of epithelial cells, stromal cells, myeloid cells, and lymphoid cells, with spot colors representing relative abundance of each compartment

samples were most enriched for NE, LP, and CRPC51 signatures (Figs. 2F). This scoring pattern confirms the expected functional states of epithelial subtypes and underscores that advanced, treatment-resistant disease (NEPC) is characterized by concurrent activation of neuroendocrine, lineage plasticity, and resistance pathways, a hallmark of aggressive prostate cancer variants [28]. CytoTRACE analysis further indicated that LPCs possess the highest differentiation potential among all epithelial subtypes (Supplementary Fig. 2A), suggesting their role as cellular hubs for phenotypic transitions during disease progression.

We employed inferCNV analysis, using normal prostate epithelial tissues as reference, to distinguish malignant from non-malignant epithelial cells in tumor samples based on chromosomal copy number variation (CNV) patterns (Supplementary Fig. 2B). This approach identified 152,872 epithelial cells harboring significant CNV events, which were classified as malignant. Comparative analysis of CNV scores demonstrated that malignant epithelial cells exhibited significantly higher CNV burdens than control cells (Fig. 2G). Quantification of malignant cell proportions across epithelial subtypes revealed the highest malignancy rates in neuroendocrine (NE) cells, lineage-plastic cells (LPC), and Luminal cells (Fig. 2H). In contrast, epithelial cells from healthy prostate tissues were exclusively classified as non-malignant (100%), validating the accuracy of our malignant cell identification approach. Intriguingly, we detected malignant epithelial cells within both hillock and club cell populations, despite their traditional characterization as primarily normal epithelial lineages not considered direct origins of prostate cancer [29]. Emerging literature suggests that NHT-induced treatment resistance and progression may drive luminal-derived malignant cells to acquire mixed transcriptional phenotypes, including hillock-, club-, and basal-like features [30–32].

We therefore performed separate inferCNV analyses for benign and malignant cells within tumor tissues (Supplementary Fig. 2C, 2D), and specifically for hillock (Supplementary Fig. 2E, 2F), club (Supplementary Fig. 2G, 2H), and basal (Supplementary Fig. 2I, 2J) cell subsets. Results confirmed that cells classified as malignant consistently displayed detectable CNV events, with CNV scores significantly exceeding those of benign tumor cells and healthy control epithelial cells. GSVA of benign versus malignant hillock, club (Supplementary Fig. 2K), and basal cells (Fig. 2I) revealed that malignant populations were enriched for oncogenic transcriptional signatures, including EMT, DNA repair, E2F targets, TGF- β signaling, androgen response, and MYC target pathways. Furthermore, literature reports implicate 23 transcription factors (TFs) in maintaining the mixed hillock/club-like phenotype associated with CRPC progression and therapy resistance [30]. Analysis of these TFs in benign versus malignant hillock (Fig. 2J) and club cells (Supplementary Fig. 2L) showed significantly elevated expression in malignant cells, with minimal expression in benign counterparts. Across clinical groups, these 23 TFs were most highly expressed in the On-ADT cohort (Fig. 2K), suggesting their activation is linked to ADT-induced progression and resistance. At the epithelial subtype level, basal-like cells exhibited high expression of TP63 and FOSL1, whereas luminal-like cells showed elevated levels of IRF1, JUNB, RUNX1, and STAT3 (Supplementary Fig. 2M). This expression dichotomy delineates the fundamental regulatory divergence between these lineages: the basal program is orchestrated by master regulators of stemness and plasticity (TP63) coupled with stress/AP-1 signaling (FOSL1), while the luminal program is driven by a network integrating cytokine signaling (IRF1, STAT3), immediate-early stress response (JUNB), and lineage-determining factors (RUNX1). These analyses support the interpretation that the observed mixed

hillock- and club-like malignant epithelial populations represent an NHT-induced, adaptation-associated state driven by tumor progression and treatment resistance, rather than artifacts of our inferCNV classification.

To spatially resolve these molecular signatures in the tissue context, we mapped the four gene signatures (AR, NE, CRPC51 and NE_Sig_UP [28]) onto our 8 in-house spatial transcriptomics samples and the public dataset GSE230282 (Fig. 2L). As anticipated, primary prostate cancer samples (P01, P02) exhibited the strongest AR signal enrichment. Specimens P08 and the NE region of GSE230282 showed predominant NE signature activity. Notably, while sample P07 lacked prominent NE signature expression, it demonstrated marked co-enrichment of CRPC51 and NE_Sig_UP signatures, with a spatial distribution pattern resembling the NE region of GSE230282. This molecular profile suggests that P07 likely represents a transitional state between CRPC and NEPC, characterized by pronounced lineage plasticity and enhanced proliferative potential, potentially capturing the critical phase of neuroendocrine transdifferentiation.

Molecular subtyping and trajectory analysis reveals distinct malignant epithelial states in prostate cancer

To systematically delineate the heterogeneity within prostate cancer epithelial cells, we performed non-negative matrix factorization (NMF) on malignant epithelial cells from 46 prostate cancer samples (Fig. 3A). This analysis defined ten meta-programs (MPs), each characterized by its top 50 genes (Supplementary Table 9). These included the Luminal epithelial identity Program (MP1: KLK3, NKX3-1, AR, FOLH1), the PCa proliferation Program (MP2: TOP2A, STMN1, AR, PSCA), the Neuronal Signaling Program (MP3: ROBO2, CNTNAP2, SCN2A, RBFOX1), the Luminal secretory Program (MP4: SCGB3A1, XBP1, SCNN1A, UPK1A), the Mucosa stress Program (MP5: SLPI, RARRES1, MMP7, PIGR), the Basal/EMT transition Program (MP6: TP63, KRT5, SPARC, PDPN), the T-cell crosstalk Program (MP7: CD3E, CCL5, GZMA, CD69), the Basal stress Program (MP8: GADD45A, ITGA2, ATP2B4, BACH2), the Reactive Defense Program (MP9: MMP7, S100A6, WFDC2, TGFB2), and the Cell Cycle Program (MP10: CDK1, TOP2A, BIRC5, AURKA). The expression patterns of these MPs across the malignant cells are visualized in a UMAP heatmap (Fig. 3B). Spatial mapping of selected MPs revealed high expression of MP1 in samples P01 and P02, while MP10 was enriched in samples P08 and GSE230282, suggesting these samples harbor proliferative, aggressive disease states (Fig. 3C).

Building on the initial dimensionality reduction and clustering of malignant epithelial cells, we performed a secondary integrative analysis. This analysis consolidated

the 28 initial clusters into four clearly defined major categories (Fig. 3D). Projecting these categories onto the UMAP revealed that clusters belonging to the same molecular subtype were spatially adjacent, forming distinct and cohesive groups (Fig. 3E). Notably, each subtype exhibited a shared meta-program (MP) signature: subtype 1 was defined by high expression of MP1 (Luminal Epithelial Identity); subtype 2 by MP4 and MP5; subtype 3 by MP3 and MP10 (Neuronal Signaling and Cell Cycle); and subtype 4 by MP6 and MP8 (Basal/EMT transition and Basal stress program). These conserved MP profiles formed the basis for defining these four groups as distinct molecular subtypes of prostate cancer.

To characterize their biological and clinical relevance, we analyzed the distribution of clinical groups and epithelial cell types across the subtypes. Subtype 1 was predominantly composed of cells from Primary and a subset of CRPC samples, Subtypes 2 and 4 were almost exclusively enriched in On-ADT samples, while the proportion of Subtype 3 cells was significantly elevated in NEPC samples (Supplementary Fig. 3A, 3B). Analysis of epithelial cell type composition revealed that Subtype 1 was overwhelmingly luminal, Subtype 3 was dominated by basal-like cells, Subtype 4 by neuroendocrine (NE) cells, and Subtype 2 displayed a mixed cellular phenotype (Supplementary Fig. 3C). Ratio of Observed to Expected (Ro/e) analysis further confirmed a significant enrichment of NE cells in Subtype 3 (Fig. 3D). This distribution suggests that Subtype 3 likely represents NEPC, Subtype 1 represents classic AR-positive, luminal-derived prostate cancer, while Subtypes 2 and 4 emerge following ADT. Subtype 4, with its high expression of basal-lineage markers (KRT5, KRT14, TP63), points towards an ADT-induced, basal-like, double-negative prostate cancer (DNPC) state with pronounced lineage plasticity and therapy resistance. Based on the dominant subtype (comprising > 50% of malignant epithelial cells) within each tumor sample, we classified the samples into Subtype1-, Subtype2-, Subtype3-, Subtype4-dominant, or Mixed groups (for samples lacking a single dominant subtype) (Supplementary Fig. 3E). CytoTRACE analysis indicated that Subtypes 3 and 4 possessed the highest differentiation potential, significantly greater than Subtypes 1 and 2 (Supplementary Fig. 3F, 3G). This further supports the notion that Subtypes 3 (NEPC) and 4 (DNPC) represent advanced states with high plasticity and stemness, whereas Subtypes 1 and 2 reside at earlier stages of the disease spectrum. InferCNV analysis applied separately to each subtype confirmed that Subtypes 3 and 4 harbored the most significant CNV burdens, indicating they occupy the most genomically unstable, terminal branches of the prostate cancer lineage (Supplementary Fig. 3H, 3I). Furthermore, we mapped the four molecular subtypes onto the UMAP embedding of all epithelial cells

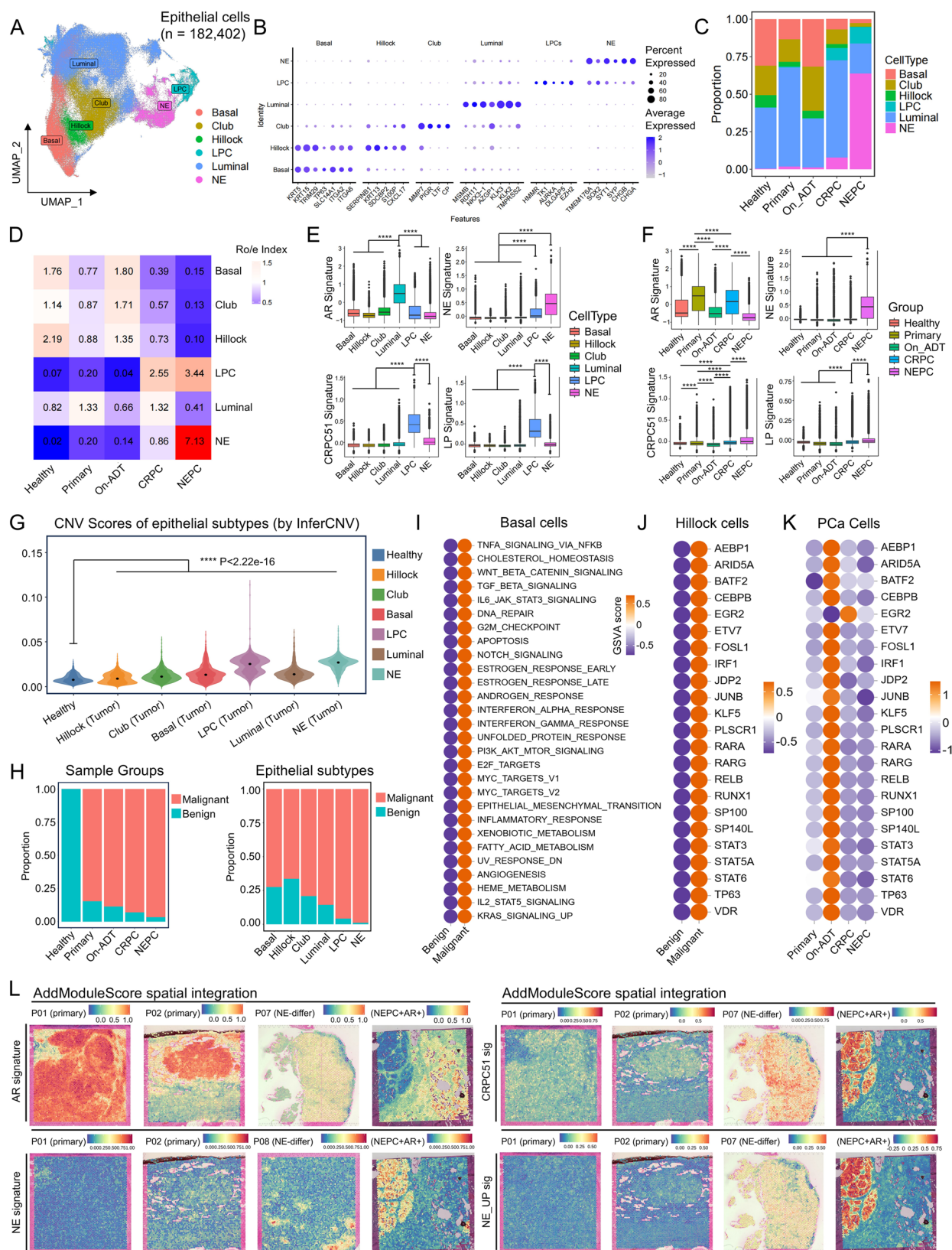


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Fig. 2 Characterization of epithelial cell heterogeneity and lineage plasticity in prostate cancer. **A** UMAP visualization of epithelial cell subsets. UMAP projection of 182,402 epithelial cells following dimensionality reduction and clustering, colored by annotated epithelial cell type. **B** Marker gene expression across epithelial cell types. Dot plot displaying the expression of canonical marker genes in each epithelial cell cluster. The color gradient (white to blue) indicates the scaled average expression level, while dot size represents the percentage of cells expressing the gene within each cluster. **C** Compositional analysis of epithelial subsets across clinical groups. Bar plot showing the relative proportion of each epithelial cell type within the Healthy, Primary, On-ADT, CRPC and NEPC clinical cohorts. **D** Enrichment of epithelial subsets in clinical groups. Ro/e (Ratio of observed to expected) index heatmap quantifying the over- (red) or under-representation (blue) of each epithelial cell type within each clinical group. **E** Gene signature activity in epithelial subsets. Violin plots depicting the enrichment scores (AddModuleScore) for four gene signatures - Androgen Receptor (AR) response, Neuroendocrine (NE), CRPC51, and Luminal Progenitor (LP) - across different epithelial cell types. Statistical significance was assessed using the Wilcoxon rank-sum test. In this and subsequent panels, significance levels are denoted as follows: ns, not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$. **F** Gene signature activity across clinical groups. Violin plots showing the distribution of enrichment scores for the four gene signatures (AR, NE, CRPC51, LP) among epithelial cells from the Healthy, Primary, On-ADT, CRPC and NEPC cohorts. **G** Malignant cell identification by inferCNV. Violin plot comparing the inferCNV-predicted copy number variation (CNV) scores between healthy epithelial cells and malignant epithelial cells (classified by inferCNV) across different epithelial subtypes. **H** Proportion of malignant cells across contexts. Stacked bar charts showing the percentage of cells classified as malignant (red) or benign (green) by inferCNV analysis within (Left) each clinical group and (Right) each epithelial cell type. **I** Pathway activity in benign vs. malignant Basal cells. Heatmap displaying the results of Gene Set Variation Analysis (GSVA) for selected Hallmark pathways in benign versus malignant Basal cells. The color scale (blue to red) represents low to high pathway activity scores. **J** Key transcriptional regulator expression in Hillock cells. Heatmap showing the expression levels of 23 key transcription factors critical for maintaining CRPC cell state plasticity in benign versus malignant Hillock epithelial cells. Expression is scaled from low (blue) to high (red). **K** Expression of key transcriptional regulators across disease stages. Violin plots illustrating the expression levels of the 23 transcription factors (aggregated) in epithelial cells across the four clinical stages: Primary, On-ADT, CRPC, and NEPC. **L** Spatial mapping of gene signatures. Representative spatial transcriptomics sections showing the in-silico mapping of the four gene signature scores (AR, NE, CRPC51, NE_UP) onto tissue architecture using the AddModuleScore method. For each spot, the color gradient (blue to red) indicates low to high signature enrichment

to explicitly link established epithelial cell types with the newly defined molecular subtypes (Supplementary Fig. 3J). This mapping revealed distinct compositional profiles: Subtype 1 was predominantly composed of Luminal cells (80%), Subtype 2 consisted of a mixed population of Club (65.1%) and Luminal (29.9%) cells, Subtype 3 was clearly defined by LPC (24.6%) and NE (71.5%) cells, and Subtype 4 was primarily composed of Basal-like (74.5%) with a minor proportion of Hillock-like (17%) cells. Notably, the molecular profile of Subtype 4 is fully consistent with the contemporary mechanistic understanding of lineage plasticity in double-negative prostate cancer (DNPC). As defined by Guo et al. [33], DNPC is molecularly characterized by the absence of AR signaling and neuroendocrine marker expression, rather than by a specific cellular origin. Critically, this state typically arises through the lineage transdifferentiation of luminal tumor cells under sustained AR pathway inhibition. This transdifferentiation is accompanied by a profound shift in epithelial identity, resulting in a mixed transcriptional state that adopts basal-, club-, and hillock-like gene programs [30]—including the upregulation of canonical basal markers such as KRT5, KRT6A, KRT14, and TP63. Consequently, the basal-like and hillock-like features of Subtype 4 do not signify an origin from normal basal or hillock epithelia. Instead, they reflect the acquired, plastic epithelial phenotype that is a hallmark of therapy-induced DNPC. Collectively, these observations support the conclusion that Subtype 4 represents a luminal-derived, lineage-plastic DNPC state, rather than a cancer subtype originating from normal basal cells.

Following subtype identification, we performed trajectory analysis to infer their evolutionary relationships. While Subtype 3 appeared spatially isolated on the

UMAP, potentially due to a scarcity of intermediate transitional cells in our cohort, Monocle3 analysis suggested a differentiation trajectory originating from Subtype 1, progressing through Subtype 2, and culminating in Subtype 4 (Supplementary Fig. 3K). This trajectory aligns with the hypothesis of ADT-induced progression from a luminal phenotype towards a basal-like, DNPC state. Notably, Subtype 3 was not placed on this primary path. This finding was corroborated by Slingshot analysis (Supplementary Fig. 3L). Subsequent application of scTOUR software also supported the Subtype 1→2→4 trajectory, although it positioned Subtype 3 closer to the root (Supplementary Fig. 3M). Re-running Monocle3 and scTOUR analyses after excluding Subtype 3 cells consistently reinforced the Subtype 1→2→4 trajectory (Supplementary Fig. 3N, 3O). These results suggest that Subtype 4 represents one terminal differentiation state, originating from Subtype 1 under the selective pressure of ADT. Integrating all evidence, we propose a branched evolutionary model for prostate cancer (Supplementary Fig. 3P): the disease initiates as a luminal-identity state (Subtype 1). Under ADT pressure, cells adapt via lineage diversification, with one branch differentiating into a basal-like, resilient Subtype 4 (DNPC), and another branch potentially through distinct intermediate states, ultimately giving rise to the highly proliferative, neurally-differentiated Subtype 3 (NEPC).

To functionally validate these subtypes, we performed GSVA. Examination of the top 20 marker genes revealed characteristic profiles (Supplementary Fig. 4A): Subtype 1 expressed PCA3, KLK2/3/4, and AR; Subtype 2 expressed VNN2 and MMP7; Subtype 3 expressed SYT5 and CHGA; and Subtype 4 expressed TP63 and KRT5/14/17. GSVA confirmed that Subtype 1 was

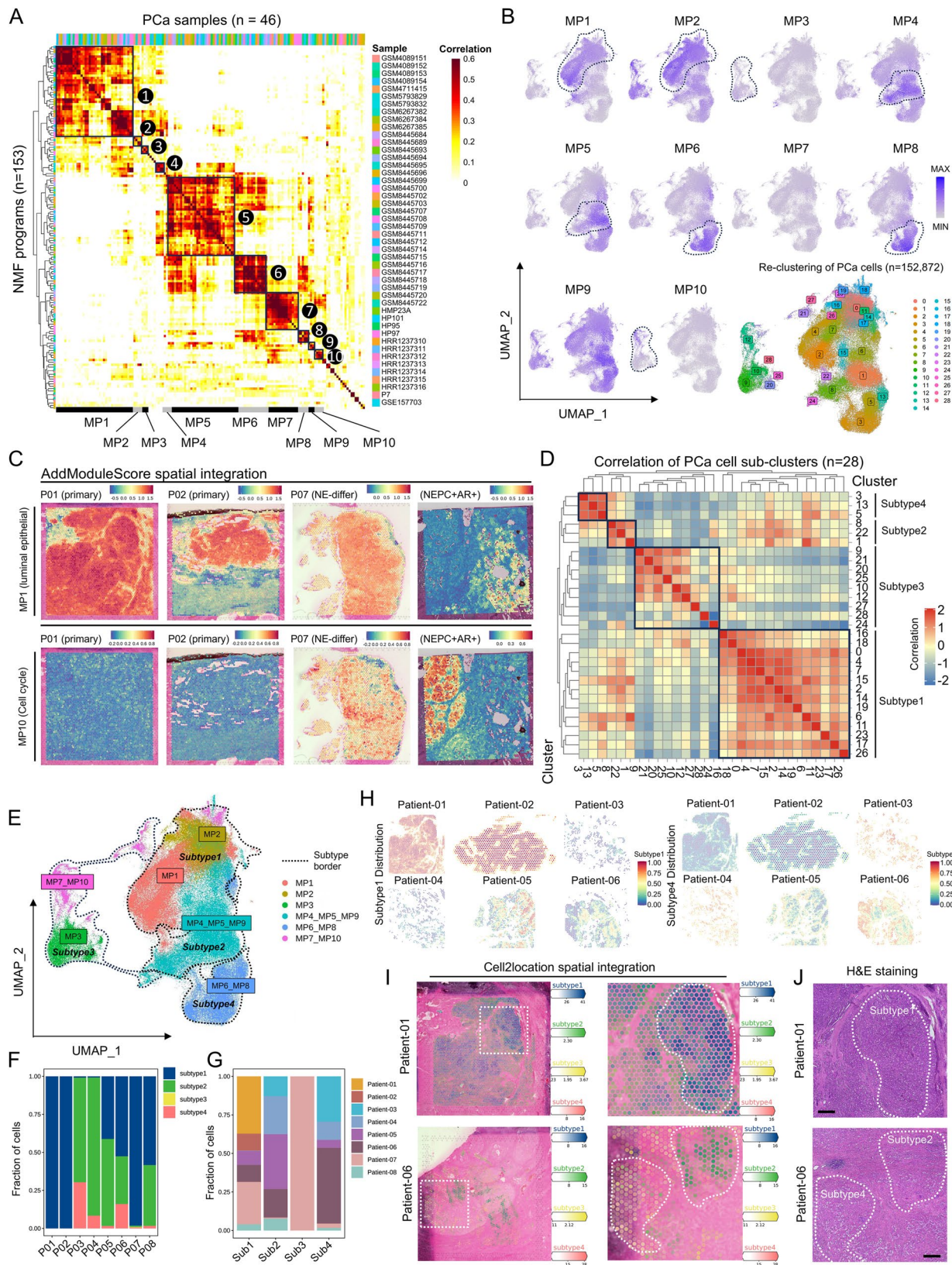


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Fig. 3 Meta-program analysis delineates malignant epithelial subtypes with spatial context. **A** Identification of meta-programs in the epithelial compartment. Non-negative Matrix Factorization (NMF) was performed on 46 samples, yielding 153 initial programs. These were aggregated based on correlation to define 10 biologically relevant meta-programs (MPs): MP1 (Luminal Epithelial), MP2 (PCa Proliferation), MP3 (Neuronal Signaling), MP4 (Luminal Secretory), MP5 (Mucosal Stress), MP6 (Basal/EMT Transition), MP7 (T-cell Crosstalk), MP8 (Basal Stress), MP9 (Reactive Defense), and MP10 (Cell Cycle). **B** Meta-program activity landscapes in malignant epithelium. UMAP visualization of 152,872 malignant epithelial cells, re-clustered into 28 subclusters. The activity scores (AddModuleScore) of the 10 meta-programs are projected onto the UMAP, with a gradient from white to blue indicating low to high expression. Dashed circles highlight regions where the respective MP is predominantly active. **C** Spatial mapping of luminal and proliferative programs. Representative spatial transcriptomics sections showing the spatial distribution of MP1 (Luminal Epithelial) and MP10 (Cell Cycle) activity scores. Per-spot scores (blue to red) are calculated using AddModuleScore. **D** Correlation analysis of malignant epithelial subclusters. Hierarchical clustering and heatmap depicting the correlation between the 28 malignant epithelial subclusters. Strongly correlated clusters, grouped within black rectangles, form the basis for subtype definition. Color scale indicates correlation coefficient from low (blue) to high (red). **E** Definition of malignant epithelial subtypes. UMAP of malignant epithelial cells, annotated with four major subtypes (Subtype 1–4). Dashed circles delineate subtype boundaries. The dominant, hallmark meta-program for each subtype is indicated with a corresponding color. **F** Subtype composition across samples. Stacked bar chart showing the relative proportion of each malignant epithelial subtype within each individual patient sample, as inferred by cell2location deconvolution of spatial transcriptomics data. Subtypes are color-coded. **G** Sample distribution across subtypes. Bar chart illustrating the contribution of cells from each patient sample to the four defined malignant epithelial subtypes. **H** Spatial patterns of Subtype 1 and Subtype 4. Heatmaps displaying the spatial enrichment scores of Subtype 1 (left) and Subtype 4 (right) across serial sections from Patient 01–06. Color gradient (blue to red) represents low to high subtype abundance scores. **I** Co-localization of subtypes in tissue architecture. Cell2location maps overlaid on H&E backgrounds for representative samples Patient 01 (top) and Patient 06 (bottom). The four subtypes are rendered in distinct colors (intensity indicates abundance). The left panels show the whole transcriptome array (6.5 × 6.5 mm). The white dashed boxes are magnified in the right panels, highlighting regions enriched for Subtype 1 (blue, top), Subtype 2 (red, bottom), and Subtype 4 (yellow, bottom). **J** Histopathological correlation of malignant subtypes. High-magnification H&E images corresponding to the regions identified in (I). White dashed boxes and labels indicate areas mapped to Subtype 1 (Patient 01), Subtype 2, and Subtype 4 (Patient 06). Scale bar represents 200 μ m

enriched for Androgen Response and MYC Targets pathways. Subtype 3 showed activation of Cell Cycle and DNA Repair pathways. Subtype 4 exhibited the strongest enrichment for Inflammatory Response pathways and the highest EMT activity (Supplementary Fig. 4B). Further signature scoring aligned Subtype 1 with an AR-driven phenotype, Subtype 3 with neuroendocrine and lineage plasticity signatures, and Subtype 4 with squamous/basal features, prostate cancer epigenetic programs, and stemness (Supplementary Fig. 4C).

To assess clinical relevance, we employed the Scissors algorithm on four bulk transcriptomic datasets: GSE70769 [34], GSE107299 [35], the MSKCC cohort [36], and TCGA-PRAD [37] to identify cells associated with biochemical recurrence (BCR). Scissors-positive cells were predominantly concentrated in the sub3 subtype, consistent with the poor prognosis of NEPC (Supplementary Fig. 4D). An intersection of results across cohorts yielded 73 robust BCR-associated genes (Supplementary Table 11), which were predominantly overexpressed in sub3 (Supplementary Fig. 4E – 4G). Survival analysis within the TCGA-PRAD dataset confirmed that patients enriched for the sub3 signature had the worst prognosis (Supplementary Fig. 4H), and stratification of TCGA samples by sub3 abundance revealed significantly poorer outcomes for the high-abundance group (Supplementary Fig. 4I).

Finally, we aimed to delineate the spatial distribution of these four subtypes within prostate cancer tissues. To this end, malignant epithelial regions in spatial transcriptomics samples were first identified via a combined strategy of spaCET analysis and clinicopathological assessment. Within these demarcated regions, we performed spatial mapping of the four subtypes using

Cell2location. Subsequent quantification of subtype proportions across samples revealed distinct distribution patterns (Fig. 3F and G). Subtype 1 was most abundant in samples P01, P02, and P07, with the highest proportion detected in P01. In contrast, Subtypes 2 and 4 were predominantly enriched in post-NHT samples, and P06 exhibited the highest proportion of Subtype 4. Notably, Subtype 3 was exclusively observed in the NE-differentiated sample (P07). Owing to the low abundance of Subtype 3, subsequent spatial distribution analyses focused on Subtypes 1, 2, and 4. We generated heatmaps to visualize the localization of Subtypes 1 and 4 within malignant epithelial regions of samples P01–P06 (Fig. 3H). Integration of these spatial transcriptomic data with hematoxylin and eosin (H&E) staining results enabled high-resolution spatial annotation (Fig. 3I and J). Regions with marked Subtype 1 enrichment corresponded to classic prostatic adenocarcinoma morphology. This morphology is characterized by well-formed glandular structures, columnar epithelial lining, and minimal nuclear atypia. Distinct histopathological features were observed in regions cohabited by Subtypes 2 and 4. In Subtype 2-dominant areas, epithelial cells were poorly differentiated but retained partial epithelial morphology, with increased pleomorphism. In contrast, Subtype 4-enriched regions displayed more aggressive dedifferentiation. Cells in these regions lost epithelial characteristics, adopted spindle or oval shapes, showed prominent pleomorphism, and occasionally exhibited sarcomatoid features. This rigorous spatial-morphological integration provides a critical link between molecular states and tissue pathology. The distinct cytology and spatial pattern of Subtype 4 (DNPC) reinforce its identity as a highly plastic, treatment-emergent, and aggressive state. The

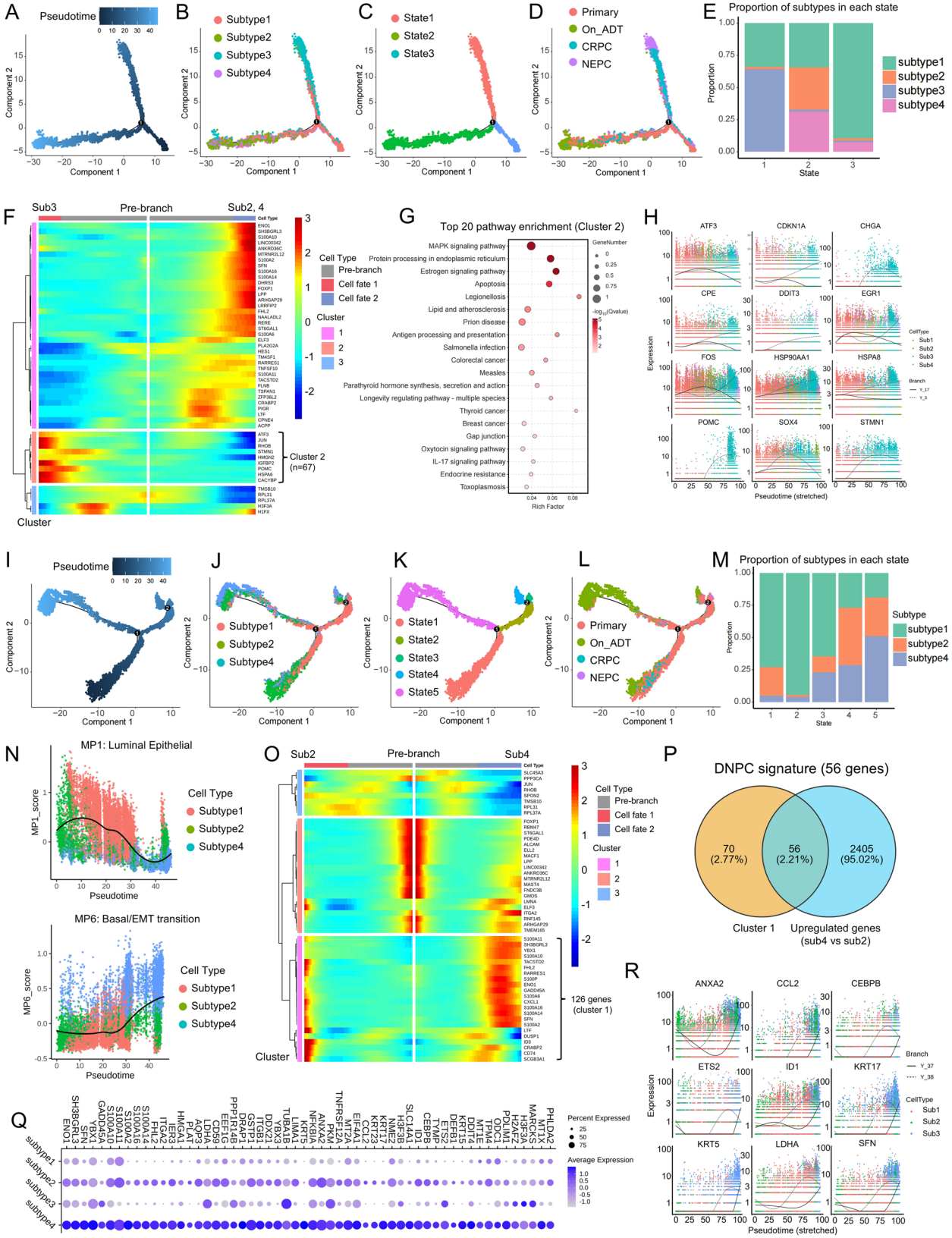


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Fig. 4 Lineage trajectory analysis reveals molecular drivers of prostate cancer progression. **A** Monocle2 trajectory analysis of sub1-sub4 cells colored by pseudotime, with gradient from dark blue (start) to light blue (end). **B** Same trajectory as A colored by molecular subtypes: sub1 (pink), sub2 (green), sub3 (cyan), sub4 (purple). **C** Same trajectory as A colored by Monocle2 states: state1 (red), state2 (green), state3 (blue). **D** Same trajectory as A colored by clinical groups: Primary (red), On-ADT (green), CRPC (cyan), NEPC (purple). **E** Stacked bar plot showing subtype composition within each Monocle2 state: sub1 (green), sub2 (orange), sub3 (purple), sub4 (pink). **F** Heatmap of genes differentially expressed along pseudotime during trajectory bifurcation. The central region represents pre-branch cells, with genes showing increased expression toward the sub2/sub4 branch (blue direction) or sub3 branch (red direction). Cluster 2 (67 genes, red box) shows progressive upregulation toward sub3 differentiation. **G** KEGG pathway enrichment analysis of 67 genes in Cluster 2, displaying top 20 significantly enriched pathways. **H** Expression dynamics of 12 representative genes from Cluster 2 along pseudotime, showing progressive upregulation during sub3 differentiation. **I** Monocle2 trajectory analysis of sub1, sub2, and sub4 cells colored by pseudotime, with gradient from dark blue (start) to light blue (end). **J** Same trajectory as J colored by molecular subtypes: sub1 (pink), sub2 (green), sub4 (blue). **K** Same trajectory as J colored by Monocle2 states: state1 (red), state2 (khaki), state3 (green), state4 (blue), state5 (pink). **L** Same trajectory as J colored by clinical groups: Primary (red), On-ADT (green), CRPC (cyan), NEPC (purple). **M** Stacked bar plot showing subtype composition within each Monocle2 state: sub1 (green), sub2 (orange), sub4 (purple). **N** Pseudotemporal dynamics of meta-program activities. Scatter plots showing the changes in activity scores for (Top) MP1 (Luminal Epithelial) and (Bottom) MP6 (Basal/EMT Transition) along the inferred pseudotime trajectory. Each point represents a cell, colored by its assigned subtype (red for sub1, green for sub2, blue for sub4). The x-axis represents pseudotime, and the y-axis represents the meta-program enrichment score (AddModuleScore). The black curve represents the overall trend of MP activity across pseudotime, fitted using a generalized additive model (GAM) on all cells. **O** Heatmap of genes differentially expressed along pseudotime during sub1-sub2-sub4 trajectory bifurcation. Cluster 1 (126 genes, pink box) shows progressive upregulation toward sub4 differentiation (blue direction). **P** Venn diagram showing the intersection between 126 genes from Cluster 1 and 2461 genes differentially expressed in sub4 versus sub2, yielding 56 genes defined as the DNPC signature. **Q** Dot plot displaying expression patterns of the 56 DNPC signature genes across sub1-sub4. Dot size represents percentage of expressing cells, color intensity indicates average expression level from light blue (low) to dark blue (high). **R** Expression dynamics of 9 representative genes from the DNPC signature along pseudotime, showing progressive upregulation during sub4 differentiation

co-localization of Subtype 2 and Subtype 4 in regions of poor differentiation visually encapsulates the transitional stress-response and lineage plasticity axis central to our model. These morphological observations are consistent with existing literature on advanced prostate cancer variants [38]. However, further validation in larger, dedicated spatial pathology cohorts is warranted to confirm their clinical and biological relevance.

Developmental trajectory analysis reveals molecular drivers of lineage diversification

To delineate the developmental relationships among the four molecular subtypes, we performed trajectory inference using Monocle2. The resulting trajectory tree originated from sub1 and bifurcated into two distinct branches: one leading to sub3 (neuroendocrine) and another to sub2 and sub4 (Fig. 4A – 4 C). This bifurcation pattern is analogous to the trajectory inferred by Monocle3, indicating a divergent evolutionary path from sub1 towards either sub3 or the sub2/sub4 branch. Given our previous finding positioning sub4 as a terminal state, we re-analyzed the trajectory after excluding sub4 cells, performing fresh dimensionality reduction and clustering on sub1, sub2, and sub3 (Supplementary Fig. 5A, 5B). Monocle3 analysis of this refined dataset revealed a linear trajectory originating from sub1, progressing through sub2, and culminating in sub3 (Supplementary Fig. 5C). This linear progression supports a model where ADT-induced cellular stress and adaptation drive lineage evolution ultimately leading to neuroendocrine differentiation. Slingshot analysis identified two independent lineage trajectories: one tracing a near-direct path from sub1 to sub4, and another terminating at sub2, closely mirroring the bifurcation observed in the Monocle2

analysis (Supplementary Fig. 5D). Mapping clinical groups onto the Monocle2 trajectory confirmed its biological relevance, showing progression from primary prostate cancer along two paths: one advancing through CRPC to NEPC (sub3), and another branching toward post-neoadjuvant treatment states (sub2/sub4) (Fig. 4D). State analysis within the Monocle2 framework revealed that state1 primarily contained sub1 cells (root), state3 was dominated by sub3 cells, while state2 contained a mixture of sub1, sub2, and sub4 cells (Fig. 4E).

Analysis of dynamically expressed genes along the trajectory identified three major gene clusters (Fig. 4F, Supplementary Fig. 5E). Notably, cluster 2 (67 genes) exhibited significant upregulation specifically along the sub3 differentiation branch. Expression validation confirmed these genes were predominantly expressed in sub3 (Supplementary Fig. 5F). KEGG pathway analysis revealed their involvement in MAPK signaling, multiple malignancies (colorectal, breast, and thyroid cancers), estrogen signaling, and antigen processing and presentation (Fig. 4G). This suggests that neuroendocrine differentiation may be driven by MAPK pathway activation and involves metabolic reprogramming and immune modulation, potentially facilitating immune evasion.

We visualized expression dynamics of 12 key differentiation-related genes (ATF3, CDKN1A, CHGA, CPE, DDIT3, EGR1, FOS, HSP90AA1, HSPA8, POMC, SOX4, STMN1) across the trajectory (Fig. 4H). These genes, including established neuroendocrine markers (CHGA, POMC) and stress-responsive transcription factors (ATF3, DDIT3, EGR1, FOS), showed progressive upregulation along the sub3 branch. Their coordinated expression suggests involvement in cellular stress response,

neuronal differentiation, and proliferation control during neuroendocrine transdifferentiation.

Our prior analyses using Monocle3 and scTOUR had already established a differentiation continuum from sub1 through sub2 and terminating at sub4. To further refine this trajectory, we applied Monocle2, which identified five distinct states and two bifurcation points, with sub1 cells concentrated in states 1–2 and sub4 cells predominantly in state 5 (Fig. 4I and L). We focused on the branch leading to state 5 as the major route for sub4 differentiation (Fig. 4M). Along this branch, we observed a progressive downregulation of MP1 (Luminal epithelial identity) concurrent with a rise in MP6 (Basal/EMT transition), aligning with the acquisition of basal-like transcriptional features and loss of luminal identity during sub4 differentiation (Fig. 4N, Supplementary Fig. 5G). Analysis of genes varying along this branch identified 126 genes (cluster 1) significantly upregulated during sub4 differentiation (Fig. 4O, Supplementary Fig. 5H).

Intersecting these 126 trajectory-specific genes with 2405 differentially expressed genes between sub4 and sub2 yielded a 56-gene signature, designated the “DNPC signature” (Fig. 4P, Supplementary Table 11). Bubble plot visualization confirmed specific enrichment of these genes in sub4, effectively distinguishing it from sub2 (Fig. 4Q). Key signature genes included ANXA2, CCL2, CEBPB, ETS2, ID1, KRT17, KRT5, LDHA, and SFN, which have established roles in basal lineage biology, inflammation (CCL2), metabolic reprogramming (LDHA), and treatment resistance, consistent with the double-negative, therapy-resistant phenotype of sub4 (Fig. 4R).

Spatial mapping of the DNPC signature across our 9 spatial transcriptomics samples revealed predominant localization to stromal regions, with minimal overlap with AR-positive luminal or NEPC regions (Supplementary Fig. 5I). This unexpected spatial distribution, despite the derivation of the signature from epithelial subtyping, may reflect several factors: the strong basal cell character of the signature (KRT5, KRT17), which often localizes at stromal-epithelial interfaces and shares transcriptional features with stromal cells [39, 40]; possible epithelial-mesenchymal transition features where tumor cells acquire mesenchymal characteristics; or substantial stromal contamination in the sub4 transcriptome. Alternatively, it may indicate active epithelial-stromal crosstalk in double-negative prostate cancer development, where stromal niches support the emergence and maintenance of this aggressive subtype through paracrine signaling.

Functional validation of FOSL1 in double-negative prostate cancer progression

Given the limited understanding of molecular drivers in double-negative prostate cancer (DNPC) pathogenesis

[33], we focused on identifying key transcriptional regulators in the sub4 subtype. Through transcription factor activity analysis, we discovered that both FOSL1 and NR2F6 exhibited significantly elevated activity in sub4 (Fig. 5A). While NR2F6 has been implicated in immune modulation and therapy resistance [41, 42], we selected FOSL1 for further investigation due to its established role in promoting EMT, cellular invasion, and treatment resistance across various cancer types [43, 44], making it a more promising candidate for DNPC progression.

Transcriptomic analysis of single-cell data revealed that both the expression and transcriptional activity of FOSL1 were most pronounced in the On-ADT group (Supplementary Fig. 6A, 5B), significantly exceeding levels observed in healthy epithelium, primary prostate cancer, CRPC, and NEPC. FOSL1 exhibited elevated expression particularly in basal-like and hillock-like epithelial cells, aligning with the transcriptional signature of cellular stress and heterogeneity induced by NHT (Supplementary Fig. 6C, 5D). Notably, subtype 4 demonstrated the highest FOSL1 expression and activity (Fig. 5B, Supplementary Fig. 6E, 5 F), positioning FOSL1 as a key regulator associated with the treatment-emergent, double-negative phenotype.

To validate these findings *in vitro*, we assessed FOSL1 protein levels across prostate cancer cell lines. Western blot analysis confirmed high FOSL1 expression in AR-negative PC-3 cells, significantly lower expression in AR-positive LNCaP cells, and intermediate levels in AR/AR-V7-positive 22Rv1 cells (Supplementary Fig. 6G). This expression pattern corroborates the single-cell data, suggesting that FOSL1 is preferentially active in AR-independent or low-AR contexts. We therefore selected PC-3 as an AR-negative DNPC model and LNCaP as an AR-positive control for subsequent functional studies.

Efficient FOSL1 knockdown was achieved in both cell lines using siRNA (Supplementary Fig. 6H, 6I). Functional assays demonstrated that FOSL1 depletion significantly inhibited proliferation (as assessed by CCK-8 and colony formation assays, Fig. 5C and D) and induced apoptosis (measured by Annexin-V/PI staining, Fig. 5E) specifically in PC-3 cells, with no comparable effects observed in LNCaP cells (Supplementary Fig. 6G – 6 L). Furthermore, FOSL1 knockdown led to cell cycle inhibition in PC-3 cells, consistent with the observed suppression of proliferation (Supplementary Fig. 6M). Concurrently, the migratory and invasive capacities of PC-3 cells were also markedly attenuated following FOSL1 knockdown (Supplementary Fig. 6N, 6O). FOSL1 knockdown led to a significant reduction in cell viability, proliferation, and long-term clonogenic survival. Consistent with the prominent EMT signature of DNPC cells and the known role of FOSL1 in regulating EMT in other cancers, we also observed a pronounced decrease in

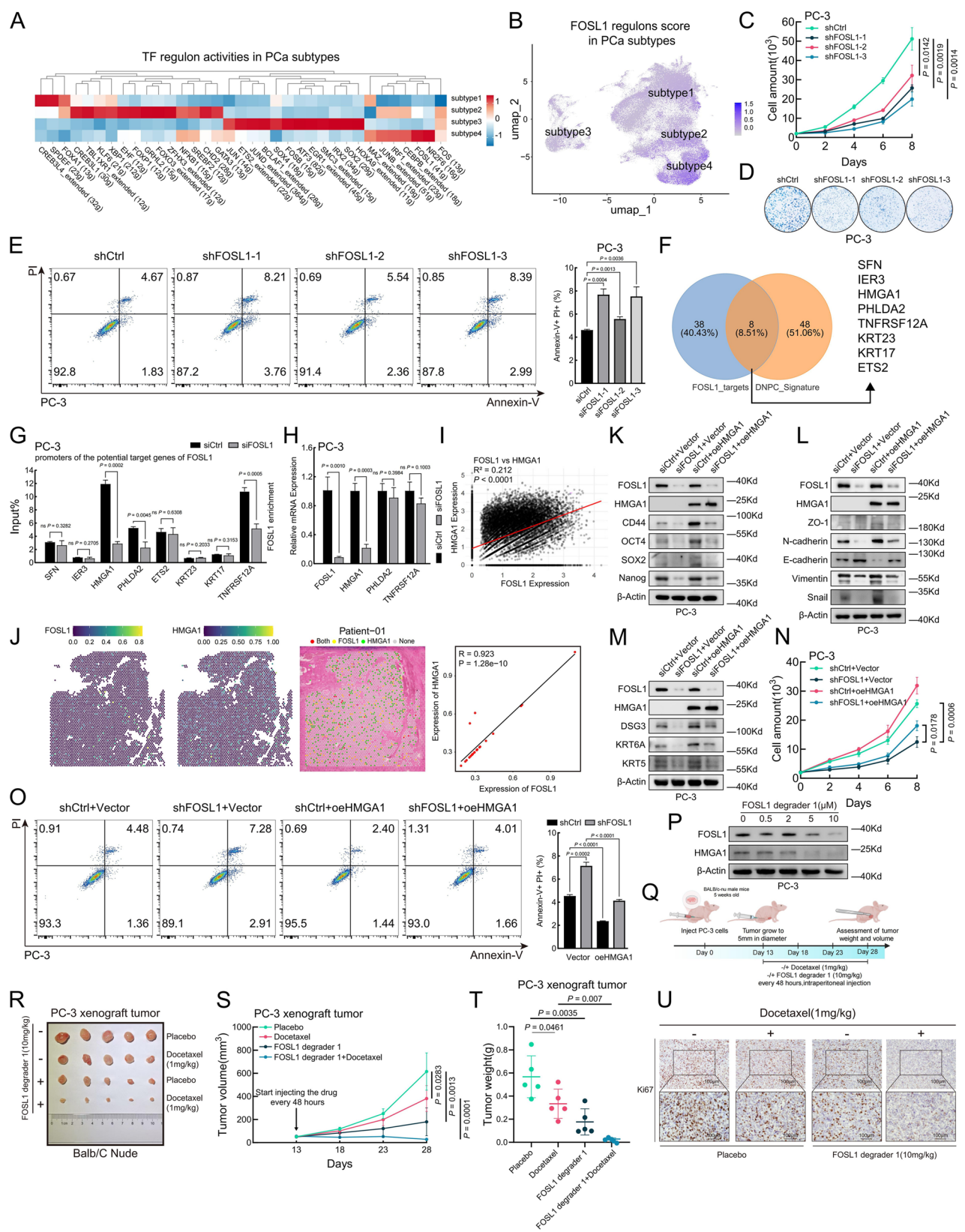


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Fig. 5 Functional characterization of FOSL1 as a key regulator in double-negative prostate cancer subtype. **A** Transcription factor activity in malignant epithelial subtypes. Heatmap displaying the top transcription factors with the highest inferred activity (based on SCENIC analysis) in each of the four malignant epithelial subtypes. Color scale (blue to red) represents low to high regulon activity. **B** Spatial activity of FOSL1 across subtypes. UMAP of malignant epithelial cells colored by the SCENIC-derived regulon activity score of FOSL1, showing its specific enrichment in Subtype 4. Gradient from white to blue indicates low to high activity. **C** Effect of FOSL1 knockdown on cell proliferation (CCK-8 assay). Growth curves of PC-3 cells transfected with control siRNA or three independent siRNAs targeting FOSL1. Data are presented as mean $\pm$ SD; *** P < 0.001, ** P < 0.01 (two-way ANOVA). **D** Effect of FOSL1 knockdown on clonogenic survival. Representative images of colony formation assays for PC-3 cells treated as in (C). **E** Effect of FOSL1 knockdown on apoptosis. (Left) Representative flow cytometry plots of Annexin V/PI staining. Quadrants: viable cells (Annexin V⁻/PI⁻), early apoptotic (Annexin V⁺/PI⁻), late apoptotic (Annexin V⁺/PI⁺), and necrotic (Annexin V⁻/PI⁺). (Right) Quantification of total apoptotic cells (early + late apoptosis). Data are mean $\pm$ SD; *** P < 0.001, ** P < 0.01 (one-way ANOVA). **F** Identification of FOSL1-regulated DNPC genes. Venn diagram showing the overlap between predicted FOSL1 target genes (from SCENIC) and the DNPC gene signature, yielding 8 candidate genes. **G** FOSL1 binding to candidate gene promoters (ChIP-qPCR). ChIP-qPCR analysis showing enrichment of FOSL1 at the promoters of the 8 candidate genes in control vs. FOSL1-knockdown PC-3 cells. Results are presented as % input. Data are mean $\pm$ SD; *** P < 0.001, ** P < 0.01, ns = not significant (two-tailed Student's *t*-test). **H** Expression of candidate genes upon FOSL1 knockdown (qRT-PCR). mRNA expression levels of four selected candidate genes in PC-3 cells after FOSL1 knockdown. Data are normalized to control and presented as mean $\pm$ SD; *** P < 0.001, ** P < 0.01, ns = not significant (two-tailed Student's *t*-test). **I** Correlation between FOSL1 and HMGA1 expression. Scatter plot showing a significant positive correlation between FOSL1 and HMGA1 expression across all malignant epithelial cells (n = 152,872). The red line indicates the linear regression fit (R^2 = 0.212, P < 0.0001, Pearson correlation). **J** Spatial co-expression of FOSL1 and HMGA1. (Left three panels) Spatial mapping of FOSL1 and HMGA1 expression and their co-localization in a representative section (Patient 01). Red spots indicate high co-expression, yellow indicates high FOSL1 alone, green indicates high HMGA1 alone, and gray indicates low expression of both. (Right panel) Correlation analysis of FOSL1 and HMGA1 expression specifically within the co-expressing spots (red spots, left panel), showing a strong positive correlation (R = 0.923, P = 1.28e-10, Pearson correlation). **K–M** Western blot analysis of downstream pathways. Protein levels of (K) stemness markers (CD44, OCT4, SOX2, NANOG), (L) EMT markers (ZO-1, N-cadherin, E-cadherin, Vimentin, SNAIL), and (M) DNPC markers (DSG3, KRT6A, KRT5), along with FOSL1 and HMGA1, in PC-3 cells under four conditions: Control, FOSL1 knockdown (siFOSL1), HMGA1 overexpression (oeHMGA1), and FOSL1 knockdown combined with HMGA1 rescue (siFOSL1 + oeHMGA1). GAPDH served as a loading control. **N** Rescue of proliferation by HMGA1 overexpression. CCK-8 proliferation assay of PC-3 cells under the four conditions described in (K–M). Data are mean $\pm$ SD; *** P < 0.001, ** P < 0.01, ns = not significant (two-way ANOVA). **O** Rescue of apoptosis by HMGA1 overexpression. Quantification of total apoptosis (Annexin V⁺ cells) by flow cytometry under the four conditions described in (K–M). Data are mean $\pm$ SD; *** P < 0.001, ** P < 0.01, ns = not significant (one-way ANOVA). **P** Pharmacological inhibition of FOSL1 reduces HMGA1. Western blot showing dose-dependent decrease of FOSL1 and its downstream target HMGA1 in PC-3 cells treated with increasing concentrations (0, 0.2, 2, 5, 10 μ M) of the FOSL1 degrader T-5224. **Q** In vivo combination therapy schematic. Workflow of the xenograft study. Nude mice bearing PC-3 subcutaneous tumors were treated starting at day 13 post-inoculation with vehicle, Docetaxel (1 mg/kg), FOSL1 degrader T-5224 (10 mg/kg), or the combination via intraperitoneal injection every 48 h. Treatment continued until day 28. **R** Representative images of resected tumors. Photographs of excised tumors from each treatment group at the study endpoint. **S** Tumor growth curves. Tumor volume (mm^3) was measured over time for each treatment group. Data are presented as mean $\pm$ SEM; *** P < 0.001 (two-way ANOVA). **T** Tumor weight at endpoint. Final tumor weights (g) for each group. Data are mean $\pm$ SEM; *** P < 0.001, ** P < 0.01 (one-way ANOVA). **U** Assessment of tumor cell proliferation (IHC). Representative immunohistochemistry images showing Ki67 expression in tumor sections from each treatment group. Scale bar, 100 μ m

migratory and invasive capacity; however, this effect may be compounded by the overall reduction in proliferative fitness.

To identify FOSL1 target genes relevant to DNPC differentiation, we intersected 46 established FOSL1 target genes with our DNPC signature, yielding 8 candidates: SFN, IER3, HMGA1, PHLDA2, TNFRSF12A, KRT23, KRT17, and ETS2 (Fig. 5F). Chromatin immunoprecipitation (ChIP) assays revealed direct FOSL1 binding to promoter regions of HMGA1, PHLDA2, and TNFRSF12A (Fig. 5G). However, only HMGA1 expression was consistently downregulated following FOSL1 knockdown, suggesting it as a key downstream effector (Fig. 5H). Supporting this regulatory relationship, existing literature documents FOSL1-mediated transcriptional regulation of HMGA1 across multiple cancer types [45, 46]. Furthermore, analysis of public FOSL1 ChIP-seq datasets confirms the presence of binding peaks at the HMGA1 promoter in various cancer cell lines [47], corroborating the direct regulatory interaction between FOSL1 and HMGA1.

The regulatory relationship between FOSL1 and HMGA1 was further supported by integrated single-cell

and spatial transcriptomic analysis. A significant positive correlation was observed between FOSL1 and HMGA1 expression in single-cell data (R^2 = 0.212, P < 0.0001, Fig. 5I). Spatial mapping across 8 samples revealed co-localization patterns, with expression levels of both genes demonstrating significant correlation within co-localized regions (Fig. 5J, Supplementary Fig. 5P). This correlation was consistently observed across individual samples and clinical groups (Primary, post-NHT, NE-differentiation), confirming FOSL1 as a spatial regulator of HMGA1 in the prostate cancer microenvironment.

Single-cell analysis revealed that subtype 4 cells exhibited significantly elevated EMT activity, and DNPC cells are recognized for their pronounced lineage plasticity. To validate these observations and investigate the role of the FOSL1/HMGA1 axis, we performed rescue experiments by transfecting HMGA1 plasmid into FOSL1-knockdown PC-3 cells. Western blot analysis showed that FOSL1 knockdown suppressed stemness factors (CD44, OCT4, SOX2, Nanog; Fig. 5K), EMT markers (ZO-1, N-Cadherin, E-Cadherin, Vimentin, Snail; Fig. 5L), and Basal-like proteins (KRT5, KRT6A, DSG3; Fig. 5M), HMGA1 overexpression substantially reversed these phenotypic

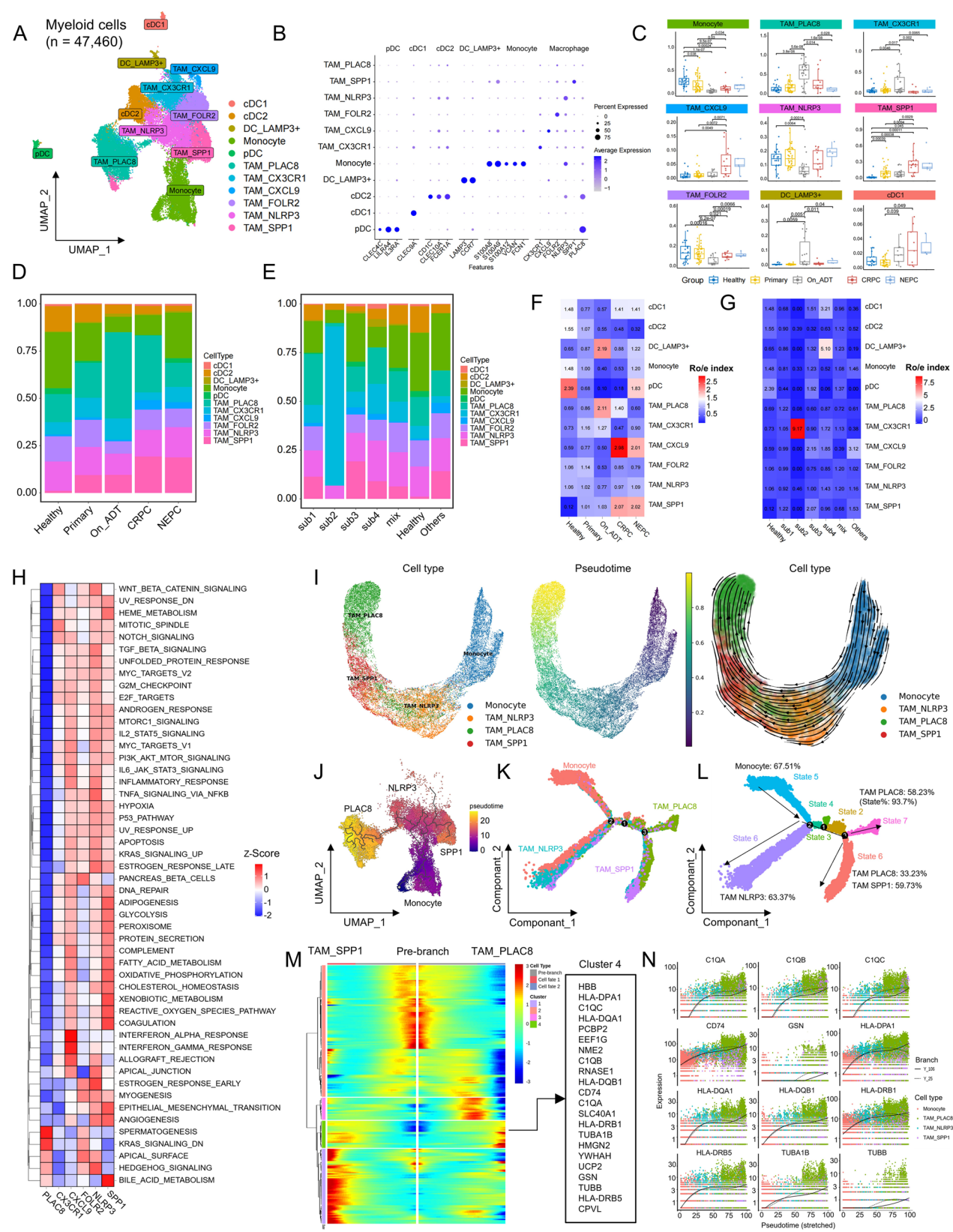


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Fig. 6 Myeloid cell heterogeneity and differentiation dynamics in prostate cancer. **A** UMAP visualization of 40,847 myeloid cells, annotated into 11 distinct subsets: cDC1, cDC2, DC LAMP3+, Monocyte, pDC, TAM PLAC8+, TAM CX3CR1+, TAM CXCL9+, TAM FOLR2+, TAM NLRP3+, and TAM SPP1+. **B** Dot plot displaying expression patterns of canonical marker genes across myeloid subsets. Dot size represents the percentage of cells expressing each gene, while color intensity indicates average expression level from light blue (low) to dark blue (high). **C** Box plots showing proportional differences of myeloid subsets across clinical groups. Statistical significance was determined using Kruskal-Wallis test with Dunn's post-hoc correction (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Boxes represent interquartile range with center lines indicating median values. **D-E** Stacked bar plots illustrating the compositional distribution of myeloid subsets across clinical groups (D) and molecular subtypes (E). Each colored segment represents the percentage of a specific myeloid subset. **F-G** Ratio of Observed to Expected (Ro/e) analysis heatmaps depicting enrichment patterns of myeloid subsets across clinical groups (F) and molecular subtypes (G). Color scale represents Ro/e index from blue (depletion) to red (enrichment). **H** GSVA heatmap showing Hallmark pathway activities across TAM subsets. Color gradient from blue (low activity) to red (high activity) represents enrichment scores. **I** scTOUR trajectory analysis of monocyte-to-TAM differentiation. Left panel: cell type distribution (monocytes-blue, TAM NLRP3+-orange, TAM SPP1+-purple, TAM PLAC8+-green). Middle panel: pseudotime trajectory from purple (start) to yellow (end). Right panel: vector field analysis showing differentiation directions. **J** Monocle3 trajectory analysis (UMAP visualization) of monocyte-to-TAM differentiation. Monocytes represent the starting point, TAM NLRP3+ cells occupy the branching position, differentiating toward TAM SPP1+ and TAM PLAC8+ lineages. **K-L** Monocle2 trajectory analysis of monocyte-to-TAM differentiation. (K) Colored by cell types: monocytes (red), TAM NLRP3+ (cyan), TAM PLAC8+ (green), TAM SPP1+ (purple). (L) Colored by Monocle2 states (state1-state7). Black arrows indicate differentiation directions. **M** Heatmap of genes differentially expressed along pseudotime during monocyte-to-TAM differentiation. Gray indicates pre-branch region, blue represents PLAC8+ differentiation branch, red shows SPP1+ differentiation branch. Cluster 4 (22 genes, green box) shows progressive upregulation during PLAC8+ TAM differentiation, with representative genes listed. **N** Expression dynamics of 12 representative genes from Cluster 4 along pseudotime, showing progressive upregulation during PLAC8+ TAM differentiation

changes. Subsequent CCK-8 (Figure 5N), Annexin-V/PI apoptosis (Fig. 5O), and Transwell assays (Supplementary Fig. 6Q) confirmed that HMGA1 restoration rescued the proliferative, apoptotic, migratory, and invasive alterations induced by FOSL1 knockdown. These results demonstrate that FOSL1 regulates EMT, basal-like and stemness marker expression through HMGA1, thereby promoting DNPC-like differentiation in AR-negative prostate cancer.

For translational investigation, we employed the FOSL1 degrader T-5224 [48, 49], which dose-dependently reduced both FOSL1 and HMGA1 expression in PC-3 cells (Fig. 5P). To evaluate therapeutic efficacy in vivo, PC-3 xenografts were established in nude mice. Tumor-bearing mice received low-dose docetaxel (simulating prostate cancer chemotherapy) via intraperitoneal injection, alone or in combination with the FOSL1 degrader or placebo (Fig. 5Q). While both low-dose docetaxel and the FOSL1 degrader monotherapy slowed tumor growth, the combination therapy produced the most potent inhibitory effect, resulting in the smallest final tumor volumes (Fig. 5R and S) and weights (Fig. 5T), and the lowest Ki67 proliferation index (Fig. 5U) by IHC. These findings demonstrate that FOSL1 degradation effectively suppresses AR-negative tumor growth, and combination with low-dose chemotherapy provides enhanced antitumor efficacy while potentially maintaining a more favorable toxicity profile associated with reduced chemotherapy dosing.

Myeloid cell heterogeneity and PLAC8+ TAM dynamics in prostate cancer progression

From our integrated single-cell dataset, we isolated 40,847 myeloid cells and identified 11 distinct subsets based on canonical marker expression (Fig. 6A and B, Supplementary Table 12): one monocyte population, six tumor-associated macrophage (TAM) subsets (FOLR2+,

PLAC8+, CX3CR1+, CXCL9+, SPP1+, and NLRP3+), and four dendritic cell (DC) populations. Compositional analysis across disease stages revealed that monocytes and FOLR2+ TAMs were predominantly enriched in healthy and primary prostate samples, with their proportions significantly declining as tumors progressed (Fig. 6C and D, Supplementary Fig. 5A). In contrast, PLAC8+ TAMs and CX3CR1+ TAMs were markedly expanded in the On-ADT group, while CXCL9+ TAMs and SPP1+ TAMs became increasingly prevalent in CRPC and NEPC samples. This dynamic redistribution suggests that specific TAM subsets are recruited or reprogrammed in response to therapeutic pressure and tumor evolution, with inflammatory (CXCL9+) and matrix-remodeling (SPP1+) TAMs dominating late-stage disease, consistent with their established roles in treatment resistance and metastatic progression [50, 51].

We further stratified samples based on the predominant malignant epithelial subtype (sub1-sub4 or mixed). CX3CR1+ TAMs were significantly enriched in subtype 2 (sub2)-dominant samples (Fig. 6E, Supplementary Fig. 5B), suggesting their potential involvement in post-therapy inflammatory responses and tissue repair processes following androgen deprivation. Ratio of Observed to Expected (Ro/e) analysis corroborated these findings, confirming significant enrichment of PLAC8+ TAMs in the On-ADT group, SPP1+ and CXCL9+ TAMs in CRPC/NEPC, and CX3CR1+ TAMs in sub2 samples (Fig. 6F and G).

The significant enrichment of both CX3CR1+ TAMs and LAMP3+ DCs in the On-ADT cohort suggests their coordinated involvement in adaptive immune responses to therapeutic pressure. Further analysis through Ro/e quantification revealed distinct specialization patterns: CX3CR1+ TAMs were predominantly enriched in sub2, while LAMP3+ DCs showed specific accumulation in sub4. This differential distribution implies specialized

functional roles in distinct resistance pathways. CX3CR1 + TAMs, known for their tissue-repair, inflammatory resolution and tumor-promoting capabilities [52–54], likely contribute to the establishment of an immunosuppressive microenvironment in sub2 tumors, facilitating survival under continuous androgen deprivation. Conversely, LAMP3 + DCs, recognized as mature dendritic cells with potent antigen-presenting capacity [55, 56], may promote alternative immune activation pathways in sub4 tumors, potentially driving the double-negative phenotype through mechanisms of immune evasion or chronic inflammation. This compartmentalization of myeloid subsets across molecular subtypes highlights the complexity of therapy-induced immune remodeling and suggests distinct therapeutic vulnerabilities in different resistance contexts.

The pronounced expansion of TAMs exhibiting a PLAC8-high signature in the On-ADT cohort suggests a potential role in modulating therapy response. While a distinct PLAC8-positive TAM subset has not been widely defined, this gene is implicated in tumor-promoting processes such as drug resistance [57, 58] and EMT [59]. Here, we observed a TAM subpopulation characterized by a gene signature that includes PLAC8, TNFAIP8L2, PKIB, and others related to immunomodulation, cytoskeletal dynamics, and metabolic stress. Based on the central role and high expression of PLAC8 within this signature—a gene with established links to cancer pathology—we refer to this subpopulation as PLAC8 + TAMs for further discussion. GSVA revealed enrichment for KRAS signaling downregulation and spermatogenesis-related pathways, though overall pathway activity was low (Fig. 6H). GSEA, however, demonstrated significant enrichment for DNA duplication, drug metabolism (cytochrome P450 and other enzymes), and mismatch repair pathways (Supplementary Fig. 5C, 5D). We further characterized PLAC8 + TAM function through KEGG and GO analyses (Supplementary Fig. 5E – 5H), which revealed predominant enrichment in ribosome-related pathways (KEGG: Ribosome; MF: Structural constituent of ribosome; BP: Cytoplasmic translation; CC: Cytosolic ribosome). Combined with GSEA results showing drug metabolism pathway enrichment, these findings suggest that PLAC8 + TAMs may undergo extensive translational reprogramming to support their adaptive response to therapeutic stress. Additionally, analysis of P450 pathway activity across TAM subsets showed the highest activity in both SPP1 + and PLAC8 + TAMs (Supplementary Fig. 5I), indicating that these two subsets may collaboratively participate in P450-mediated drug metabolism or be coordinately influenced by therapeutic agents, potentially contributing to treatment resistance mechanisms through metabolic adaptation.

Trajectory analysis using Monocle3 and scTOUR revealed a differentiation path where monocytes give rise to NLRP3 + TAMs, which subsequently bifurcate into PLAC8 + and SPP1 + TAM lineages (Fig. 6I and J, Supplementary Fig. 5J). Monocle2 analysis further refined this trajectory, identifying three branching points with PLAC8 + TAMs predominantly in state 7 and SPP1 + TAMs in state 6 (Fig. 6K and L, Supplementary Fig. 5K). Analysis of genes dynamically expressed along this trajectory identified a 22-gene cluster (cluster 4) associated with PLAC8 + TAM differentiation (Fig. 6M), including HBB, HLA-DPA1, C1QC, and CD74 (Fig. 6N). These genes are involved in oxygen transport, antigen presentation, and complement activation, suggesting a specialized immunomodulatory phenotype.

To assess clinical relevance, we applied the Scissors algorithm to identify myeloid cells associated with BCR across multiple cohorts (GSE70769, GSE107299, MSKCC, TCGA). Scissors-positive cells were predominantly concentrated in the PLAC8 + TAM subset (Supplementary Fig. 5L). Intersection of BCR-associated genes across datasets yielded an 8-gene signature (COL1A1, BIRC5, UBE2C, BGN, CENPF, HAGHL, C4orf48, LAMP5) (Supplementary Fig. 5M, 5N, Supplementary Table 11). These genes have established roles in extracellular matrix remodeling (COL1A1, BGN), cell survival (BIRC5), cell cycle regulation (UBE2C, CENPF), and lysosomal function (LAMP5), suggesting their collective involvement in promoting treatment resistance and disease progression through modulation of the tumor microenvironment.

Fibroblast heterogeneity and immunosuppressive niche formation in prostate cancer

We identified 16,204 fibroblast cells and categorized them into six functionally distinct subsets using established marker genes (Fig. 7A and B, Supplementary Table 13): apCAF, CLDN1 + CAF, CXCL12 + iCAF, IL6 + iCAF, SLC2A1 + meCAF, and ACTA2 + myCAF. These subsets represent diverse functional states within the tumor microenvironment: apCAFs (antigen-presenting CAFs) demonstrate immunomodulatory capabilities [60]; CXCL12 + iCAFs contribute to inflammatory signaling and stem cell maintenance [61]; IL6 + iCAFs promote tumor growth through IL6-STAT3 signaling; ACTA2 + myCAFs drive extracellular matrix remodeling and metastasis [62]; while CLDN1 + CAFs and SLC2A1 + meCAFs represent specialized metabolic states.

Compositional analysis revealed dynamic changes across disease progression (Fig. 7C and D). IL6 + iCAFs were significantly enriched in the On-ADT group, while ACTA2 + myCAFs progressively increased with disease advancement, peaking in NEPC samples. Conversely, CXCL12 + iCAFs exhibited a declining trend during

progression, reaching their lowest levels in NEPC, suggesting a potential balance between CXCL12 + iCAF and ACTA2 + myCAF populations. Molecular subtype analysis showed ACTA2 + myCAF enrichment in subtype 3 (NEPC), while CXCL12 + iCAFs were most abundant in subtype 1 (Fig. 7E, Supplementary Fig. 6A). Ro/e analysis confirmed these patterns and revealed co-enrichment of apCAFs and ACTA2 + myCAFs in NEPC/subtype 3 (Fig. 7H and G), suggesting their collaborative role in establishing aggressive tumor microenvironments through complementary mechanisms - apCAFs in immune regulation and myCAFs in matrix remodeling [55].

GSVA revealed distinct functional specializations: apCAFs were enriched for immune regulatory pathways (interferon response, inflammation, androgen response, IL2/IL6 signaling) and cell cycle regulation, while ACTA2 + myCAFs exhibited pro-tumorigenic pathways including TGF- β signaling, EMT, angiogenesis, WNT, and Notch signaling (Fig. 7H), indicating their central role in fostering immunosuppressive and pro-metastatic niches.

Application of the Scissors algorithm identified ACTA2 + myCAFs as the primary fibroblast subset associated with biochemical recurrence (Supplementary Fig. 6B). Intersection across five datasets yielded 42 BCR-associated genes that were predominantly expressed in myCAFs (Supplementary Fig. 6C, 6D, Supplementary Table 11), further supporting their link to poor prognosis.

Spatial mapping using AddModuleScore revealed potential co-localization of CXCL12 + iCAFs, ACTA2 + myCAFs, FOLR2 + TAMs, and PLAC8 + TAMs. Correlation analysis showed the strongest spatial association between CXCL12 + iCAFs and ACTA2 + myCAFs (Fig. 7I and J, Supplementary Fig. 6E, 6 F), suggesting potential cooperative interactions or plasticity between these subsets that may contribute to immunosuppressive niche formation.

CellChat analysis of communication networks among all CAF and TAM subsets, and specifically among five key subsets (CXCL12 + iCAF, ACTA2 + myCAF, FOLR2 + TAM, PLAC8 + TAM, and tumor cells), demonstrated extensive intercellular crosstalk significantly altered by neoadjuvant therapy (Fig. 7K). NHT enhanced CAF-tumor cell communication while attenuating TAM-tumor cell interactions. Pathway analysis revealed NHT-induced enhancement of TGF- β , GAS, EGF, IGF, IGF, VEGF, and IL6 signaling, with concurrent suppression of KLF, WNT, TNF, CXCL, and GALECTIN pathways (Supplementary Fig. 6G – 6I) [63]. This signaling rewiring suggests that while NHT may promote immune cell infiltration, the resulting stromal activation creates a suppressive microenvironment that impedes effective T-cell function, particularly through TNF pathway down-regulation and TGF- β upregulation.

Spatial mapping demonstrated a trend toward elevated TGF- β pathway activity in post-NHT samples, with the most pronounced activation observed in P06 compared to the pre-treatment sample P01 (Fig. 7L). Single-cell analysis revealed that NHT significantly enhanced TGF- β signaling specifically in FOLR2 + TAMs and CX3CR1 + TAMs (Fig. 7M), with no significant activation observed in CXCL12 + iCAFs or ACTA2 + myCAFs. These findings suggest that elevated TGF- β activity and subsequent immunosuppression in the tumor microenvironment are primarily driven by these TAM subsets. The balance between CXCL12 + iCAFs and ACTA2 + myCAFs may instead facilitate TAM recruitment and indirectly shape the immunosuppressive landscape through stromal-macrophage crosstalk.

Lymphoid cell dynamics and spatial organization in prostate cancer microenvironment

We analyzed 192,197 lymphoid cells and identified 13 distinct subsets through established marker genes (Fig. 8A and B, Supplementary Fig. 7A, 7B, Supplementary Table 14), including CD4 + T cells (Tn CCR7+, Tcm, Tem, Treg, Tfh, Trm ZNF683+), CD8 + T cells (Tn CCR7+, Teff, Tem, Tex LAG3+, Trm CXCR6+), ILCs, MAIT cells, and NK cells (CD56bright, CD56dim). Compositional analysis across disease stages revealed significant redistribution of lymphoid populations (Fig. 8C and D, Supplementary Fig. 7C). CD4 + Tregs were markedly enriched in NEPC samples, while CD8 + Tex LAG3+ cells progressively increased during disease progression, peaking in CRPC. Conversely, CD8 + Teff cells were significantly downregulated in tumor tissues, reaching their lowest levels in NEPC. CD4 + Tn CCR7+ and CD4 + Trm ZNF683+ populations declined significantly following neoadjuvant therapy and continued to decrease with disease advancement. Interestingly, CD8 + Trm CXCR6+ cells were most abundant in the On-ADT group and subsequently decreased with progression. Molecular subtype analysis showed CD8 + Trm CXCR6+ enrichment in subtype 2, while CD4 + Tregs and Tfh cells were predominantly enriched in subtype 3 (Fig. 8E). Ro/e analysis confirmed these patterns, demonstrating CD8 + Tex elevation in NHT and CRPC groups, CD8 + Trm CXCR6+ enrichment in On-ADT, and CD4 + Treg/Tfh accumulation in neuroendocrine subtypes (Fig. 8F and G). These findings reveal progressive T-cell exhaustion and regulatory T-cell expansion during disease progression, with distinct lymphoid signatures associated with treatment response and neuroendocrine transformation.

Functional characterization through cytotoxicity, exhaustion, and regulation scoring (Fig. 8H – 8 J, Supplementary Fig. 7D), demonstrated that NK CD56dim and CD8 + Teff cells exhibited the highest cytotoxic potential, while CD4 + Tregs showed predominant exhaustion and

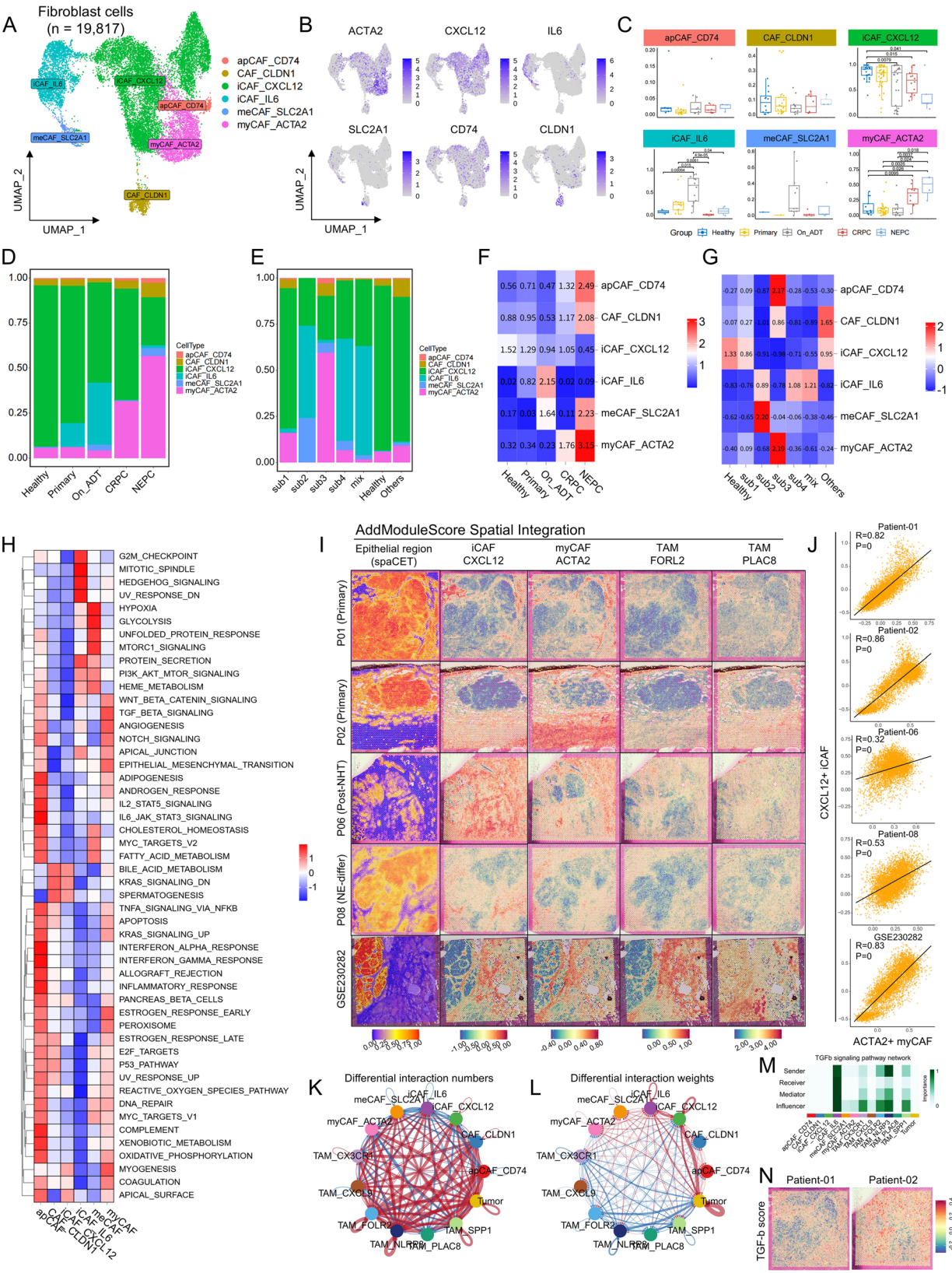


Fig. 7 (See legend on next page.)

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Fig. 7 Fibroblast heterogeneity and stromal-immune interactions in prostate cancer. **A** UMAP visualization of 16,204 fibroblast cells, annotated into six distinct subsets: apCAF, CLDN1+ CAF, CXCL12+ iCAF, IL6+ iCAF, SLC2A1+ meCAF, and ACTA2+ myCAF. **B** UMAP feature plots displaying expression patterns of major marker genes across CAF subsets. Expression levels are represented by color gradient from light blue (low expression) to dark blue (high expression). **C** Box plots showing proportional differences of CAF subsets across clinical groups. Statistical significance was determined using Kruskal-Wallis test with Dunn's post-hoc correction (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Boxes represent interquartile range with center lines indicating median values. **D-E** Stacked bar plots illustrating the compositional distribution of CAF subsets across clinical groups (D) and molecular subtypes (E). Each colored segment represents the percentage of a specific CAF subset. **F-G** Ratio of Observed to Expected (Ro/e) analysis heatmaps depicting enrichment patterns of CAF subsets across clinical groups (F) and molecular subtypes (G). Color scale represents Ro/e index from blue (depletion) to red (enrichment). **H** GSVA heatmap showing Hallmark pathway activities across CAF subsets. Color gradient from blue (low activity) to red (high activity) represents enrichment scores. **I** Spatial mapping using AddModuleScore showing the distribution of tumor boundary regions, CXCL12+ iCAF, ACTA2+ myCAF, FOLR2+ TAM, and PLAC8+ TAM across representative samples: P01-P02 (primary), P06 (On-ADT), P08 (NEPC), and GSE230282. **J** Correlation heatmap displaying Pearson correlation coefficients between CXCL12+ iCAF and ACTA2+ myCAF across corresponding samples. Asterisks indicate statistical significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). **K-L** Cell-cell communication analysis showing interaction patterns among all TAM, CAF subsets and tumor cells. Left panel: differential network of communication numbers between primary and post-NHT conditions (K). Right panel: differential network of communication strength between the two conditions (L). **M** Box plots showing TGF- β pathway activity in FOLR2+ TAM (left) and CX3CR1+ TAM (right) subsets across clinical groups. Boxes represent interquartile range with center lines indicating median values. **N** Spatial mapping of TGF- β pathway activity using AddModuleScore, comparing pathway activation between P01 (primary) and P06 (post-NHT) samples

regulatory scores, consistent with their established functional roles.

Given the relatively sparse lymphoid infiltration in prostate cancer, we employed Seurat spatial mapping to analyze the spatial relationships between key stromal subsets (CXCL12+ iCAFs, ACTA2+ myCAFs) and lymphoid populations across 2 primary and 4 post-NHT samples. Analysis revealed that CXCL12+ iCAFs and ACTA2+ myCAFs predominantly co-localized at tumor-stroma boundaries (Fig. 8K, Supplementary Fig. 7E). Post-NHT samples showed significantly increased T/NK and B-cell infiltration, consistent with treatment-induced lymphoid recruitment. However, this increased infiltration appeared functionally compromised, suggesting “ineffective recruitment” where lymphocytes are present but exhibit impaired anti-tumor activity.

CellChat analysis of communication among these subsets revealed that NHT significantly enhanced CypA, FGF, IGFBP, EGF, and IGF pathway activities (Fig. 8L and M, Supplementary Fig. 7F, 7G). The upregulation of CypA signaling correlated with increased T-cell recruitment, while the suppression of TGF- β , IL16, and KLK pathways indicated impaired T-cell function and altered tumor growth dependencies. Analysis of signaling directionality showed that CypA signaling, primarily originating from B cells, CXCL12+ iCAFs, and tumor cells in treatment-naïve conditions, shifted to predominantly CXCL12+ iCAF and tumor cell-derived signaling post-NHT (Supplementary Fig. 7H). This rewiring suggests stromal cells become the primary architects of immune cell recruitment following therapy.

Spatial mapping of chemokine signaling pathways demonstrated moderate enhancement in post-NHT samples, particularly in P06 (Fig. 8N). Single-cell analysis revealed that both FOLR2+ TAMs and CX3CR1+ TAMs upregulated chemokine signaling and T-cell receptor signaling pathways, likely contributing to the observed T-cell recruitment following NHT (Fig. 8O).

Collectively, these results paint a complex picture of NHT-induced microenvironmental remodeling: while therapy enhances lymphoid infiltration through stromal-derived chemokine signals, it simultaneously fosters an immunosuppressive niche through T-reg expansion, exhausted T-cell accumulation, and altered cell-cell communication that ultimately compromises anti-tumor immunity. This paradoxical response may underlie the limited efficacy of immunotherapies in prostate cancer and highlights the need for combinatorial strategies that target both lymphoid recruitment and function.

Discussion

This multi-omics investigation provides a comprehensive decoding of prostate cancer progression through integrated single-cell and spatial transcriptomic profiling of 127 samples across the disease continuum. By simultaneously resolving epithelial plasticity programs and microenvironmental rewiring, we establish a unified model of treatment resistance development. Our work significantly extends beyond previous molecular classifications by incorporating spatial context and cellular interactions, revealing previously unrecognized therapeutic vulnerabilities.

Our delineation of four molecularly distinct epithelial subtypes (sub1-sub4) reveals a continuous lineage hierarchy from luminal-identity through treatment-emergent states to lethal neuroendocrine carcinoma. This classification system integrates and extends several previously proposed taxonomies. While the TCGA consortium identified seven molecular subtypes primarily based on genomic alterations [64], and subsequent studies described transcriptional subtypes with prognostic significance [65, 66], our work demonstrates how these discrete categories actually represent interconnected states along a plasticity continuum. The ten meta-programs (MPs) identified through NMF analysis provide mechanistic insight into the biological processes governing

these transitions. Particularly, the opposing MP1 (Luminal Epithelial Identity) and MP6 (Basal/EMT transition) mirror the luminal-proliferative and basal-like compartments recently described in treatment-resistant prostate cancer [67], while the neuronal signaling program (MP3) aligns with neuroendocrine differentiation pathways identified in multiple studies [68, 69].

Our integrated subtyping approach reveals critical state transitions under therapeutic pressure that were not captured by previous classification systems. For instance, the PAM50 classifier originally developed for breast cancer and adapted to prostate cancer [70] primarily distinguishes luminal versus basal differentiation, but fails to capture the treatment-emergent double-negative state (sub4) identified in our study. Similarly, the PCS1-PCS3 classification system [71] effectively stratifies primary prostate cancer but shows limited utility in advanced disease where lineage plasticity dominates. The spatial mapping of these MPs across disease stages provides direct histological validation of their distribution patterns and reveals previously unappreciated intra-tumoral heterogeneity that may explain differential treatment responses within individual tumors.

The rising incidence of double-negative prostate cancer following novel hormonal therapies represents a critical clinical challenge, with recent studies reporting DNPC incidence rates of 15–20% in post-treatment cohorts [39, 40]. Our identification of the FOSL1-HMGA1 axis as a master regulator of DNPC progression provides mechanistic insight into this treatment-emergent phenotype. While FOSL1 has been implicated in EMT and stemness maintenance across various malignancies [43, 44], its specific role in prostate cancer lineage plasticity has remained incompletely characterized. Our findings position FOSL1 upstream of HMGA1, a chromatin regulator known to facilitate epigenetic reprogramming in cancer stem cells [72].

The FOSL1-HMGA1 axis promotes multiple aggressive features including proliferation, invasion, stemness, and treatment resistance, effectively orchestrating the transition to the double-negative state. This aligns with recent work demonstrating AP-1 transcription factor family involvement in prostate cancer progression [73], though our study specifically establishes the dominance of FOSL1 in the DNPC context. Importantly, emerging FOSL1 inhibitors such as T-5224 have shown promising results in preclinical models of other solid tumors [74, 75], suggesting immediate translational potential for prostate cancer. The development of HMGA1-targeting strategies remains challenging due to its structural characteristics, but indirect targeting through FOSL1 inhibition represents a promising alternative approach.

Our analysis reveals an elegantly coordinated stromal-immune rewiring following neoadjuvant therapy, with

five key subsets—CXCL12 + iCAF, ACTA2 + myCAF, FOLR2 + TAM, PLAC8 + TAM, and CX3CR1 + TAM—collaboratively establishing an immunosuppressive niche. This network extends beyond previously reported individual stromal subsets in prostate cancer [76] by demonstrating their spatial organization and functional cooperation. The CXCL12 + iCAF/ACTA2 + myCAF balance appears crucial for maintaining stromal architecture, reminiscent of the iCAF/myCAF equilibrium described in pancreatic cancer [61], while FOLR2 + and CX3CR1 + TAMs drive TGF- β -mediated immunosuppression through mechanisms distinct from the well-characterized SPP1 + macrophages dominant in advanced disease [50].

Our spatial analysis reveals the paradoxical increase in lymphoid infiltration coupled with functional impairment observed post-therapy, providing mechanistic insight into why immune checkpoint inhibitors have shown limited efficacy in prostate cancer [77]. This “immune-privileged” niche, characterized by simultaneous T-cell recruitment and functional suppression, represents a sophisticated immune evasion mechanism that may explain the disappointing results of recent combination trials [78, 79]. The identification of this coordinated network provides multiple potential intervention points for overcoming immunosuppression, including targeting specific TAM subsets or disrupting stromal-immune crosstalk.

Several limitations warrant consideration. While our spatial transcriptomics covers key disease stages, the sample size remains modest, particularly for rare subtypes like pure NEPC. The integration of additional multi-omics data, including epigenomic and proteomic profiles, could further elucidate regulatory mechanisms driving lineage transitions. Furthermore, our functional validation focused primarily on the FOSL1-HMGA1 axis, leaving other identified pathways for future investigation. Future studies should explore therapeutic targeting of the stromal-immune network identified here, particularly in combination with existing AR-directed therapies. The clinical translation of our findings will require validation in larger prospective cohorts and development of more specific stromal-targeting agents.

Conclusion

This study establishes a comprehensive roadmap of prostate cancer progression that integrates epithelial plasticity with microenvironmental reprogramming. By identifying the FOSL1-HMGA1 axis as a key driver of double-negative prostate cancer development and characterizing the cellular network responsible for therapy-induced immunosuppression, we provide both fundamental biological insights and translational opportunities. The integrated single-cell and spatial framework developed here serves

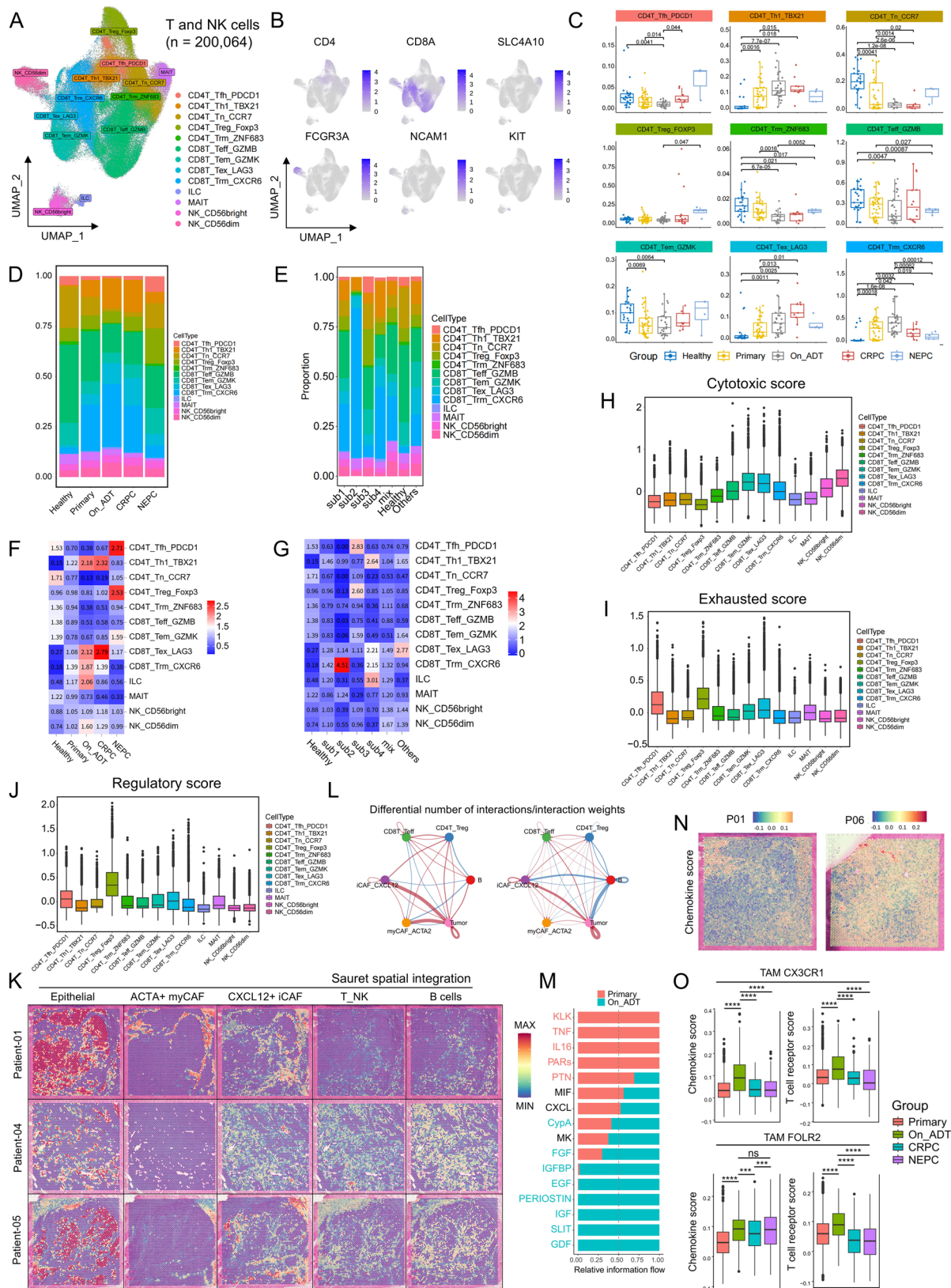


Fig. 8 (See legend on next page.)

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Fig. 8 Lymphoid cell dynamics and spatial organization in prostate cancer microenvironment. **A** UMAP visualization of 192,197 lymphoid cells, annotated into 13 distinct subsets: CD4+T cells (Tfh, Th1, Tn, Treg, Trm), CD8+T cells (Teff, Tem, Tex, Trm), ILCs, MAIT cells, and NK cells (CD56bright, CD56dim). **B** UMAP feature plots displaying expression patterns of major marker genes (CD4, CD8A, SLC4A10, FCGR3A, NCAM1, KIT) across lymphoid subsets. Expression levels are represented by color gradient from light blue (low expression) to dark blue (high expression). **C** Box plots showing proportional differences of lymphoid subsets across clinical groups. Statistical significance was determined using Kruskal-Wallis test with Dunn's post-hoc correction (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Boxes represent interquartile range with center lines indicating median values. **D-E** Stacked bar plots illustrating the compositional distribution of lymphoid subsets across clinical groups (D) and molecular subtypes (E). Each colored segment represents the percentage of a specific lymphoid subset. **F-G** Ratio of Observed to Expected (Ro/e) analysis heatmaps depicting enrichment patterns of lymphoid subsets across clinical groups (F) and molecular subtypes (G). Color scale represents Ro/e index from blue (depletion) to red (enrichment). **H-J** Box plots displaying functional scores across lymphoid subsets: (H) cytotoxicity score, (I) exhaustion score, and (J) regulation score. Boxes represent interquartile range with center lines indicating median values. **K** Spatial mapping using Seurat showing the distribution of CD8+Teff, CD4+Treg, CXCL12+iCAF, ACTA2+myCAF, B cells, and tumor cells in primary (P01) and Post-NHT (P04, P06) samples. **L** Cell-cell communication analysis showing interaction patterns among selected cell populations. Left panel: differential network of communication numbers between primary and On-ADT conditions. Right panel: differential network of communication strength between the two conditions. **M** Bar plot displaying significantly altered signaling pathways between treatment conditions. Red bars indicate pathways upregulated in primary samples, green bars represent pathways upregulated in On-ADT samples. Pathway names are colored according to their differential expression patterns, with significantly different pathways marked. **N** Spatial mapping of chemokine pathway activity using AddModuleScore, comparing pathway activation between P01 (primary) and P06 (post-NHT) samples. **O** Box plots showing chemokine signaling (left) and T cell receptor signaling (right) pathway activities in CX3CR1+TAM (upper) and FOLR2+TAM (lower) subsets across clinical groups. Boxes represent interquartile range with center lines indicating median values. Statistical significance was assessed using the Wilcoxon rank-sum test. ns, not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$

as a foundation for future studies exploring cellular dynamics in cancer progression and for developing novel therapeutic strategies targeting treatment resistance in advanced prostate cancer.

Materials and methods

Ethical statement

Animals were housed in a specific pathogen-free (SPF) environment with controlled humidity and temperature, provided by the Model Animal Research Center of China Medical University, under a 12-h light/12-h dark cycle. Throughout the in vivo experiments, animals were closely monitored to minimize discomfort, pain, or injury. Animals were removed from the study and euthanized if any signs of pain and distress were detected or if the tumor volume reached 1500 mm³. All animal experimental procedures were approved and supervised by the Institutional Animal Care and Use Committee (IACUC) of China Medical University, under approval numbers CMU20241328. Four-week-old male BALB/c nude mice were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd.

Patient samples and spatial transcriptomics

This study utilized 8 in-house prostate cancer samples obtained from patients undergoing diagnostic or therapeutic procedures at our institution. The cohort included 2 primary prostate cancer cases, 4 neoadjuvant hormone therapy-treated specimens, and 2 neuroendocrine prostate cancer cases. All samples were collected with informed consent under institutional review board-approved protocols (Ethics Committee approval number: AF-SOP-07-1.2-01). Fresh tissue specimens were immediately embedded in OCT compound and stored at -80°C until sectioning. For spatial transcriptomics, 10 µm thick cryosections were prepared and processed using the 10X

Genomics Visium platform with CytAssist instrumentation according to manufacturer's specifications. Tissue sections were stained with H&E and imaged before cDNA synthesis and library preparation. Sequencing was performed on an Illumina NovaSeq 6000 platform with a target of 50,000 reads per spot. Experiments complied with the principles outlined in the World Medical Association's "Declaration of Helsinki" and the US Department of Health and Human Services' "Belmont Report."

Single-cell RNA sequencing data processing

Raw sequencing data from 127 public single-cell RNA sequencing samples were processed using a uniform pipeline. Fastq files were aligned to the GRCh38 reference genome and quantified using Cell Ranger (v7.1.0). Quality control metrics included removal of cells with fewer than 200 genes or more than 20% mitochondrial content. Doublets were identified and removed using Scrublet [80]. Batch effects across different datasets were corrected using Harmony integration [81], with subsequent clustering performed via the Seurat (v4.3.0) pipeline. Cell types were annotated using established marker genes with manual curation.

Spatial transcriptomics data analysis

Spatial gene expression data from both in-house (8 samples) and public (GSE230282) datasets underwent standardized quality control to remove spots with fewer than 100 genes or located in tissue-free areas. Cell type deconvolution of major compartments (e.g., immune, stromal, epithelial) was performed using Cell2location [82]. Tumor region identification and cancer cell fraction estimation were conducted using the spaCET package [83]. Spatial domains were annotated by integrating gene expression patterns with morphological features from H&E staining visualized through the 10X Genomics

Loupe Browser. General cell type mapping and visualization were performed using Seurat's spatial analysis functions. Gene signature activities across tissue sections were spatially mapped using the AddModuleScore method.

Spatial deconvolution and mapping of molecular subtypes

For the specific spatial mapping of the four malignant epithelial subtypes (sub1-sub4), we employed a sequential analytical pipeline. First, epithelial regions were initially identified using the spaCET tool and subsequently reviewed and refined by a clinical pathologist based on matched H&E staining using the 10X Genomics Loupe Browser. The curated epithelial masks were applied to filter the spatial data, retaining only spots within confirmed epithelial regions for subtype analysis.

We then performed subtype-specific deconvolution using Cell2location v0.1.3 (Kleshchevnikov et al., *Nature Biotechnology*, 2022). A dedicated single-cell reference was constructed by aggregating gene expression counts from the four annotated malignant epithelial subtypes (sub1-sub4). The filtered spatial count matrices (from FFPE tissue sections processed with the 10X Visium CytAssist protocol) served as input. The model was trained with default parameters (20,000 epochs, learning rate 0.01) to infer subtype abundance at the spot level.

Results were visualized using two complementary approaches in Squidpy: (1) Single-subtype spatial heatmaps depicting the absolute abundance of each subtype, and (2) Multi-subtype composite heatmaps overlaying all four subtypes to visualize co-localization and heterogeneity. The spatial distribution of inferred subtypes was validated against corresponding H&E histology.

Non-negative matrix factorization and meta-program definition

Malignant epithelial cells were subsetted and analyzed using non-negative matrix factorization (NMF) implemented in the R NMF package (v0.26). The optimal factorization rank ($k=10$) was determined based on cophenetic correlation coefficient and dispersion metrics. Meta-programs were defined by the top 50 genes ranked by loadings in each factor, with biological interpretation through pathway enrichment analysis.

Molecular subtyping of malignant epithelial cells

Molecular subtypes were defined through an integrative analysis of non-negative matrix factorization (NMF) meta-programs and cluster correlation patterns. First, correlation-based hierarchical clustering was applied to the 28 initial malignant epithelial clusters, revealing four major groupings. We then examined the NMF meta-program (MP) expression profiles and spatial distribution on the UMAP for cells within each grouped

category. This analysis demonstrated that clusters within the same category shared dominant MP signatures: subtype 1 was characterized by MP1 (Luminal epithelial) and MP2 (PCa proliferation); subtype 2 by MP4 (Luminal secretory), MP5 (Mucosa stress), and MP9 (Reactive Defense); subtype 3 by MP3 (Neuronal signaling), MP7 (T-cell crosstalk) and MP10 (Cell Cycle); and subtype 4 by MP6 (Basal/EMT transition) and MP8 (Basal stress). These distinct MP profiles indicated fundamentally different functional states: subtype 1 represents a classic luminal, AR-positive adenocarcinoma; subtype 2 an epithelial stress-responsive state; subtype 3 a neuroendocrine phenotype with dysregulated cell cycle; and subtype 4 a basal-lineage altered phenotype. These four categories were thus formally designated as molecular subtypes (sub1-sub4) for all subsequent analyses.

Lineage trajectory analysis

Cell lineage trajectories were constructed using multiple complementary approaches. Monocle2 [84] was employed with DDRTree algorithm for dimension reduction, while Monocle3 [85] was used for more complex trajectory inference. Additionally, scTOUR [86] was applied as an independent method to validate trajectory structures. Genes with differential expression across pseudotime were identified using the BEAM test in Monocle2, with consistent patterns verified across all three methods.

Functional enrichment and pathway analysis

Gene set variation analysis (GSVA) [87] was performed to estimate pathway activity in single-cell populations using the Hallmark gene sets from MSigDB. Gene Set Enrichment Analysis (GSEA) [88] was conducted for bulk RNA-seq data comparisons. KEGG pathway and Gene Ontology (GO) term enrichment analyses were performed using the clusterProfiler package [89] with adjusted p-value cutoff of 0.05.

Ratio of observed to expected (Ro/e) analysis

The Ro/e method was implemented to identify cell type enrichments across clinical conditions. For each cell type and clinical group combination, the ratio of observed cell count to expected count (based on overall cellular composition) was calculated. Statistical significance was assessed using chi-square tests with Bonferroni correction for multiple testing.

Copy number variation inference

InferCNV (v1.14.1) was used to infer large-scale chromosomal copy number alterations in epithelial cells. Healthy epithelial cells from benign tissues served as reference controls. The hidden Markov model (HMM) was employed to predict chromosomal regions with

significant copy number alterations, with a cutoff of 0.1 for CNV calling.

Cell-Cell communication analysis

CellChat (v2.1.2) [90] was utilized to infer intercellular communication networks from single-cell transcriptomic data. The method employs a mass action-based model to identify significantly over-expressed ligand-receptor interactions. Differential communication analysis between clinical groups was performed using a Wilcoxon rank-sum test with Bonferroni correction.

Transcription factor activity analysis

We employed the SCENIC (Single-Cell rEgulatory Network Inference and Clustering) method [91] to systematically analyze transcription factor activities across the four molecular subtypes. Initially, we constructed regulatory networks for each transcription factor and its potential target genes based on co-expression patterns. Subsequently, the AUCell algorithm was applied to calculate activity scores for each regulatory network at the single-cell level, reflecting the relative activity of transcription factors in individual cells. We specifically focused on transcription factors showing significant activation in the sub4 subtype, including FOSL1 and NR2F6. The activity scores of these transcription factors were normalized using z-score transformation and compared across different molecular subtypes. Statistical significance of differences was assessed using the Wilcoxon rank-sum test, with multiple testing correction performed using the false discovery rate (FDR) method.

Gene signature scoring

Four established gene signatures were employed for functional characterization: AR signaling signature, neuroendocrine signature, lineage plasticity signature, and CRPC51 signature. Scoring was performed using the AddModuleScore function in Seurat, which calculates the average expression of signature genes subtracted by the average expression of control genes.

Biochemical recurrence-associated gene identification

The Scissors algorithm [92] was applied to four bulk RNA-seq datasets (GSE10769, GSE107299, MSKCC, and TCGA-PRAD) to identify cells associated with biochemical recurrence. Genes consistently associated with recurrence across multiple datasets were identified through intersection analysis, with functional annotation via KEGG and GO enrichment.

CytoTRACE analysis

CytoTRACE [93] was used to assess cellular differentiation potential from single-cell transcriptomic data. Analysis was performed using the CytoTRACE R package

(v0.3.3) following standard workflow. The algorithm computes a differentiation score for each cell based on expressed gene number and transcriptional state continuity, with higher scores indicating greater differentiation potential. Scores were compared across epithelial subtypes using Wilcoxon rank-sum test with Bonferroni correction.

Cell lines

Human PC-3 cell lines were purchased from ATCC (American Type Culture Collection) and authenticated by STR (Short Tandem Repeat) profiling. Cells were tested negative for mycoplasma contamination prior to experiments. Cells were cultured in an incubator at 37°C with 5% CO₂ and RPMI-1640 medium (Thermo Fisher Scientific 11875093) supplemented with 10% FBS and 1% penicillin-streptomycin. Cells were passaged at a ratio of 1:3 or 1:2 every 3 to 5 days.

siRNA, lentivirus, and plasmids

Specific siRNA sequences targeting the FOSL1 gene and control siRNA (siCtrl) were purchased from Sigma Aldrich. The FOSL1 siRNA sequences used were as follows:

siFOSL1 - 1: 5' - GCUCAUCGCAAGAGUAGCA - 3';
siFOSL1 - 2: 5' - ACTCATGGTGTGATGCTTGG - 3';
siFOSL1 - 3: 5' - CTGTACCTTGTATCTCCCTT - 3';
siCtrl: 5' - CA TTCTCCGAACGTGTCACGT - 3' (Human Non-Target 1).

siRNA was transfected into PC-3 cell using jet-PRIME transfection reagent (Polyplus 101000046) according to the manufacturer's instructions. For lentivirus-mediated RNAi, lentivirus (shFOSL1) identical to the siFOSL1-3 sequence and control lentivirus (shCtrl) identical to the siCtrl sequence were custom-ordered from Shanghai GeneChem Co., Ltd. The infection process followed GeneChem's lentivirus infection protocol. Two days after lentiviral infection, puromycin (Beyotime ST551) was added to the medium at a concentration of 2 µg/ml to select stably transduced cells. HMGA1 overexpression plasmid (Cat: HG11613-NF), and the empty vector plasmid pCMV3-N-FLAG were purchased from SinoBiological. Plasmid transfection was also performed following the jet-PRIME transfection reagent protocol. PC-3 cells were treated with FOSL1-degrader-1 (MedChemExpress, HY-162723) at the indicated concentrations of 0, 0.5, 2, 5, and 10 µM.

Protein extraction and Western Blotting (WB)

Western blot experiments were performed according to previously described standard methods [94]. Briefly, samples were lysed in lysis buffer containing protease inhibitors, centrifuged, and the supernatant was collected;

proteins were denatured by heating in a boiling water bath; proteins were separated by SDS-PAGE; after electrophoresis, proteins were transferred to PVDF membranes; membranes were then incubated with primary antibodies overnight at 4°C, followed by incubation with corresponding secondary antibodies at room temperature for 1 h; finally, corresponding protein bands were visualized by chemiluminescence using ECL reagent. All data were derived from at least three independent experiments. Antibodies used in this experiment included:

anti-FOSL1 (Cell Signaling Technology, cat#17470, 1:1000),

anti-HMGA1 (Cell Signaling Technology, cat# 4513, 1:1000),

anti-OCT4 (Abmart, cat#T55781, 1:1000),

anti-Vimentin (Abmart, cat#T55134, 1:1000),

anti-ZO1 (Abmart, cat#TA5145, 1:1000),

anti-SNAIL (Abmart, cat#TA6032, 1:1000),

anti-N Cadherin (Abmart, cat#T55015, 1:1000),

anti-E Cadherin (Abmart, cat#P90408R1, 1:1000),

anti-Nanog (Proteintech, cat#14295-1-AP, 1:5000),

anti-SOX2 (Proteintech, cat#11064-1-AP, 1:1000),

anti-OCT4 (Proteintech, cat#11263-1-AP, 1:1000),

anti-CD44 (Proteintech, cat#15675-1-AP, 1:20000),

anti-DSG3 (Invitrogen, cat#32-6200, 1:500),

anti-KRT5 (Proteintech, cat#66727-1-Ig, 1:20000),

anti-KRT6A (Proteintech, cat#10590-1-AP, 1:5000),

anti- β -actin (Proteintech, cat# 66009-1-Ig, 1:5000),

anti-FLAG (Abmart, cat#R20008, 1:5000).

RNA isolation and quantitative real-time PCR (qPCR)

After transfecting cells with siCtrl or siFOSL1, cells were collected, and total RNA was extracted using the TaKaRa MiniBEST Universal RNA Extraction Kit (TAKARA 9767) according to the manufacturer's instructions. 1 μ g of total RNA was used for reverse transcription to synthesize cDNA using the PrimeScript™ RT reagent Kit (TAKARA RR037A). Subsequently, qPCR analysis was performed on the LightCycler96 system (Roche) using TB Green® Premix Ex Taq™ II FAST qPCR reagents (TAKARA CN830S), with three technical replicates per reaction. Data were analyzed using the delta-delta Ct ($2^{-\Delta\Delta Cq}$) method, and the expression levels of all target genes were normalized to the internal reference gene 18S mRNA. The fold change in gene expression difference was normalized to the control group (siCtrl). Primer sequences used for qPCR are listed in Supplementary Table 15. Experiments were performed in three independent cell cultures (biological replicates), and data are expressed as mean $\pm$ SEM.

Chromatin Immunoprecipitation (ChIP)

Chromatin immunoprecipitation (ChIP) assays were performed using the Beyotime ChIP Assay Kit (Beyotime,

P2078) according to the manufacturer's instructions. Briefly, cells were cross-linked with 1% formaldehyde for 10 min at room temperature and quenched with 0.25 M glycine. After washing with cold PBS, cells were lysed in SDS lysis buffer and sonicated on ice to shear chromatin to an average size of 200–500 bp. The lysate was centrifuged at 12,000 rpm for 15 min at 4°C to remove insoluble material.

The supernatant was diluted and incubated overnight at 4°C with anti-FOSL1 antibody (Cell Signaling Technology, #17470; dilution 1:100) or normal rabbit IgG as a negative control. Immune complexes were captured with Protein A Agarose/Salmon Sperm DNA beads (Beyotime) for 4 h at 4°C with gentle rotation. Beads were washed sequentially with low salt, high salt, LiCl, and TE buffers. Bound complexes were eluted with elution buffer (1% SDS, 0.1 M NaHCO₃), and cross-links were reversed by incubation at 65°C overnight in the presence of 200 mM NaCl. DNA was purified by Proteinase K treatment, followed by phenol-chloroform extraction and ethanol precipitation. Precipitated DNA was resuspended in TE buffer and analyzed by quantitative PCR using primers specific to candidate target gene promoter regions (primer sequences are provided in Supplementary Table 16). All ChIP experiments were performed in three independent biological replicates; data are presented as mean $\pm$ SEM.

Cell viability, colony formation, and flow cytometry analysis

In the cell viability assay, for cell lines stably infected with shCtrl or shFOSL1 lentivirus, cells were seeded at a density of 2×10^3 cells per well in 96-well plates (3 replicate wells per treatment group). The number of viable cells was assessed by measuring absorbance at 490 nm at different time points using an MTS assay kit (Promega G3580). For the colony formation assay, 2×10^3 cells were seeded in six-well plates and cultured for 10 days. Colonies were then fixed with 4% paraformaldehyde for 15 min, stained with crystal violet (MCE HY-B0324A), and counted.

For flow cytometric analysis of the cell cycle, PC-3 cells stably expressing shCtrl or shFOSL1 were seeded in 6-well plates (3 replicate wells per treatment group). For rescue tests, when density reached 60%–70%, they were transfected with empty vector plasmid (pCMV-3-FLAG) or HMGA1 overexpression plasmid. Cell cycle synchronization was achieved using serum starvation; after synchronization, complete medium was replaced and culture continued for 8 h. Cells were collected by digestion with EDTA-free trypsin, fixed overnight (≥ 12 h) in 75% ethanol at -20°C, washed with ice-cold PBS, and stained in the dark with a propidium iodide (BD Biosciences 550825) staining solution containing RNase for 15 min. Cell cycle

distribution was finally analyzed using a flow cytometer. Apoptosis was detected using the Annexin V-FITC Apoptosis Detection Kit (KeyGEN BioTECH, KGA105) according to the manufacturer's instructions. Briefly, after treatment, cells were harvested by digestion with EDTA-free trypsin, washed twice with ice-cold PBS, and resuspended in 500 μ L of Binding Buffer. The cell suspension was then incubated with 5 μ L Annexin V-FITC and 5 μ L propidium iodide (PI) for 15 min at room temperature in the dark. Stained cells were detected by using a flow cytometer within 1 h after staining. All experiments were performed in triplicate, and results are presented as mean $\pm$ SD.

Cell migration and invasion assays

Cell migration and invasion abilities were assessed using Transwell chambers (Corning, 8- μ m pore size). For the invasion assay, the membranes were pre-coated with Matrigel (BD Biosciences, 354230) diluted in serum-free medium at 37°C for 4 h. Cells transfected with shFOSL1, shCtrl, HMGA1-overexpressing plasmid, or empty vector were serum-starved for 12 h. Subsequently, 5×10^4 cells in 200 μ L serum-free medium were seeded into the upper chamber. The lower chamber was filled with 500 μ L complete medium containing 10% FBS. After incubation for 24 h at 37°C, non-invading cells on the upper surface were removed with a cotton swab. Cells that migrated to the lower membrane were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet, and imaged under a microscope. Three random fields were captured per well, and cell numbers were quantified using ImageJ software. The migration assay was performed similarly, but without Matrigel coating.

Wound healing assay

Cells were seeded in 6-well plates and cultured until reaching 90% confluency. A sterile 200- μ L pipette tip was used to create a straight scratch wound. After washing with PBS to remove detached cells, the cells were cultured in serum-free medium. Wound closure was photographed at 0 and 24 h under an inverted microscope. The migration rate was calculated as follows: Migration rate (%) = $(1 - [\text{wound area at 24 h} / \text{wound area at 0 h}]) \times 100\%$.

Animal experiments

All animal procedures were performed in accordance with the institutional guidelines approved by the Animal Ethics Committee of China Medical University. Four-week-old male BALB/c nude mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. and housed under specific pathogen-free conditions.

For the xenograft tumor model, 5×10^6 lentivirus-transduced PC-3 cells (expressing shCtrl or shFOSL1) were

resuspended in an injection mixture composed of 50% Matrigel (BD Biosciences, 356237) and 50% serum-free medium, and subcutaneously inoculated into the flanks of 5–6 week-old male BALB/c nude mice. When tumor volumes reached approximately 5 mm³ (on day 15 post-inoculation), mice were randomly divided into treatment groups. Docetaxel and/or FOSL1-degrader1 (MedChemExpress, Cat: HY-162723) were administered via intraperitoneal injection every two days (both at 10 mg/kg). The compounds were dissolved in a vehicle consisting of 40% PEG300, 5% DMSO, 5% Tween-80, and 50% saline. Control mice received the vehicle only. Tumor dimensions were measured every 5 days, and volume was calculated using the formula: $V = 0.5 \times \text{width}^2 \times \text{length}$. All mice were euthanized on day 30. Tumors were excised, weighed, and snap-frozen for protein extraction.

Statistical analysis

All statistical analyses were performed in R (v4.2.2). Differential expression analysis used the Wilcoxon rank-sum test with false discovery rate correction. Survival analysis was conducted using the Kaplan-Meier method with log-rank test. Spatial correlation analysis employed Pearson correlation coefficients with permutation testing. Multiple testing corrections were applied using the Benjamini-Hochberg procedure where appropriate.

Abbreviations

PCa	Prostate Cancer
AR	Androgen Receptor
ARSIs	Androgen Receptor Signaling Inhibitors
scRNA-seq	Single-Cell RNA Sequencing
ST	Spatial Transcriptomics
NHT	Novel Hormone Therapy
CRPC	Castration-Resistant Prostate Cancer
NEPC	Neuroendocrine Prostate Cancer
DNPC	Double-Negative Prostate Cancer
Ro/e	Ratio of Observed to Expected
CAFs	Cancer-Associated Fibroblasts
TAMs	Tumor-Associated Macrophages
iCAF	Inflammatory Cancer-Associated Fibroblast
myCAF	Myofibroblastic Cancer-Associated Fibroblast
apCAF	Antigen-Presenting Cancer-Associated Fibroblast
EMT	Epithelial-Mesenchymal Transition
MP	Meta-Program
NMF	Non-negative Matrix Factorization
GSVA	Gene Set Variation Analysis
GSEA	Gene Set Enrichment Analysis
KEGG	Kyoto Encyclopedia of Genes and Genomes
GO	Gene Ontology
ChIP	Chromatin Immunoprecipitation
FDR	False Discovery Rate

Supplementary Information

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Supplementary Material 1.

Supplementary Material 2.

Supplementary Material 3.

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Authors' contributions

H. Yu, Y. Wang and M. He contributed equally to this work. H. Yu, Y. Wang and M. He performed the majority of bioinformatic analyses, data interpretation, and figure preparation. Y. Wang and M. He conducted functional validation experiments and data analysis. Y. Chen collected and curated clinical data from prostate cancer patients. X. Lin provided pathological expertise, performed histological assessment, and interpreted spatial transcriptomics data in the context of clinical pathology. X. Cui coordinated data integration, curated all figures and tables, and wrote the original manuscript. X. Cui, X. Lin and J. Bi supervised the research, provided funding support, and revised the manuscript. All authors read and approved the final manuscript.

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Data availability

The single-cell RNA sequencing data generated and analyzed during this study are available in the Gene Expression Omnibus (GEO) repository under accession numbers GSE181294, GSE137829, GSE193337, HRA002145, GSE206962, GSE157703, GSE264573, HRA004818, and GSE274229. The public spatial dataset GSE230282 was obtained from the GEO repository. The raw spatial transcriptomic sequencing data generated in this study have been deposited in the China National Center for Bioinformation (CNCB, <https://www.cncb.ac.cn/>) under accession number HRA016876. All other data supporting the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

All procedures involving human participants in this study were conducted in accordance with the ethical standards of the China Medical University Institutional Review Board (approval number: AF-SOP-07-1.201) and with the 1964 Helsinki declaration and its later amendments. Informed consent was obtained from all individual participants included in this study. All animal experiments were approved by the China Medical University Animal Care and Use Committee (approval number: CMU20241328) and were performed in accordance with the institutional guidelines for the care and use of laboratory animals. All procedures involving animals complied with the ARRIVE guidelines and were conducted in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments.

Competing interests

The authors declare no competing interests.

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