Targeting Pre-existing Club-Like Cells in Prostate Cancer Potentiates Androgen Deprivation Therapy

Short title: Targeting Club Cells in Prostate Cancer

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Abstract

A critical knowledge gap in prostate cancer research is understanding whether castration-tolerant progenitor-like cells that reside in treatment-naïve tumors play a direct role in therapy resistance and tumor progression. Herein, we reveal that the castration tolerance of LSC^{med} (Lin^{-}, $Sca\text{-}1^{+}$, $CD49f^{med}$) progenitor cells, the mouse equivalent of human prostatic Club cells, arises not from intrinsic properties but from significant transcriptional reprogramming. Utilizing single-cell RNA sequencing of LSC^{med} cells isolated from prostate-specific Pten-deficient ($Pten^{pc-/-}$) mice, we identify the emergence of castration-resistant LSC^{med} cells enriched in stem-like features, driven by the transcription factor Fosl1/AP-1. We demonstrate that cells exhibiting $Pten^{pc-/-}$ LSC^{med} characteristics are prevalent in the aggressive mesenchymal stem-like prostate cancer (MSPC) subtype recently identified in human castration-resistant prostate cancer (CRPC). Furthermore, our findings show that the dual-targeting agents JQ-1 and CX-6258—focused on Fosl1/AP-1 and PIM kinases, respectively—effectively suppress both the progenitor properties and the growth of mouse and human MSPC surrogates *in vitro* and *in vivo*. Thus, early eradication of castration-tolerant Club-like cells presents a promising therapeutic strategy to mitigate prostate cancer progression toward CRPC.

Keywords

Castration-resistance / Club cells / MSPC / Fosl1 / LSCmed cells

Introduction

Prostate Cancer (PCa) is the second most frequent male cancer worldwide and the fifth in terms of death (Bray *et al*, 2018). PCa is androgen-dependent and the gold-standard treatment of advanced PCa is androgen deprivation therapy (ADT), i.e. chemical castration. A clinical response is initially observed in virtually all patients, reflecting the death of androgen-dependent tumoral cells. Within 1 to 3 years, however, castration-resistant PCa (CRPC) develops in most patients, leading to metastatic dissemination to distant sites such as bone and liver (Bubendorf *et al*, 2000). Although CRPC can be further treated with second-generation androgen receptor (AR) pathway inhibitors (ARPi, e.g. abiraterone, enzalutamide, apalutamide, and darolutamide), tumors eventually become resistant within a few months of treatment (Wang *et al*, 2021), which has been linked to the emergence of aggressive forms of CRPC such as mesenchymal stem-like PCa (MSPC) and neuroendocrine PCa (NEPC) (Han *et al*, 2022). JAK/STAT and FGFR signaling (Chan *et al*, 2022), FOSL1/YAP (Tang *et al*, 2022), ONECUT2 (Qian *et al*, 2024) and FOXA2 (Wang *et al*, 2024) were recently identified as drivers of these aggressive forms of CRPC via lineage plasticity, opening new hopes for the treatment of advanced stages of the disease. Therapeutic interventions earlier in the course of the disease to eradicate tumor cells that drive the shift from hormone-sensitive tumors to CRPC may improve the prognosis of PCa patients. Intriguingly, the potential contribution of intrinsic androgen-independent prostatic cells that preexist in naïve prostate tumors is ill-explored.

Club and Hillock cells have been recently identified in the epithelium of healthy human prostate by single-cell RNA sequencing (scRNAseq) (Henry *et al*, 2018). Club-like cells have also been identified in treatment-naïve primary prostate tumors using scRNAseq and spatial transcriptomics (Chen *et al*, 2021; Kiviaho *et al*, 2024; Song *et al*, 2022). These two cell types exhibit very similar transcriptomic profiles and can be discriminated by the differential expression of lineage markers reflecting a more basal (Hillock) *versus* luminal (Club) phenotype (Henry *et al.*, 2018). Club/Hillock cells are poorly differentiated cells that are enriched in stem/progenitor-like programs (Baures *et al*, 2022a; Henry *et al.*, 2018). In addition, they both exhibit low androgen signaling, hence they are predicted to be ADT-tolerant. Accordingly, the transcriptomic signatures of Club/Hillock cells are enriched in the MSPC molecular subtype of PCa (Han *et al.*, 2022). MSPC largely overlaps with PCa subtypes identified in other studies as stem-cell like (SCL) (Tang *et al.*, 2022) and double-negative PCa (DNPC) (Bluemn *et al*, 2017), all of which exhibit a reduced AR expression signature and no neuroendocrine features. Although MSPC is frequently observed at the CRPC stage i.e. post-castration (Han *et al.*, 2022), analysis of The Cancer Genome Atlas (TCGA), CPC-GENE (Fraser *et al*, 2017) and DFKZ (Gerhauser *et al*, 2018) datasets reveals that 27-74% of

treatment-naïve localized tumors exhibit mixed MSPC and AR-positive PCa (ARPC) features (Han *et al.*, 2022). Moreover, up to 11% of the tumors in the early-onset (<55 years of age) DFKZ dataset were identified as MSPC subtype (Han *et al.*, 2022). These *de novo* MSPC tumors are associated with enrichment for *PTEN* deletions, more advanced pathology (Gleason score, T/N stages) and premature patient death after treatment compared to ARPC or mixed ARPC/MSPC tumors. Together, these findings converge on the question whether Club cells pre-existing in treatment-naïve tumors may also contribute to therapeutic resistance and tumor progression towards aggressive forms of CRPC. To our knowledge, however, these cells have not been functionally characterized.

The *Pten*$^{pc-/-}$ mouse is an appropriate preclinical model to address these key questions as its developing prostatic tumors are highly populated by the mouse equivalent of human prostatic Club/Hillock cells termed LSC^{med} (Lin^{-}, Sca-1^{+}, CD49f^{med}) cells (Baures *et al.*, 2022a; Baures *et al*, 2022b; Sackmann Sala *et al*, 2017). In this acknowledged mouse model of CRPC, the tumor suppressor gene *Pten* is deleted in prostatic luminal epithelium, recapitulating one of the most frequent mutations observed in the human disease (Mulholland *et al*, 2011; Wang *et al*, 2003), especially in the MSPC subtype (Han *et al.*, 2022). According to their low AR-signaling profile, the proliferation rate of *Pten*$^{pc-/-}$ LSC^{med} cells is unaffected by castration (Sackmann Sala *et al.*, 2017). In addition, they exhibit progenitor properties *in vitro* and tumor-initiating capacity *in vivo* (Baures *et al.*, 2022b; Sackmann Sala *et al.*, 2017). Together, these findings support a key role of these Club-like cells in castration resistance and progression towards CRPC.

In this study, we show that *Pten*$^{pc-/-}$ LSC^{med} cells are not intrinsically castration-tolerant, but undergo transcriptomic reprogramming after androgen deprivation that enhances their aggressiveness. Using scRNAseq specifically applied to *Pten*$^{pc-/-}$ LSC^{med} cells sorted from intact *versus* castrated mice, we show that castration favors the emergence of a LSC^{med} cell subpopulation enriched in stem-like features. We identified the transcription factor Fosl1/AP-1 as the major driver of this transcriptomic phenotype switch. Combined pharmacological inhibition of Fosl1/AP-1 and its transcriptional target PIM1 kinase abrogated the progenitor properties of *Pten*$^{pc-/-}$ LSC^{med} cells and markedly reduced the growth of CRPC in castrated *Pten*$^{pc-/-}$ mice. In line with the transcriptional resemblance of *Pten*$^{pc-/-}$ mouse LSC^{med} cells and the human MSPC subtype, our drug combination also reduced the growth of human MSPC-like PC-3 cells xenografted in castrated host mice. These results suggest that early eradication of castration-tolerant Club-like cells presents a promising therapeutic strategy to mitigate PCa progression toward CRPC.

Results

Pten$^{pc-/-}$ LSC^{med} cells share transcriptomic features with human MSPC/SCL subtypes

LSC^{med} cells isolated from *Pten*$^{pc-/-}$ mice exhibit castration tolerance *in vivo* and form organoids in an androgen-independent manner (Baures *et al.*, 2022b; Sackmann Sala *et al.*, 2017). To investigate whether castration impacts the fate of LSC^{med} cells, we conducted Smart-seq2-based scRNAseq analysis of 384 LSC^{med} sorted cells from dissociated prostates of intact and 2-month-castrated mice (Fig. 1A and Fig. S1A). After selecting for high-quality single-cell-transcriptomes, we performed dimension reduction using Uniform Manifold Approximation and Projection (UMAP) and annotated the resulting single-cell space according to the corresponding castration profiles (Fig. 1B). The identity of the analyzed cells was assessed by the mRNA expression of the LSC^{med} marker *Krt4* (Sackmann Sala *et al.*, 2017) and of the surface markers used in cell sorting (Fig. S1, B to E). The intrinsically low AR signaling of LSC^{med} cells (Sackmann Sala *et al.*, 2017) was further reduced after castration (Fig. 1C). Differential gene expression (DEG) analysis identified 206 genes that exhibited at least a 2-fold (adj. pval<0.001) difference after castration (Table S1). The 20 most discriminative genes (based on adj. pval and fold-change (FC)) per experimental condition were plotted as heatmap (Fig. 1D). Notably, post-castration, we observed the decreased expression of androgen-regulated genes (*Fxyd3*, *Mme*) (Mevel *et al*, 2020), and concomitant increased expression of *Krt6b* (a marker of suprabasal layers in many epithelia) (Volkel *et al*, 2022) and *Krt13* (a Hillock cell marker) (Henry *et al.*, 2018). Various stem-related genes (*Lgr4*, *Klf4*, *Tgfb2*, *Ly6d*) and inflammatory genes (*ifi202b*, *Cxcl5*) were also upregulated. This first experiment shows that *Pten*$^{pc-/-}$ LSC^{med} cells are actually sensitive to mouse castration.

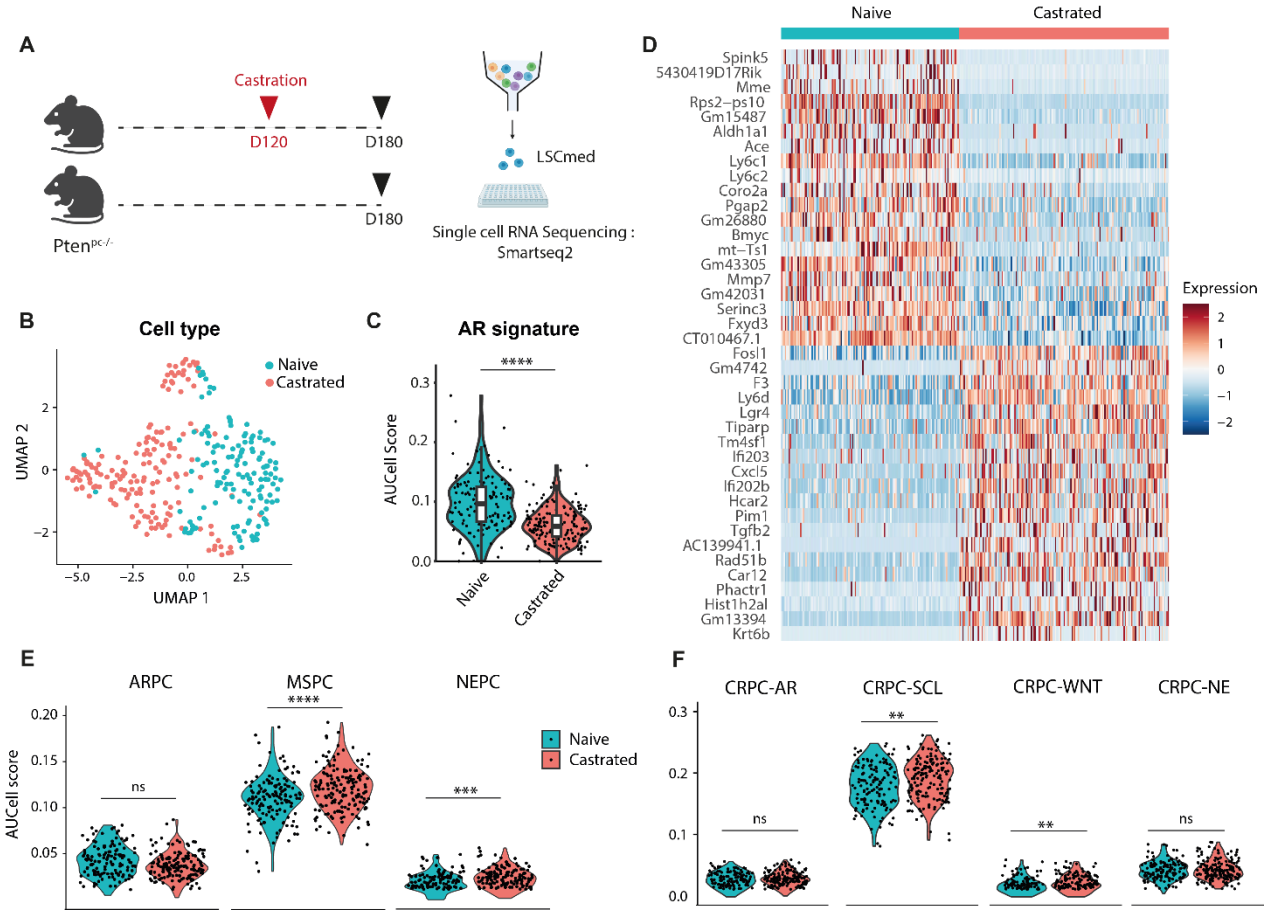

Fig. 1. *Pten*$^{pc-/-}$ LSC^{med} cells as a robust surrogate of human MSPC subtype. (A) Schematic representation of LSC^{med} single-cell RNA sequencing protocol. Two 6-month-old *Pten*$^{pc-/-}$ mice were used in each group. Created in BioRender. BAURES, M. (2024) BioRender.com/f61z820. **(B)** UMAP space of *Pten*$^{pc-/-}$ LSC^{med} cells colored according to the castration status. **(C)** AR signature expression intensities (AUCell score) (Sackmann Sala *et al.*, 2017) in LSC^{med} cells per *Pten*$^{pc-/-}$ mouse, stratified by castration. **(D)** The 20 most discriminative (FC and adj. pval) genes of LSC^{med} cells from intact *versus* castrated *Pten*$^{pc-/-}$ mice are shown. **(E,F)** The AUCell scores of the MSPC/ARPC/NEPC (Han *et al.*, 2022) and CRPC-WNT/-SCL/-AR/-NE (Tang *et al.*, 2022) human CRPC subtype signatures in LSC^{med} cells per *Pten*$^{pc-/-}$ mouse**,** stratified by castration.

We then compared the signatures of *Pten*$^{pc-/-}$ LSC^{med} cells in intact and castrated conditions to recently reported signatures of human PCa subtypes (Table S2) (Han *et al.*, 2022; Tang *et al.*, 2022). This analysis revealed that MSPC (Han *et al.*, 2022) and its counterpart in the Tang *et al.* study, CRPC-SCL, are markedly enriched in typical LSC^{med} cell genes compared to other PCa subtypes (Fig. 1E and F). The enrichment was further enhanced when using post-castration LSC^{med} cell signatures. This was mainly due to the upregulation of genes such as *Anxa1*, *Tm4sf1*, *Ltbp1*, *Msn*, *Lgals3*, *Arhgdib* (for the MSPC phenotype) and *Plat*, *Sdc4*, *Cstb*, *Cd44*, *Plau*, *Epha2*, *S100a14*, *Pttg1ip* and *Hif1a* (for the SCL phenotype).

Together, these results highlight that *Pten*$^{pc-/-}$ LSC^{med} cells are sensitive to castration and exhibit high molecular similarity with human MSPC/SCL tumors, i.e. PCa subtypes that are enriched in stemness features and associated with ADT-resistance and metastatic potential.

Castration strengthens the stem-like characteristics of one *Pten*$^{pc-/-}$ LSC^{med} cell subpopulation

To better characterize the impact of castration on *Pten*$^{pc-/-}$ LSC^{med} cells, we conducted unsupervised Louvain-clustering of these cells and identified three subpopulations (Fig. 2A, Table S3). Hereafter these three LSC^{med} clusters are referred to as LSC^{med}-0, LSC^{med}-1 and LSC^{med}-2. The LSC^{med}-0 subpopulation, highly predominant before castration, was drastically reduced after castration when LSC^{med}-1 subpopulation became predominant (16-fold increased) (Fig. 2B). LSC^{med}-2 subpopulation exhibited a more modest increase (3-fold) after castration (Fig. 2B). According to the observation reported above for the bulk LSC^{med} cell pool (Fig. 1C), AR signaling was lower in LSC^{med}-1 cells, and to a lesser extent, in LSC^{med}-2 cells, compared to LSC^{med}-0 cells (Fig. 2C).

LSC^{med}-0 cells express *Psca*, a well-described androgen-dependent marker in both murine and human luminal progenitors of the prostate (Crowell *et al*, 2019) (red arrow on Fig. 2D, Table S3). Gene Ontology (GO) enrichment analysis highlighted hallmarks of protein secretion, oxidative phosphorylation, DNA repair and prostate development (red arrows in Fig. S2, A and B). With the exception of MYC and E2F targets, most of the pathways in the Hallmark 50 gene dataset were less enriched in LSC^{med}-0 cells compared to the two other subpopulations (Fig. S2A).

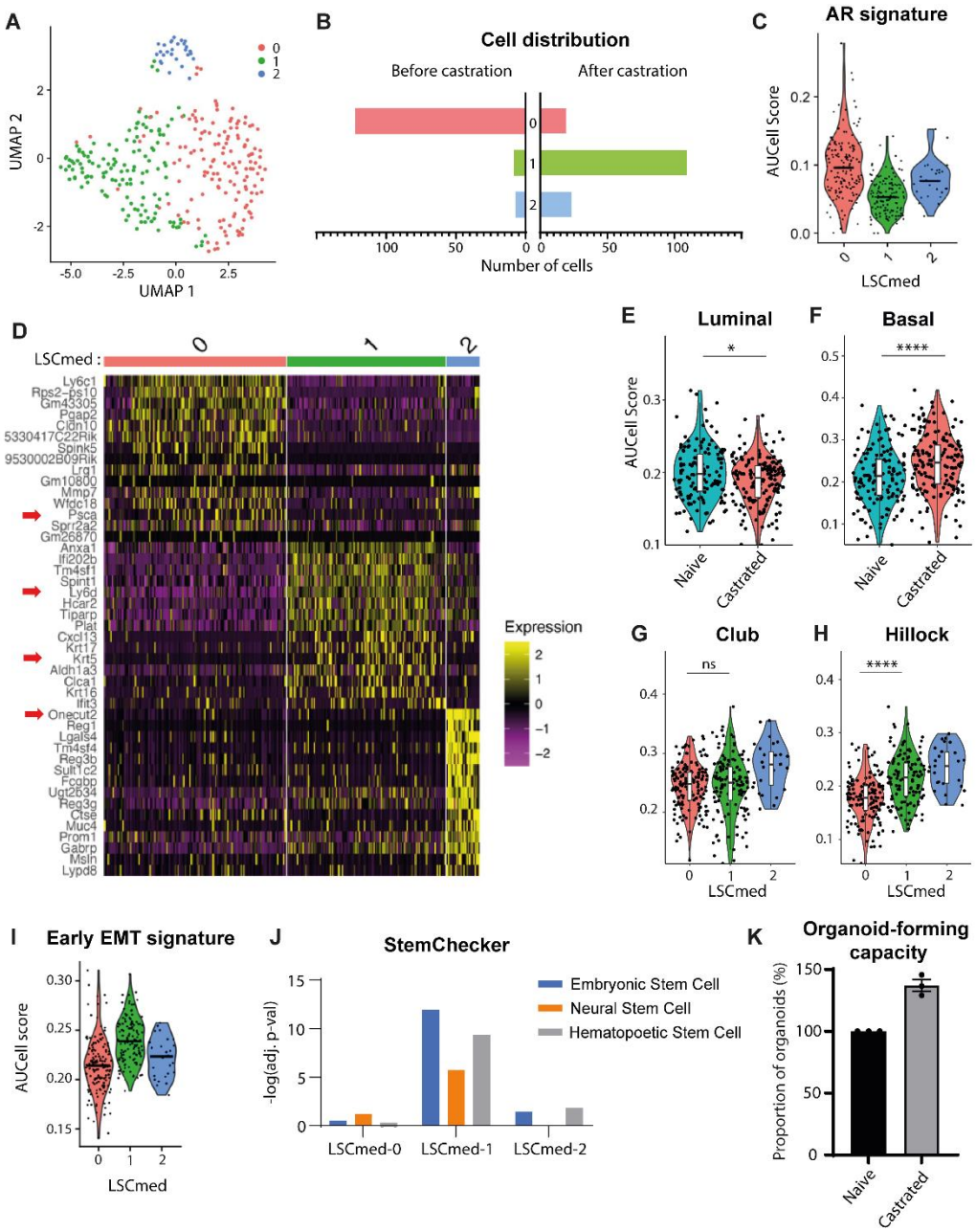

Fig. 2. Transcriptomic heterogeneity of *Pten*$^{pc-/-}$ LSC^{med} cells. (**A)** The UMAP analysis (unsupervised Louvain-clustering) of *Pten*$^{pc-/-}$ LSC^{med} cells analyzed by scRNAseq identified 3 distinct LSC^{med} cell clusters named LSC^{med}-0, -1 and -2. (**B)** Distribution of the three LSC^{med} cell subpopulations in prostates from intact versus castrated mice (**C)** The AUCell score of the AR signature (Sackmann Sala *et al.*, 2017) in the three LSC^{med} cell subpopulations is shown. **(D)** Discriminative marker genes (top 15) of the three LSC^{med} cell subpopulations. **(E-H)** Representation of the AUCell scores of the luminal (**E**) and basal (**F**) signatures (Joseph *et al*, 2020) per castration status, and of the Club (**G**) and Hillock (**H**) signatures (Henry *et al.*, 2018) per cluster. (**I)** The AUCell score of early EMT signature (Meyer-Schaller *et al*, 2019) per LSC^{med} cell subpopulations. (**J)** StemChecker analysis in each LSC^{med} cell subpopulations (-log (adj. p-values) are represented). (**K)** Organoid-forming capacity of LSC^{med} cells sorted from 10-month-old *Pten*$^{pc-/-}$ mice, intact versus 2-month after castration (n = 3 independent experiments).

LSC^{med}-1 cells represent the most amplified subpopulation after castration. It expresses *Ly6d* (red arrow on Fig. 2D, Table S3), a marker of castration-resistant luminal progenitors that is detected in primary PCa, amplified in mCRPC and associated with biochemical relapse and decreased overall survival (Barros-Silva *et al*, 2018; Steiner *et al*, 2023). Interestingly, we also noted the upregulation of *Krt5* (red arrow on Fig. 2D, Table S3), associated with an increase in the global basal signature and a slight decrease in the luminal score in response to castration (Fig. 2, E and F), as previously observed in human MSPC (Han *et al.*, 2022). According to the enrichment in basal features, LSC^{med}-1 cells showed a stronger Hillock cell profile, while the Club cell profile was unchanged (Fig. 2, G and H). LSC^{med}-1 cells were also associated with enrichment in basal breast cancer signature, as well as in other oncogenic pathways (e.g. pancreatic and lung cancer) (Fig. S2C). Typical features of human MSPC/SCL subtypes (Han *et al.*, 2022; Tang *et al.*, 2022), including EMT (Fig. 2I), migration and stem signatures (Fig. S2, A and C), were also enriched in LSC^{med}-1 cells. The enrichment of stemness features was further highlighted by StemChecker analysis (Fig. 2J). This analysis allows the association of any input gene list with a curated stemness signature database by calculating enrichment values based on shared genes (hypergeometric test based -log adj. p-value) (Pinto *et al*, 2015). The StemChecker analysis indicated a higher stemness-associated transcriptional identity of LSC^{med}-1 cells as suggested by the identification of various stemness-associated genes such as *Klf4* or *Cd44* (Table S4). Accordingly, GO enrichment analysis highlighted several stemness-associated pathways, e.g. Wnt, Tgfβ, Notch and Hedgehog signaling (green arrows in Fig. S2A). The trend towards the higher organoid-forming capacity of LSC^{med} cells sorted from the prostates of castrated mice (enriched in LSC^{med}-1 cells) *versus* intact mice (enriched in LSC^{med}-0 cells) agrees with stemness enrichment in LSC^{med} cells post-castration (Fig. 2K). Together, the molecular features of LSC^{med}-1 cells suggest increased aggressiveness of this particular subpopulation amplified following castration.

Finally, the most differentially expressed gene of LSC^{med}-2 cells is *Onecut2* (red arrow on Fig. 2D). Onecut2 is known to be a driver of neuroendocrine differentiation in PCa (Chan *et al.*, 2022; Guo *et al*, 2019; Qian *et al.*, 2024; Rotinen *et al*, 2018). However, in LSC^{med}-2 cells, *Onecut2* expression was not associated with the enrichment of a neuroendocrine signature (Han *et al.*, 2022) (Fig. S2E), and typical neuroendocrine markers such as Chromogranin A and Synaptophysin were not detected in LSC^{med}-2 cell transcriptome. In contrast to LSC^{med}-1 cells, LSC^{med}-2 cells were not enriched in stemness-associated programs (Fig. 2J). GO enrichment analysis highlighted the increased oxidative stress and hypoxia in LSC^{med}-2 cells. Of note, the hypoxia-associated enzyme transglutaminase 2 (*Tgm2*), which is a marker of LSC^{med}-2 cells (Table S3), was recently identified

as a marker of malignant progression (Abu El Maaty *et al*, 2022). This suggests that the LSC^{med}-2 subpopulation could have the potential to promote cancer progression, which could be facilitated by various oncogenic pathways associated with tumor aggressiveness (Fig. S2D).

Together, these results demonstrate that castration of *Pten*$^{pc-/-}$ mice promotes the emergence of LSC^{med} cells presenting with a transcriptomic profile enriched in basal, EMT and stemness features and lineage plasticity drivers. All these features are known to correlate with cancer cell aggressiveness.

Transcriptomic plasticity of *Pten*$^{pc-/-}$ LSC^{med} cells as an adaptive mechanism to castration

The transcriptomic analysis described above identified three LSC^{med} cell subpopulations whose ratios considerably evolve in response to castration (Fig. 2B). We hypothesized that this could result from a phenomenon of i) positive selection, i.e. robust proliferation of LSC^{med}-1 cells possibly associated to death of LSC^{med}-0 cells, and/or ii) cell plasticity, i.e. a transcriptional switch from LSC^{med}-0 to LSC^{med}-1 and LSC^{med}-2 profiles (Fig. 3A). To address these hypotheses, we first quantified Ki-67 positive epithelial cells in various fields of prostates harvested from *Pten*$^{pc-/-}$ mice sacrificed prior to castration or at 5, 21 or 60 days after castration. Despite a slight increase in the proportion of proliferative cells at day 5 post-castration, cell proliferation was not altered at any time point following castration (Fig. 3B). The pattern of dead cells (TUNEL assay) was focal and the global level remained particularly low (~2.5%), again with no significant variation after castration (Fig. 3C). In contrast, we showed by RT-qPCR that the expression of DEGs associated with each LSC^{med} cell subpopulation quickly evolved after castration. Downregulation of LSC^{med}-0 markers (e.g. *Hoxb13*) concomitant to upregulation of LSC^{med}-1 (e.g. *Ifi202b*) and LSC^{med}-2 (e.g. *Onecut2*) markers was detected as soon as 5 days after castration (Fig. 3, D to F).

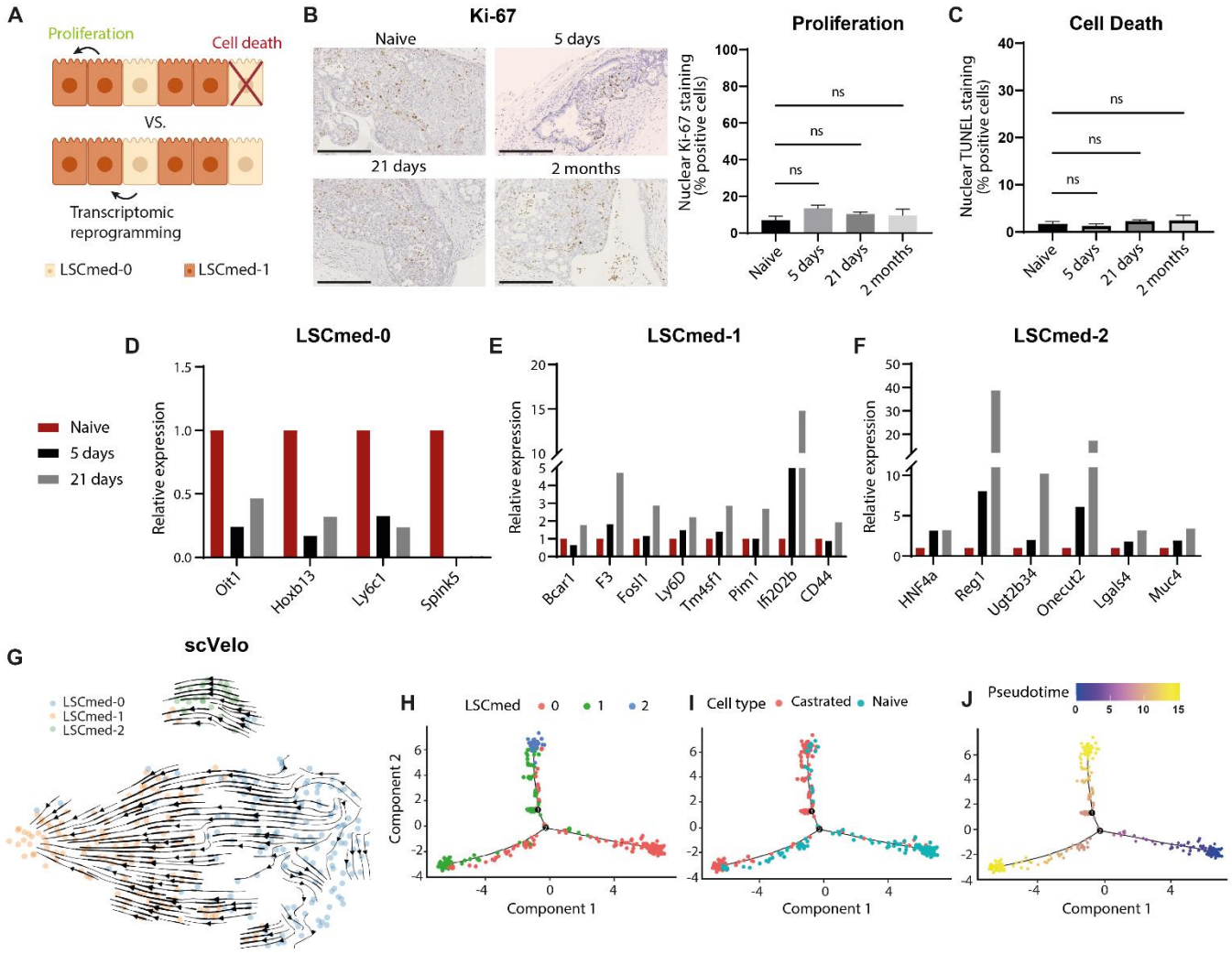

Fig. 3. Transcriptional plasticity dynamics of *Pten*$^{pc-/-}$ LSC^{med} cells by castration. (A) Schematic representation of positive selection (upper panel) and transcriptional reprogramming (lower panel) as two non-mutually exclusive mechanisms that could promote amplification of LSC^{med}-1 and LSC^{med}-2 at the expense of LSC^{med}-0 cell subpopulations after castration (see text for explanations). Created BioRender. BAURES, M. (2024) BioRender.com/k26b175. **(B,C)** Images and proportion of Ki67-positive cells quantified from immunohistochemistry (scale bar: 250 µm) **(B)** and TUNEL-positive cells **(C)** in prostates from intact *Pten*$^{pc-/-}$ mice versus 5 days, 21 days and 2 months after castration (n = 4 mice per condition). Comparison between groups was performed using ANOVA analysis and Dunnett's *post hoc* test (ns: not significant). **(D-F)** The expression of selected marker genes of LSC^{med}-0 **(D)**, LSC^{med}-1 **(E)** and LSC^{med}-2 **(F)** cells was measured by RT-qPCR in bulk LSC^{med} cells sorted from intact *Pten*$^{pc-/-}$ mice and 5 days and 21 days after castration (n= 4 mice per time point). Data are normalized to the expression in intact mice. **(G)**. RNA velocity analysis (scVelo) in *Pten*$^{pc-/-}$ LSC^{med} cells analyzed by scRNAseq, based on the inference of directed dynamic information by leveraging splicing kinetics. **(H-J)** Pseudotime-ordering analysis (Monocle): projection of cells per cluster **(H),** castration status **(I)** and pseudotime **(J)**.

The rapidity of these transcriptional changes coupled with the unaltered rates of cell proliferation and cell death favors a transcriptional reprogramming trajectory from LSC^{med}-0 towards the two other subpopulations shortly after castration. RNA velocity analysis (scVelo), based on the inference of directed dynamic information by leveraging splicing kinetics, predicted LSC^{med}-0 cells as a likely root cell population (Fig. 3G). This prediction was further supported by pseudotime-ordering analysis (Monocle) (Fig. 3H-J). The transcriptional heterogeneity of LSC^{med}-0 and LSC^{med}-1 cells was evident from the presence of "intermediate" cells distributed along the lower trajectory before castration (Fig. 3H-J). In contrast, the grouping of all LSC^{med}-2 cells at the upper branch extremity suggests higher transcriptional homogeneity. Trajectory analyses predicted that post-castration LSC^{med}-1 cells evolve from pre-castration LSC^{med}-0 cells, while post-castration LSC^{med}-2 cells may originate from LSC^{med}-0 and/or LSC^{med}-1 cells.

These results collectively support the hypothesis that cellular plasticity underlies an adaptive resistance mechanism of LSC^{med} cells, which is initiated shortly after castration.

AP-1 complex is a major regulator of *Pten*$^{pc-/-}$ LSC^{med} cells emerging post-castration

We next performed gene regulatory inference using SCENIC (Aibar *et al*, 2017) to identify transcriptional upstream regulators of the three LSC^{med} cell subpopulations. This analysis predicted *Hoxb13* as a major regulator of LSC^{med}-0 cells (Fig. 4A and Fig. S3A). *Hoxb13* was described as key driver of the prostatic cell luminal differentiation (Huang *et al*, 2007). Other highlighted transcription factors regulating LSC^{med}-0 cells included *Ddit3*, *Bmyc*, *Nr2f2*, *Atf3*, *Grhl2*, and *Ehf* (Fig. 4A and Fig. S3A). *DDIT3* was recently shown to be upregulated in CRPC patients and proposed to be associated with progression to CRPC (Jung *et al*, 2024). EHF is a transcription factor of the ETS family that controls epithelial cell differentiation and its loss promotes prostate tumors enriched in EMT signature and mesenchymal/stem-like features (Albino *et al*, 2012). Finally, while *Bmyc* may be correlated to the upregulation of MYC targets (Fig. S2A), *Nr2f2* has been shown to cooperate with *Pten* deletion to promote malignant progression (Qin *et al*, 2013).

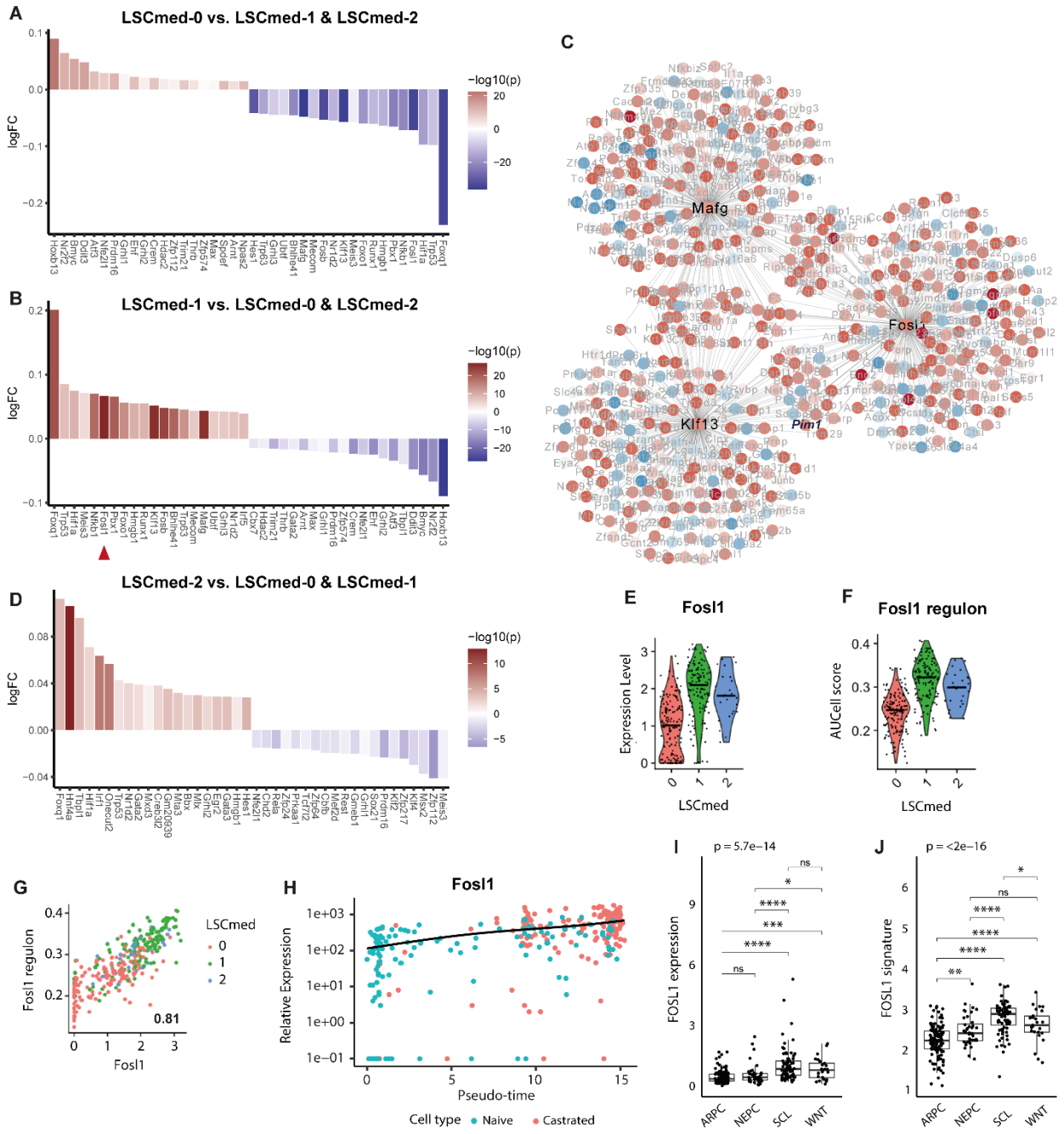

Fig. 4. Fosl1/AP-1 complex is a major transcriptional hub for *Pten*$^{pc-/-}$ LSC^{med} cells emerging post-castration. **(A to D)** Identification of the main upstream regulators of the three LSC^{med} cell subpopulations by SCENIC analysis. The top-20 (fold change) activated (red) and inactivated (blue) transcription factors in LSC^{med}-0 **(A)**, LSC^{med}-1 **(B)** et LSC^{med}-2 **(D)** cells are represented as waterfall plots. The red arrow in B identifies Fosl1. The TF network of the 3 most pertinent regulons of LSC^{med}-1 cells is represented in **C** (see Fig. S3 for LSC^{med}-0 and LSC^{med}-2 cells). **(E,F)** Fosl1 expression (**E**) and AUCell Score of Fosl1 regulon (**F**) in the three LSC^{med} cell subpopulations. **(G)** Correlation of Fosl1 expression and Fosl1 regulon in the three LSC^{med} cell subpopulations. **(H)** Projection of Fosl1 expression during pseudotime (pval = 5.01e-21, qval = 6.37e-19). **(I,J)** Expression of FOSL1 **(I)** and FOSL1 regulon **(J)** in SU2C patient cohort (Tang *et al.*, 2022), according to the tumor molecular subtype.

Remarkably, the transcription factor profile of LSC^{med}-1 cells (Fig. 4B) was the near-perfect inverse mirror image of that of LSC^{med}-0 cells (Fig. 4A). This observation further supports that castration triggers cell plasticity of LSC^{med}-0 towards LSC^{med}-1 cells by switching on/off selected transcription factors. We identified *Fosl1* as the most pertinent (based on -log10(pval)) regulator of the LSC^{med}-1 cell signature (Fig. 4B). Fosl1 is a transcription factor that acts in the AP-1 complex mainly as a heterodimer involving Jun proteins (c-Jun, JunB, JunD) (Eferl & Wagner, 2003). Other typical transcription factors of LSC^{med}-1 cells included *Mafg*, *Klf13*, *Foxq1*, *Nfκb1*, *Pbx1*, *Ets1* and *Fosb* (Fig. 4B). Of note, both *Mafg* and *Klf13* share several common target genes with Fosl1 (Fig. 4C).

Finally, we identified *Hnf4a* as a master regulator of LSC^{med}-2 cell signature (Fig. 4D and Fig. S3B). Hnf4a is a nuclear receptor known for its role as a tumor suppressor in the prostate by its ability to promote senescence in prostatic cells (Wang *et al*, 2020). Hnf4a shares several target genes with Oncecut2 (Fig. S3B), suggesting a potential role of Hnf4a in the cellular plasticity leading to the LSC^{med}-2 signature. *Nr1d2*, another transcriptional regulator promoting lineage plasticity towards NEPC (He *et al*, 2021), was also identified as a top gene of LSC^{med}-2 cells (Fig. 4D). The expression of *Gata2* and *Gata3* (Fig. 4D) suggests that LSC^{med}-2 cells present a certain degree of luminal differentiation (Xiao *et al*, 2016).

Although *Fosl1* was identified as the top gene of LSC^{med}-1 cells (Fig. 4B), it was expressed at similar level in LSC^{med}-2 cells, and at consistent, though lower, level in LSC^{med}-0 cells (Fig. 4E). The Fosl1-regulated network expectedly followed the same pattern (Fig. 4, F and G). Fosl1/AP-1 is known to be involved in various processes including stemness, EMT and cell plasticity (Eferl & Wagner, 2003; Feldker *et al*, 2020; Marques *et al*, 2021). In the mouse prostate, Fosl1/AP-1 was recently identified as one of the actors of differentiated (androgen-dependent) luminal cell plasticity in both healthy (Kirk *et al*, 2024) and cancer (Tang *et al.*, 2022) contexts. To investigate further the potential role of Fosl1/AP-1 complex in progenitor/Club-like cell plasticity upon castration, we plotted the Fosl1 expression in function of pseudotime. As illustrated in Fig. 4H, we observed that Fosl1 expression progressively increased post-castration. This data suggests that the Fosl1/AP-1 regulon is a main driver of the post-castration transcriptional switch of LSC^{med} cells. Other AP-1 factors could also contribute as their transcriptional networks were also highlighted by SCENIC analysis, albeit at lower significance (*Atf3* in LSC^{med}-0 cells, *Fosl2* and *Fosb* in LSC^{med}-1 cells, *Fos* in LSC^{med}-2 cells) (Fig. 4, A, B and D; Table S3). Strikingly, in human PCa, FOSL1 and its regulon

are particularly associated with the MSPC/SCL subtype (Fig. 4, I and J). In fact, we noticed that the majority of AP-1 complex members (*FOSL1*, *FOSL2*, *FOS*, *JUN*, *JUNB*, *ATF3*, *BATF*) are expressed at higher levels in MSPC/SCL than in the ARPC tumors (Fig. 4, I and J, and Fig. S4). This data further highlights the relevance of *Pten*$^{pc-/-}$ LSC^{med} cells as a surrogate of this aggressive cancer subtype.

Together, these analyses show that members of the AP-1 complex, and in particular Fosl1, are predicted to be major drivers of the transcriptional switch occurring post-castration in LSC^{med}/Club-like cells.

Pharmacological targeting of LSC^{med} and Club/Hillock-like cancer cells *in vitro*

We next used *Pten*$^{pc-/-}$ LSC^{med} cells as a MSPC/SCL surrogate to identify relevant therapeutic targets for this incurable cancer subtype. Considering that LSC^{med}-1 cells are the most amplified after castration (Fig. 2B), the least androgen-sensitive (Fig. 2C) and the most enriched in stemness features (Fig. 2I), we reasoned that targeting this particular cell subpopulation was the most relevant strategy to deviate LSC^{med} cells from progression towards CRPC in our mouse model. Based on the results shown in the previous section, Fosl1/AP-1 was identified as the most obvious therapeutic target. The high expression of various members of the AP-1 family, including Fosl1, in the two other LSC^{med} subpopulations (Fig. S5A) suggested a wide spectrum of targeted LSC^{med} cells. We noticed that Pim1 is part of the Fosl1-regulated gene network (Fig. 4C). Pim1 is a member of the Pim serine/threonine kinase family that phosphorylates substrates controlling various tumorigenic phenotypes, including cell survival and proliferation (Rout *et al*, 2024; Wang *et al*, 2001). In PCa, it was shown to promote cell invasion and migration (Rebello *et al*, 2016; Santio *et al*, 2015) and ligand-independent androgen receptor phosphorylation associated with hormone-refractory PCa (Ha *et al*, 2013). Pim1 pharmacological targeting was shown to reduce prostate tumor growth in mouse and human preclinical settings (Hu *et al*, 2009; Rebello *et al.*, 2016). We found that Pim1 is drastically enriched in *Pten*$^{pc-/-}$ LSC^{med}-1 and LSC^{med}-2 cell subpopulations which are predominant post-castration (Fig. 5, A and B). Of the two other paralogs of the Pim kinase family, Pim3, but not Pim2, is highly expressed in all *Pten*$^{pc-/-}$ LSC^{med} cell subpopulations (Fig. S5B).

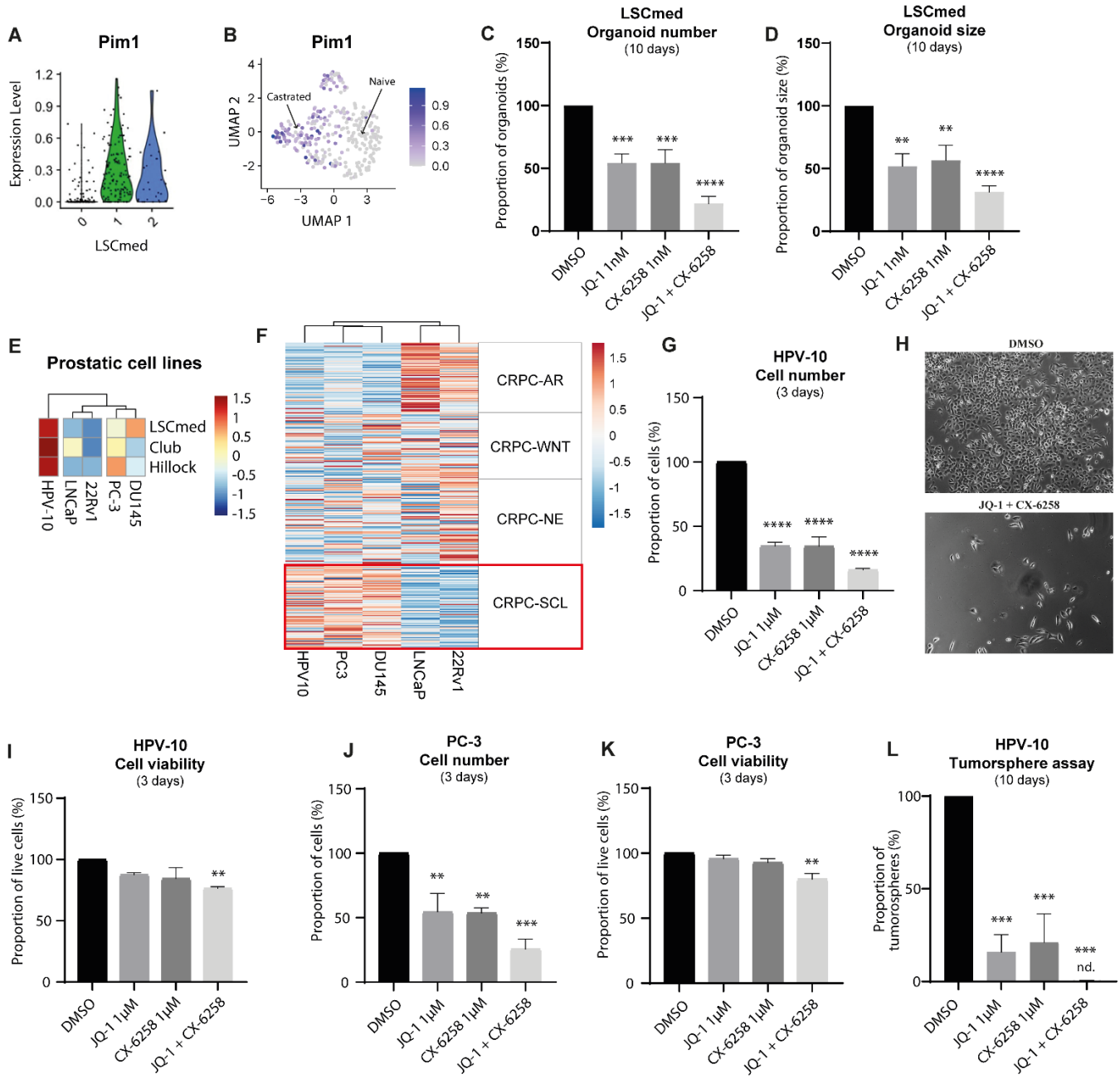

Fig. 5. Targeting Fosl1/AP-1 and Pim family prevents the growth of LSC^{med} and human Club/Hillock-like cells *in vitro*. (A,B) Expression per LSC^{med} cell subpopulation **(A)** and UMAP projection **(B)** of Pim1. **(C,D)** Number **(C)** and size **(D)** of organoids generated from sorted *Pten*$^{pc-/-}$ LSC^{med} cells after 10 days of culture in medium containing DMSO or 1nM JQ1 and 1nM CX-6258, alone or combined, as indicated. Data are normalized to the control (DMSO) condition (n=4 independent experiments). **(E)** Enrichment of LSC^{med} (Sackmann Sala *et al.*, 2017), Club and Hillock (Henry *et al.*, 2018) signatures in HPV-10, PC-3, DU145, LNCaP and 22Rv1 cell lines. **(F)** Enrichment of CRPC-AR, -WNT, -NE and -SCL human tumoral subtypes signature (Tang *et al.*, 2022) in HPV-10, PC-3, DU145, LNCaP and 22Rv1 cell lines. **(G)** Human HPV-10 cells were treated for 72 hours with 1µM JQ1 and 1µM CX-6258, alone or combined (as indicated), then the number of adherent cells was counted (n = 3 independent experiments). The data are normalized to the control (DMSO) condition. **(H)** Images of HPV-10 cells after 72h of treatment. **(I)** The viability of HPV-10 cells (adherent + in suspension) was determined by trypan blue staining (n=4 independent experiments). **(J,K)** Same as G and I with PC-3 cells **(L)** Tumorsphere-forming

capacity of HPV-10 cells in medium containing DMSO or 1µM JQ1 and 1µM CX-6258, alone or combined as indicated. Data are normalized to the DMSO condition (n=3 independent experiments). nd. = not detected. Statistical analysis between groups of treatment was performed using ANOVA analysis and Tukey's *post hoc* test.

We tested JQ-1 and CX-6258 as pharmacological inhibitors of these selected targets in functional assays. JQ-1 is a global BET/AP-1 inhibitor that has been shown to mainly target Fosl1 in lung adenocarcinomas (Casalino *et al*, 2022; Lockwood *et al*, 2012) and to phenocopy Fosl1 knockdown in other types of tumors (Baker *et al*, 2015; Bid *et al*, 2016). Importantly, JQ-1 had been validated in preclinical *in vivo* settings prior to this study (Shimamura *et al*, 2013; Shu *et al*, 2016). CX-6258 is a pan-PIM kinase inhibitor (Haddach *et al*, 2012), which prevents compensation by any other expressed Pim paralog (e.g. Pim3 in our case) as reported in other studies (Mikkers *et al*, 2002; van der Lugt *et al*, 1995). CX-6258 was recently shown to interfere with PCa progression in preclinical models (Rebello *et al.*, 2016).

We used the organoid assay to determine the functional impact of these pharmacological inhibitors on *Pten*$^{pc-/-}$ LSC^{med} cell properties. We first determined the dose-response of each drug on the organoid-forming capacity of these cells. Both JQ-1 and CX-6258 were very active in the nanomolar range, indicating high sensitivity of LSC^{med} cells to these drugs (Fig. S5, C and D). At 1 nM concentration, each drug decreased by half both the number and the size of the organoids generated from *Pten*$^{pc-/-}$ LSC^{med} cells, indicating inhibition of their progenitor and proliferation capacities, respectively. These effects were further potentiated when both drugs were combined (Fig. 5, C and D). These data indicate that targeting Fosl1/AP-1 and/or Pim kinases markedly alters the growth properties of *Pten*$^{pc-/-}$ LSC^{med} cells.

We then aimed to validate this therapeutic approach using human Club/Hillock cancer cells. In contrast to *Pten*$^{pc-/-}$ mouse LSC^{med} cells, there are currently no established procedures to sort, culture and expand Club/Hillock cells from fresh specimens of human prostate cancer. Therefore, we sought a relevant cell line matching the Club/Hillock phenotype. By comparing human PCa cell line signatures (GSE9633) to LSC^{med} (Sackmann Sala *et al.*, 2017), Club and Hillock (Henry *et al.*, 2018) cell signatures (Table S5), we found that HPV-10 cells are highly enriched in these three signatures (Fig. 5E). In comparison, androgen-independent PC-3 and DU145 cell lines exhibited an intermediate enrichment, while androgen-dependent LNCaP and 22Rv1, classically described as luminal prostate cancer cells, were the least enriched in LSC^{med}/Club/Hillock features among the

cell panel analyzed (Fig. 5E). The HPV-10 cell line is not commonly used in the field. In contrast to PC-3 and DU145 cells, which were derived from PCa metastatic sites, HPV-10 cells were isolated from a high-grade primary prostate adenocarcinoma. They grow in culture in the absence of androgens, which indicates their intrinsic castration resistance. Accordingly, no *PSCA* expression was detected by RT-qPCR (Fig. S6A). Otherwise, we confirmed the expression of various LSC^{med} markers (e.g. *KRT4*, *KRT7*) in HPV-10 cells compared to the luminal LNCaP cell line (Fig. S6A). Immunofluorescence analysis showed the co-expression of KRT4 (Club cell marker) and KRT13 (Hillock cell marker) (Fig. S6B), identifying HPV-10 as a mixed Club/Hillock cell line. This is reminiscent of murine LSC^{med} cells that match both Club and Hillock signatures (Baures *et al.*, 2022a; Baures *et al.*, 2022b). HPV-10 cells express *FOSL1* and *PIM1* at a much higher level than LNCaP cells, further supporting their Club-like cell profile (Fig. S6C). Altogether, these data identify the human HPV-10 cell line as relevant model of Club/Hillock PCa cells preexisting in prostate tumors. Finally, HPV-10 cells, but not LNCaP and 22Rv cells, expressed the genes of the CRPC-SCL signature to a similar degree as PC-3 and DU145 cells, previously identified as MSPC/SCL models (Han *et al.*, 2022; Tang *et al.*, 2022) (Fig. 5F). This identifies HPV-10 cells as another surrogate of MSPC/SCL cancer subtype.

To determine the effects of JQ-1 and CX-6258 on human Club/Hillock-like cells, we first performed dose-response assays using the HPV-10 cell line (Fig. S6D). For both drugs, we observed a significant inhibition of the number of adherent cells in the micromolar range, indicating lower sensitivity compared to *Pten*$^{pc-/-}$ LSC^{med} cells. Based on that data, we used 1 µM of each drug for subsequent experiments. As observed above with *Pten*$^{pc-/-}$ LSC^{med} cells, the combination of JQ-1 and CX-6258 potentiated the inhibition of HPV-10 cell growth in two-dimension cultures (Fig. 5G, H). The moderate (15-25%) ratio of dead cells at 72h of treatment (Fig. 5I) suggested that the drugs mainly prevented cell proliferation, as reported in former studies using JQ-1 (Li *et al*, 2019; Zhang *et al*, 2020; Zhang *et al*, 2021). A similar pattern of drug response was observed with PC-3 cells (Fig. 5, J and K). In addition, we found that the tumorsphere formation by HPV-10 cells was significantly altered by the drugs, again with almost a total inhibition when using the drug combination (Fig. 5L). This three-dimensional assay could not be performed with PC-3 cells as these cells failed to generate tumorspheres in our hands. Finally, we noticed that JQ-1 treatment decreased the level of *Fosl1* mRNA in HPV-10 cells (Fig. S6E). Since Fosl1 contributes to the regulation of its own gene expression (Casalino *et al.*, 2022), this observation further supports that JQ-1 inhibits Fosl1 activity in these cells.

Together, these results indicate that JQ-1/CX-6258 combination is an effective therapy to abolish the progenitor and growth properties of mouse LSC^{med} and human Club/Hillock-like prostate cancer cells *in vitro*.

Targeting the Fosl1/AP-1 and Pim family markedly reduces CRPC growth *in vivo*

We then aimed to validate this drug combination strategy in a CRPC context *in vivo*. We reasoned that castration-induced reprogramming of $Pten^{pc-/-}$ LSC^{med}-0 cells towards LSC^{med}-1 and LSC^{med}-2 transcriptional profiles should provide a window of opportunity for combination therapy involving JQ-1 and CX-6258. This strategy was further supported by confirming that, 21 days post-castration, sorted $Pten^{pc-/-}$ LSC^{med} cells exhibited increased expression of Fosl1 and Pim1 as determined by RT-qPCR (Fig. 6, A and B).

Two groups of experimental mice (n=8 each) were castrated and after 4-day recovery, we started treatment with JQ-1 (daily) and CX-6258 (bi-weekly) *versus* vehicle (Fig. 6C). After 28 days, mice were euthanized, then prostates were harvested, weighed, and processed for histopathological analyses or functional assays. We observed that the prostate weight in the drug-treated group was reduced by 41% compared to the vehicle-treated group (Fig. 6D). This was associated with a decreased proportion of advanced histologically-classified structures at the benefit of benign phenotypes (i.e. normal glands and prostate intraepithelial neoplasia [PINs]) (Fig. 6, E and F). In parallel to these studies, LSC^{med} cells sorted from a set of fresh prostates of vehicle- or drug-treated mice (each n=4) were cultured in organoid medium for 10 days in the absence of JQ-1 and CX-6258. Remarkably, we observed that LSC^{med} cells sorted from drug-treated $Pten^{pc-/-}$ mice had virtually lost any capacity to generate organoids compared to the vehicle-treated group (Fig. 6, G and H).

Next, we set up a xenograft assay to assess the *in vivo* efficacy of our therapeutic strategy on human MSPC-like cells. We used the PC-3 cell line as HPV-10 cells have been characterized as non-tumorigenic *in vivo* (Weijerman *et al*, 1994). Immunodeficient NSG mice were castrated and PC-3 cells were then injected subcutaneously into both flanks of the animals before the start of treatment four days later (Fig. 6I). Follow-up of tumor volume showed that tumor growth was markedly delayed in the drug-treated group compared to the vehicle-treated group (Fig. 6J). On day 24, tumors were excised (Fig. 6K) and weighed (Fig. 6L). Both the volume (- 62%) and the weight (-59%) of tumors were drastically reduced in the drug-treated group.

Together, these data demonstrate that pharmacological targeting of FOSL1/AP-1 and PIM kinases using the combination of JQ-1 and CX-6258 inhibitors markedly reduces the growth of androgen-independent MSPC/SCL-like cells in a castration context.

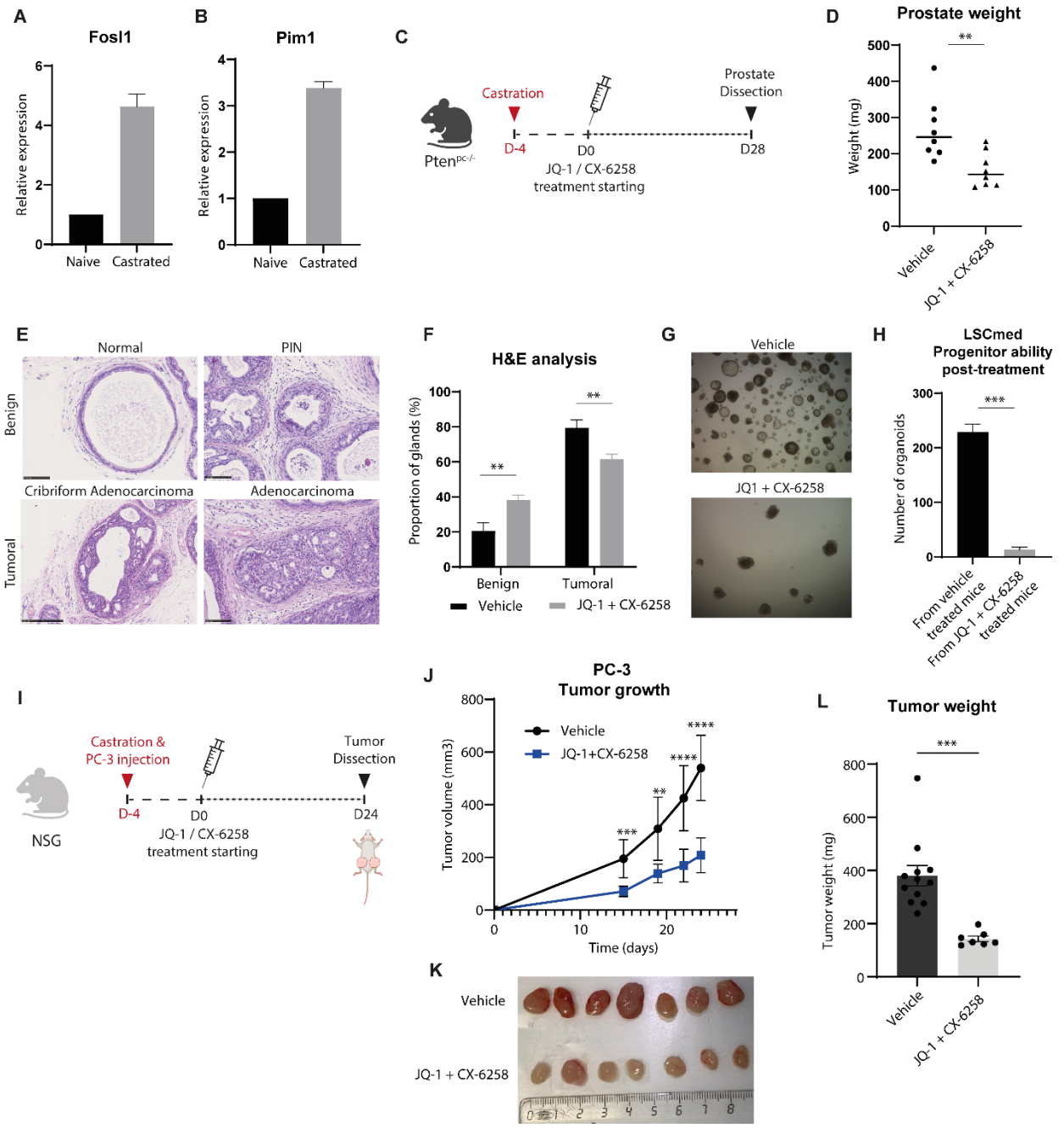

Fig. 6. Combined JQ1 and CX-6258 treatment reduce CRPC growth and aggressiveness *in vivo*. (A,B) The expression of Fosl1 **(A)** and Pim1 **(B)** was measured by RT-qPCR in bulk LSC^{med} cells sorted from intact *Pten*$^{pc-/-}$ mice versus 21 days after castration (n= 2 mice per condition). (**C**). Timeline representation of the experimental protocol used for *Pten*$^{pc-/-}$ mouse castration and pharmacological treatments. Castrated mice received vehicle (n=8) or daily JQ-1 intraperitoneal injection and bi-weekly CX-6258 oral administration (n=8) during 28 days. (**D**) After 28 days of treatment, prostates were micro-dissected and weighed. **(E)** Prostate histological structures were

classified as benign (normal glands, PINs) or tumoral (cribriform, adenocarcinoma) as described in the Materials and Methods (scale bar = 100 µm). **(F)** Quantification of the effects of pharmacological treatments on prostate histopathology. **(G,H)** After 28 days of treatment, LSC^{med} cells were sorted from the prostate of vehicle- or drug-treated animals and cultured in organoid medium in the absence of pharmacological inhibitors. Image were taken (**G**) and the number of organoids was measured (**H**) after 10 days of culture (n=3 experiments). **I**. Timeline representation of the experimental protocol used for PC-3 cell xenograft assay and pharmacological treatments. 1.10^6 PC-3 cells were injected in castrated immunodeficient NSG mice. Mice received vehicle (n=12) or daily JQ1 intraperitoneal injection and bi-weekly CX-6258 oral administration (n=7) during 24 days. **(J, K)** During the protocol, tumor size was measured manually (**J**) and after 24 days of treatment, tumors were micro-dissected (**K**) and weighed (**L**). Statistical analyses between vehicle- and drug-injected groups were performed by using two-tailed *t* test or Mann-Whitney test according to the distribution of data. Panels C and I were created in BioRender. BAURES, M. (2024) BioRender.com/p76n322

Discussion

A critical knowledge gap in prostate cancer research is to understand whether castration-tolerant progenitor-like cells that reside in treatment-naive tumors play a direct role in castration resistance, making them relevant therapeutic targets. This study uncovers the unexpected finding that pre-existing Club-like cells actually respond to castration by a transcriptional switch that increases their aggressiveness. This transcriptional plasticity is mainly orchestrated by Fosl1/AP-1 complex. This finding opens new therapeutic avenues to eradicate treatment-resistant PCa cells early in the course of the disease for suppressing progression towards CRPC. Supporting this promising therapeutic approach, combined pharmacological targeting of Fosl1/AP-1 and PIM kinases markedly reduced the *in vivo* tumor growth capacities of preclinical MSPC models, which was correlated to the suppression of their progenitor properties *in vitro*.

The concept of pre-existing CRPC cells in treatment-naïve tumors was pioneered by the group of Risbridger using patient-derived xenografts of treatment-naïve early-stage tumors (Toivanen *et al*, 2013). These authors showed that, four weeks after mouse castration, residual tumor foci contained stem-like tumor cells able to regenerate proliferating tumors upon androgen replenishment. However, the co-expression of stem-like (NANOG, ALDH1, CD44) and differentiated (NKX3.1) cell markers cannot rule out that these CRPC cells actually arose from castration-induced luminal cell plasticity, a mechanism of castration resistance recently documented in various mouse models (Chan *et al.*, 2022; Karthaus *et al*, 2020; Kirk *et al.*, 2024). More recently, the existence of stem-like/EMT-enriched CRPC cells in treatment-naïve prostate tumors has been revealed by scRNAseq approaches. These cells have been correlated with biochemical recurrence (rising PSA levels) and

distant metastasis (Cheng *et al*, 2022). Other authors (Chen *et al.*, 2021) identified one basal/intermediate epithelial cell population (referred to as cluster 10) that we subsequently showed to be enriched in typical Club/Hillock cell markers (Baures *et al.*, 2022b). This cluster was identified in each individual patient, albeit at highly variable ratio (1% to 25% of the epithelial cell pool). Song and colleagues classified PCa-associated Club-like cells into six transcriptomic states, the largest of which exhibited the highest luminal and AR signaling characteristics (Song *et al.*, 2022). This is also what we observed for the *Pten*$^{pc-/-}$ mouse LSC^{med}-0 subpopulation predominant before castration. All these scRNAseq studies corroborate the report by Han and colleagues who showed on large cohorts that the MSPC subtype, enriched in Club cell features, is present *de novo* in a significant proportion of treatment-naïve PCa (Han *et al.*, 2022).

According to the molecular similarity of mouse LSC^{med} and human Club/Hillock cells, we established that *Pten*$^{pc-/-}$ mouse LSC^{med} cells are a robust surrogate of human MSPC/SCL subtype. Based on this finding, the *Pten*$^{pc-/-}$ mouse model was attractive as it shows a highly consistent enrichment of LSC^{med} cells in tumors prior to castration (Sackmann Sala *et al.*, 2017), mimicking in an amplified way human *de novo* MSPCs. LSC^{med}-like cells have been classically considered as an end-stage of androgen-independent cells. Our study shows that this is not the case as naïve *Pten*$^{pc-/-}$ LSC^{med} cells, despite of their intrinsically low androgen-signaling, are sensitive to castration and respond to androgen deprivation by a transcriptional switch. Before castration, *Pten*$^{pc-/-}$ LSC^{med} cells mainly exhibit the LSC^{med}-0 profile enriched in developmental signaling pathways, as previously observed for wild-type LSC^{med}-like luminal progenitor cells that participate in the early steps of prostate morphogenesis (Baures *et al.*, 2022a; Mevel *et al.*, 2020). In contrast, post-castration *Pten*$^{pc-/-}$ LSC^{med} cells mainly exhibited the LSC^{med}-1 profile, which is comparatively enriched in stemness and basal features. This profile, which is typical of the human MSPC subtype resistant to ARPIs (Han *et al.*, 2022), further increases the transcriptomic similarity between post-castration LSC^{med} cells and the MSPC/SCL subtypes. The acquisition of an intermediate basal/luminal phenotype by cell plasticity has also been reported in triple-negative breast cancer and it was shown to promote resistance to chemotherapy (Marsolier *et al*, 2022). In the prostate context, the acquisition of a basal/stem cell signature identifies aggressive PCa phenotypes (Smith *et al*, 2015). This signature was also enriched in hormone-sensitive metastases compared to organ-confined prostate adenocarcinomas, suggesting that the enrichment of basal features parallels PCa progression. Therefore, the acquisition of basal features by LSC^{med}-like cells post-castration could be pivotal for their aggressiveness and the emergence of MSPC-CRPC tumors in response to treatment.

We identified Fosl1/AP-1 as a major driver of LSC^{med} cell plasticity in response to castration. The AP-1 complex, and in particular Fosl1, are involved in the development of many adenocarcinomas (Casalino *et al.*, 2022). Acting as a transcription factor, facilitator of chromatin accessibility/opening and/or super enhancer (Bi *et al*, 2020; Dong *et al*, 2021; Kadur Lakshminarasimha Murthy *et al*, 2022), Fosl1 regulates various tumor-associated processes e.g. cell proliferation (Zanconato *et al*, 2015), EMT (Feldker *et al.*, 2020; Marques *et al.*, 2021), invasion and metastases (Iskit *et al*, 2015), and stemness (Marques *et al.*, 2021). Via its association with partners such as YAP and TAZ (Feldker *et al.*, 2020; Zanconato *et al.*, 2015), Fosl1 also promotes cell plasticity towards increased stemness states (Marques *et al.*, 2021) and resistance to treatment (Bi *et al.*, 2020). Fosl1 has been recently shown to be involved in the reprogramming of mouse prostatic luminal cells towards a castration-tolerant progenitor-like state following androgen depletion (Tang *et al.*, 2022). We here identify Fosl1 as a master regulator of LSC^{med}-1 cells, i.e. the predominant LSC^{med} population after castration. The consistent expression levels of Fosl1 and of various members of the AP-1 complex in both LSC^{med}-1 and LSC^{med}-2 cells strengthens the key role of this transcriptional complex in the molecular switch of pre-existing LSC^{med} cells in response to castration. In humans, *FOSL1* has been identified as the top key transcription factor in CRPC-SCL (Tang *et al.*, 2022). It is positively associated to the chromatin remodeling that promotes CRPC-SCL subtype in castrated context by enhancing CRPC-SCL-associated enhancer accessibility, in cooperation with the YAP-TAZ complex (Tang *et al.*, 2022). As observed for post-castration LSC^{med} cells, human CRPC-SCL is enriched in various AP-1 family members. Together, these data support the key role of this transcriptional complex in the adaptation of treatment-naïve PCa luminal cells – both differentiated (Tang *et al.*, 2022) and progenitor-like (this study) – to androgen deprivation.

Based on our findings, we reasoned that inhibition Fosl1/AP-1 pathway should suppress the castration-driven mechanisms of resistance of LSC^{med} progenitor cells promoting CRPC. This therapeutic strategy was strengthened by coupling pharmacological inhibition of PIM kinase, previously shown to promote cell stemness (Gao *et al*, 2019; Jimenez-Garcia *et al*, 2016) and to affect PCa growth (Hu *et al.*, 2009; Rebello *et al.*, 2016). The combination of castration and JQ-1/CX-6258 treatment of *Pten*$^{pc-/-}$ mice markedly reduced CRPC growth and histopathology after only 28 days compared to castration alone. Similar results were obtained with xenografted MSPC-like PC-3 cells. One remarkable effect of this pharmacological treatment was to abrogate the progenitor properties of *Pten*$^{pc-/-}$ LSC^{med} cells as determined from their organoid/tumorsphere-

forming capacity. This effect was also observed with LSC^{med} cells harvested from JQ-1/CX-6258-treated *Pten*$^{pc-/-}$ mice, without adding drugs in the organoid assay (Fig. 7H). Although LSC^{med} cells are the mouse equivalent of human Club/Hillock cells (Baures *et al.*, 2022a; Baures *et al.*, 2022b), the progenitor-like properties of the latter have never been experimentally assessed. We here identified the HPV-10 cell line as the best surrogate of tumoral Club/Hillock cells, and we show that these cells are able to form tumorspheres *in vitro,* confirming their *in silico*-predicted progenitor-like capacities (Henry *et al.*, 2018). As observed with *Pten*$^{pc-/-}$ LSC^{med} cells, combined JQ-1/CX-6258 treatment completely abrogated the tumorsphere-forming capacity of HPV-10 cells while their viability was mildly affected. Taken together, the results of our *in vitro* and *in vivo* assays suggest that combined pharmacological inhibition of the Fosl1/AP-1 complex and Pim1 abrogates the progenitor capacities of mouse and human Club-like cells. Although it is at present unknown whether the drugs suppress the progenitor properties of Club-like cells or eradicate the progenitor cells *per se,* our data establish a correlation between *in situ* tumor growth and *in vitro* progenitor capacity.

Neuroendocrine differentiation (NED) is a mechanism of late-stage therapeutic resistance, increasingly observed since the generalization of ARPI treatment of advanced prostate cancer (Yamada & Beltran, 2021). Several observations suggest that LSC^{med}/Club/Hillock cells may be predisposed to NED under therapeutic pressure. *Wfdc2* and *Sox2* are part of the LSC^{med} cell signature (Baures *et al.*, 2022a); both genes are positively associated with neuroendocrine signature in human PCa (Dong *et al*, 2020), and *Sox2* is essential for NED in mice (Kwon *et al*, 2021). Similarly, human Club/Hillock cells are enriched in neuronal stem programs (Yan *et al*, 2022). In *Pten*$^{pc-/-}$ mice, we show that LSC^{med}-2 cells, which are moderately amplified after castration, are characterized by the expression of *Onecut2*. This transcription factor is a major inducer of early NED and biochemical recurrence via the inhibition of AR and FoxA1 transcriptional programs (Rotinen *et al.*, 2018). Still, LSC^{med}-2 cells showed neither enrichment in NEPC signature nor expression of late markers of neuroendocrine cells. This suggests that further genetic stress, e.g. loss of *Rb1* and/or *Tp53,* two key negative regulators of the neuroendocrine signature (Qian *et al*, 2022), may be required to drive LSC^{med}-2 cell NED. Of interest, Onecut2 was recently shown to be a broadly acting lineage plasticity facilitator as it also supports the appearance of treatment-resistant adenocarcinoma through multiple mechanisms (Qian *et al.*, 2024). In this context, strategies targeting progenitor cells presenting with the LSC^{med}-2 profile may also be relevant to prevent progression towards aggressive forms of CRPC. The consistent expression of AP-1 family members and Pim kinases in LSC^{med}-2 cells suggests that our targeting strategy using JQ-1/CX6258,

primarily designed to target LSC^{med}-1 cells, may also counteract LSC^{med}-2 cell amplification post-castration. Alternatively, the recently reported small molecule inhibitor of ONECUT2 (Qian *et al.*, 2024) should also be considered.

In conclusion, our study uncovers the critical role of transcriptional reprogramming in pre-existing Club-like cells as a novel mechanism driving castration resistance. We demonstrate that Fosl1/AP-1 functions as a key regulator of this molecular transformation, resulting in the emergence of aggressive progenitor cells enriched in basal characteristics. Furthermore, our findings align with recent research indicating that the shift from androgen-dependent luminal cells to androgen-independent progenitor-like cells during castration is similarly mediated by this transcriptional complex. This establishes Fosl1/AP-1 as a pivotal factor in the mechanisms underlying resistance to anti-androgen therapy. Therefore, our dual therapeutic approach targeting both Fosl1/AP-1 and PIM kinases presents a promising strategy to counteract these early mechanisms of castration resistance and disrupt the progression towards CRPC.

Methods

Animals

Mouse colonies were housed in controlled conditions, on a 12/12-h light/dark cycle with normal food and water provided ad libitum. Pten$^{pc-/-}$ male mice were generated by breeding *Pten*$^{loxP/loxP}$ female mice (RRID: MGI:5292551) with Pb-Cre4 (RRID: IMSR_NCIMR:01XF5)/Pten$^{loxP/loxP}$ transgenic males and maintained on a C57BL/6 and Sv/129 mixed genetic background as described previously (Baures *et al.*, 2022b; Sackmann Sala *et al.*, 2017). Experiments were performed using 7- to 11-month-old mice, i.e. when aggressive malignant phenotypes were well established. As indicated, mice were surgically castrated and prostate were analyzed between 5 days and 2 months following castration. Prostate samples were obtained by microdissection immediately after sacrifice by cervical dislocation. Under a dissection microscope, adipose tissues were removed from the urogenital tract. The bladder, the ampullary gland and the urethra were removed to isolate the four prostate lobes. All animal procedures have been extensively described in previous publications (Baures *et al.*, 2022b; Sackmann Sala *et al.*, 2017).

JQ-1 and CX-6258 treatments

JQ-1 (Cat# HY-13030) and CX6258 (Cat#HY-18095) from MedChemExpress (RRID:SCR_025062). For *in vivo* treatments, 7-month-old $Pten^{pc-/-}$ mice were castrated, then after 4-day recovery, they were randomized in two groups (n= 8 mice each) to receive either vehicle or a combination of JQ-1 (daily intraperitoneal injection, 50mg/kg solubilized in 5% DMSO, 40% PEG300, 5% Tween80 and 50% H_2O) and CX-6258 (biweekly oral administration, 100mg/kg solubilized in 15% Cremophor and PBS), for 4 weeks.

For human PCa cell xenograft experiments, we used 12-week-old NSG male mice (NOD.Cg-$Prkdc^{scid}$ $Il2rg^{tm1Wjl}$/SzJ; RRID: SCR_003792) purchased from Charles River Laboratories International Inc.. Animals were castrated and injected subcutaneously with 1.10^6 PC-3 (ATCC, cat# CRL-1435, RRID: CVCL_0035) in Matrigel on both flanks to fulfil the 3R rule. Four days later, mice were randomized in two groups of 6 mice each (n=12 tumors) to receive vehicle or combined CX-6258 and JQ-1 treatment as described above. One tumor in the treated group initially grew abnormally fast (outlier) and was therefore not included in the analysis. From day 15 of treatment, tumors were measured twice a week using a digital caliper. Tumor size was calculated using the formula (a × b2)/2, where a = length and b = width of the tumor. Two mice in the treated group experienced issues with the gavage procedure that prevented continuation of oral treatment for ethical reasons. After euthanasia, tumors were dissected, weighed and photographed. Tumors were then embedded in paraffin for further analysis. Investigators who performed mouse analyses were blinded to mouse treatments (vehicle *versus* drugs).

Breeding and maintenance of mice were carried out in the accredited animal facility of the Necker campus in compliance with French and European Union regulations on the use of laboratory animals for research. Animal experiments were approved by the local ethical committee for animal experimentation (APAFIS authorizations #40276 and #49662).

Prostate dissociation

Prostates were minced using razor blades and digested in a solution of Dulbecco's modified eagle medium (DMEM) (Thermo Fisher Scientific) containing 10% FBS (Eurobio), 1% Pen/Strep (Thermo Fisher Scientific) and 1 mg/mL Collagenase Type I solution (Thermo Fisher Scientific) for 1h at 37°C, followed by 5-minute incubation at 37°C in 0.25% Trypsin (Thermo Fisher Scientific). The digestion was stopped with a solution of DMEM containing DNase I (Roche). Cells were passed 10 times through a 20G syringe then filtered through a 40 µm cell strainer to generate

a single-cell suspension. Cells were subjected to differential centrifugation using Histopaque-1119 (Sigma-Aldrich) to reduce the overall level of secretion in the sample. An aliquot of cells was stained with Trypan blue (Thermo Fisher Scientific) and counted using a hemocytometer to assess the cell viability.

FACS-Sorting of LSC^{med} Cells

The procedure for cell sorting was performed as previously described (Lukacs *et al*, 2010; Sackmann-Sala *et al*, 2014; Sackmann Sala *et al.*, 2017). Isolated prostatic cells were stained for FACS on ice for 30 minutes. Antibody references are listed in Table S6. Dead cells were stained using SYTOX Blue (Life Technologies). Cell sorting was performed on a BD FACS Aria III (BD Biosciences) in DMEM containing 2% FBS and 1% penicillin/streptomycin (Pen/Strep). Lineage antibodies (CD31, CD45, TER-119) were used to deplete hematopoietic, endothelial and immune cells and EpCAM antibody to distinguish epithelial from stromal cells. CD49f and SCA-1 markers were used to select LSC^{med} cells from the other epithelial cell types. Sorted cells were collected in DMEM medium supplemented with 50% FBS and 1% Pen/Strep.

Single-cell RNA-Sequencing and Analyses

Sorted LSC^{med} cancer cells were dispensed (BD FACS Aria III) in 96 well plates (VWR, DNase, RNase free) containing 2 µL of lysis buffer (0.2% Triton X-100, 4U of RNase inhibitor, Takara) per well. Plates were properly sealed and spun down at 2,000 g for 1 min before storing at −80°C. Whole transcriptome amplification was performed with a modified SMART-seq2 protocol as described previously (Picelli *et al*, 2014) using 23 instead of 18 cycles of cDNA amplification. PCR purification was realized with a 0.8:1 ratio (ampureXP beads:DNA). Amplified cDNA quality was monitored with a high sensitivity DNA chip (Agilent) using the Bioanalyzer (Agilent). Sequencing libraries were performed using the Nextera XT kit (Illumina) as described previously (Picelli *et al.*, 2014) using 1/4th of the recommended reagent volumes and 1/5th of input DNA with a tagmentation time of 9 min. Library quality was monitored with a high-sensitivity DNA chip (Agilent) using the Bioanalyzer (Agilent). Indexing was performed with the Nextera XT index Kit V2 (A-D). Up to 4 × 96 single cells were pooled per sequencing lane. Samples were sequenced on the Illumina NextSeq 500 platform using 75bp single-end reads.

Single-Cell Transcriptome Analysis

The merged gene expression matrix (raw counts) containing all samples was analyzed with the Seurat3 package in R (RRID:SCR_016341). Cells were filtered by nFeature_RNA (genes detected) >3000 and <7000, percent.mt (percentage of mitochondria genes) < 10. This resulted in n=306 valid cells for downstream analysis. After filtering cells, log-normalization was performed using the default NormalizeData function in Seurat. The scaled data was regressed for cell cycle phases (S.Score and G2M.Score) and number of genes per cell (nFeature_RNA) before performing UMAP based dimension reduction (dims=1:20, resolution =0.4).

StemChecker Analysis

Seurat cluster (0,1,2) characteristic murine genes (adj.pval>0.05) were analyzed for “stemness” enrichment using the stemchecker tool (RRID:SCR_025014), while masking cell cycle genes.

Pseudotime-Ordering Analysis

The raw count matrix (n=306 cells) was used as input for Monocle analysis (2.14.0) (RRID:SCR_016339) and normalized by the M3Drop function. Dimension reduction was based on the variable genes (n=1483) (max_components = 2, method = 'DDRTree'). Ordering of the cell was performed by the f orderCells (HSMM_myo, num_paths = 2, reverse = T) function and the resulting trajectory was colored by pseudotime, cell type and Seurat cluster information. Differential gene expression analysis between groups through a likelihood ratio test for comparing a full generalized linear model with additional effects to a reduced generalized linear model based on negative binomial distributions.

Differentiation Trajectory Analysis Using Velocyto and scVelo

To infer the directionality of differentiation, we employed the Velocyto pipeline (RRID:SCR_018167) (La Manno *et al*, 2018). Utilizing the *.bam files and cell metadata, we computed the spliced and unspliced RNA matrices. Differentiation trajectories were visualized using UMAP embeddings, which were generated with Seurat in R (RRID:SCR_016341). The trajectories, represented by arrows, were plotted using ScVelo (Bergen *et al*, 2020) in Python (RRID:SCR_018168).

Gene set enrichment analyses

Gene enrichment analyses were conducted for each Seurat cluster by interrogating the C2 Chemical and Genetic Perturbations category of MsigDB via the hypeR (2.2.0) package and the GeneSetAnalysis function from the SeuratExtend package.

We employed the Hallmark 50 gene set, a collection of well-defined, biologically relevant gene sets from the Molecular Signatures Database (MSigDB, https://www.gsea-msigdb.org/gsea/msigdb/human/genesets.jsp?collection=H). This analysis was performed to identify significantly enriched pathways across the LSC^{med}-0, LSC^{med}-1, and LSC^{med}-2 clusters. The results were visualized using a heatmap, with color intensity representing z-score normalized enrichment scores.

SCENIC analysis

To investigate regulon networks, we employed a comprehensive approach combining computational analysis and visualization techniques. We utilized the SCENIC algorithm (RRID:SCR_017247) to infer gene regulatory networks and regulon activities. The analysis pipeline was implemented using the SeuratExtend package (RRID:SCR_016341) (Hua *et al*, 2024) for data processing and visualization. We generated waterfall plots to highlight differential TF regulon activities between selected clusters and other cells, with the top 10 TFs labeled for clarity. Gene regulatory networks were visualized in Cytoscape (RRID: SCR_003032), with nodes representing genes (round nodes) and TFs (square nodes). Node colors were mapped to relative gene expression or regulon activity using z-score normalization, enabling comparison of expression and activity patterns among LSCmed-0, LSCmed-1, and LSCmed-2 clusters. To manage the visualization of large regulons, we capped the number of target genes per transcription factor (TF) at 200, prioritizing the most variable genes based on their ranking from Seurat's FindVariableFeatures function. This approach ensured a balance between capturing key regulatory relationships and maintaining analytical tractability.

Cell Lines and Treatments

HPV-10 (ATCC, Cat# CRL-2220, RRID: CVCL_3495), PC-3 (ATCC, cat# CRL-1435, RRID: CVCL_0035) and LNCaP (ATCC, cat# CRL-1740; RRID: CVCL_0395) PCa cell lines were purchased from ATCC (Manassas, USA) at the beginning of this study. Frozen stocks were generated between passages 2 to 5 of initial cell cultures, then aliquots were thawed for each

experiment and used before passage 20. Therefore, no cell authentication was performed. None of the cells used in this study are listed in the ICLAC database of commonly misidentified lines.

Cells were grown at 37°C and 5% CO_2. HPV-10 cells were cultured in Keratinocyte Serum Free Medium (K-SFM) (Thermo Fisher Scientific, Cat# 17005-034) supplemented with 0.05mg/mL Bovine Pituitary Extract (BPE) (Thermo Fisher Scientific, Cat# 13028-014), 5ng/mL EGF (PeproTech, Cat# AF-100-15-500UG) and 1% Pen/Strep. PC-3 and LNCaP cells were cultured in DMEM (Thermo Fisher Scientific, Cat# 31966-021) supplemented with 10% FBS (Eurobio Scientific, Cat# CVFSVF00-01, #lot S80515) and 1% Pen/Strep. Medium was changed every other day.

HPV-10 and PC-3 cells were plated in 6-well plates at 50,000 and 30,000 cells/well, respectively. After 3 days, cells were treated with DMSO, JQ-1 and CX-6258, alone or combined, and analyzed after 72h, according to the protocols described below.

For cell number analysis, wells were rinsed in PBS and adherent cells were trypsinized before being manually counted using a Malassez counting cell. For cell viability analysis, culture medium (containing floating cells) and adherent cells (trypsinized) were collected, centrifuged (300g) and resuspended in HBSS before being counted with trypan blue as above. Cell viability was quantified as the ratio of unstained *versus* total (unstained + stained) cells.

Reverse Transcription-Quantitative PCR (RT-qPCR)

RNA extractions from *Pten*$^{pc-/-}$ LSC^{med} cells and human PCa HPV-10 cells were performed with the Nucleospin RNA XS and Nucleospin RNA (Macherey-Nagel), respectively, following the manufacturer's protocols. Reverse transcription was carried out using the SuperScript™ VILO™ cDNA Synthesis Kit (Invitrogen) for murine cells and the GoScript™ Reverse Transcriptase (Promega) for the human cell line, according to the manufacturer's instructions.

For qPCR, iTaq Universal SYBR Green Supermix (Promega) was used, and reactions were run on a qTower 2.0 real-time thermal cycler (Analytik Jena). Expression data were normalized to Cyclophilin A for murine samples and Actin B for human samples.

Primer sequences (Sigma-Aldrich) are listed in Table S7.

Organoid and tumorsphere culture and treatment

We used the reference protocol for prostate organoid culture described by Clevers' lab (Drost *et al*, 2016) with modifications. LSC^{med} cells sorted from *Pten*$^{pc-/-}$ mice and human PCa HPV-10 cells were plated in triplicate on a Low Growth Factor-containing Matrigel (Corning) layer in a 96-well plate (Sarstedt) at concentrations of 3,000 or 1,000 cells/well, respectively, and were cultured at 37°C and 5% CO_2. After one day of incubation, the culture medium was removed and the cells were covered by a new layer of Matrigel to generate 3D cell structures. The organoid-forming capacity did not differ from the efficacy obtained using 3D droplet culture earlier described (Drost *et al.*, 2016). Drugs were added at this stage and the medium (+ drugs) was changed every other day. After 10 days of culture, organoids were fixed in 4% PFA and images were taken with a 4x objective on an M5000 EVOS inverted microscope (Invitrogen) to capture the entire surface of the well. Organoid counting and surface area measurements were performed using Fiji software (RRID: SCR_002285) by manually outlining the organoid surface. Only structures with size superior to 2000μm^2 were considered as organoid. The number and size of organoids in the various experimental conditions were normalized to cognate mean value obtained in the non-treated condition.

Immunohistochemistry (IHC) and Immunofluorescence (IF)

Antibody references are listed in Table S6.

For IHC, all murine prostate samples were fixed in 4% PFA, paraffin wax-embedded, and sections underwent heat-induced antigen retrieval in citrate buffer at pH 6 (95°C, 30 min). IHC was performed as previously described (Baures *et al.*, 2022b). Signal was amplified and detected using the Vector Elite ABC HRP kit with DAB substrate (Vector Laboratories) and nuclei were counterstained with hematoxylin. Slides were scanned with a Nanozoomer 2.0 (Hamamatsu) and analyzed using NDP.view 2 software (Hamamatsu; RRID:SCR_025177). Positive nuclei were quantified in 10 fields per slide using QuPath Software.

For IF, cells were fixed in PFA 4% for 15 minutes at room temperature and permeabilized for 15 minutes in PBS supplemented with 0.2% Tween-20. Primary antibodies (Table S6) were incubated overnight at 4°C and secondary antibodies were incubated during 1h at room temperature. Nuclei were stained with Hoechst dye. Samples were analyzed using a 40× objective under an Apotome 2 microscope (Zeiss).

Terminal Deoxynucleotidyl Transferase dUTP Nick End (TUNEL) Labeling

The TUNEL assay was performed with the In Situ Cell Death Detection Kit (Roche, Cat. #11 684 809 910) according to the manufacturer's instructions. Briefly, samples underwent dewaxation and rehydratation. Slides were heated in Citrate Buffer during 5 minutes at 350W and then incubated for 1h at 37°C, with the TUNEL reaction mixture. Fluorescence was observed and images were taken using a 20x objective under an M5000 EVOS inverted microscope.

Histological Classification Post-Treatment

The prostates were immediately microdissected and weighed after *in vivo* treatment. Tissues were fixed in 4% PFA, paraffin wax-embedded, and sections were stained with hematoxylin and eosin. Analysis was performed by using the QuPath Software. Glands and lumen were manually detected and outlined. For each gland, we measured the number of lumens and the proportion of lumen to the total surface of the gland. Based on these parameters, glands were classified in 4 different categories: (1) normal gland (a single lumen representing >70% of the gland), (2) PIN structure (a single lumen representing <70% of the gland), (3) cribriform structure (two or more lumens representing >80% of the gland) and (4) malignant gland (two or more lumens representing <80% of the gland or absence of any lumen). Following this analysis, normal and PIN structures were classified as "benign" (versus "advanced").

Statistical analyses

The data describe biological replicates, and the number of independent experiments is indicated in Figure captions. Comparison between two groups was performed using a two-tailed unpaired Student's t test. Analysis of Variance (one-way ANOVA) tests were used to evaluate differences among three or more groups. Simple or multiple comparisons were performed with Dunnett's or Tukey's post hoc tests, respectively. The statistical tests performed for each biological experiment are indicated in figure captions. One, two, three or four symbols illustrating significance (*) represent p values < 0.05, <0.01, <0.001 and <0.0001, respectively. A value of $p < 0.05$ was used as significance cutoff for all tests. Error bars represent S.E.M. All analyses were performed using GraphPad Prism version 9.00 for Windows (RRID:SCR_002798). Statistical parameters used for transcriptomic data analyses are described in the corresponding Methods section.

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Competing interests

The authors declare that they have no conflict of interest.

The paper explained

Problem

The major challenge in prostate cancer treatment is to overcome resistance to castration therapy that ultimately leads to patient death. The mechanisms that drive the shift of tumors from castration-sensitivity to castration-resistance remain poorly understood. Club cells is a newly-identified progenitor-like cell type predicted to be castration-resistant. While these cells have been identified in prostate tumors prior to castration, their functional contribution to therapy resistance is unexplored.

Result

In this study, we used a mouse model of castration-resistant prostate cancer (prostate-specific deletion of the tumor suppressor gene *Pten*) that is enriched in Club-like cells. We report that the castration tolerance of these cells is not intrinsic, but results from a transcriptional shift that enhances several hallmarks of cancer aggressiveness such as epithelial-to-mesenchymal transition, basal cell features and stem-like programs. We identified the transcription factor Fosl1/AP-1 as a major driver of this process, called cell plasticity. Dual pharmacological targeting of Fosl1 and Pim1, a Fosl1-regulated kinase known to promote prostate cancer, abrogates the *in vitro* progenitor properties of the various mouse and human Club-like models that we used, and this was correlated to a marked reduction of *in vivo* tumor growth after castration.

Impact

Our findings show that targeting stemness and tumor-initiating capacities of Club-like prostatic cells by dual inhibition of Fosl1/AP-1 and PIM kinase is a promising strategy to potentiate androgen deprivation therapy and mitigate prostate cancer relapse.

Author contributions

Conceptualization: VG, FR, JEG, MB

Software: FR, MD, MR

Formal analysis: FR, JEG, MB

Supervision: VG, JEG, JCM, FR

Funding acquisition: VG, DM, JEG

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Investigation: FR, ASVA, AK, EP, FB, MB, MD, MR, YH

Methodology: VG, JCM, DM, GL, FR, ASVA, CD, JEG, FB, MB, MD, MR

Writing-original draft: VG, FR, JEG, MB, CD

Project administration: VG, JEG

Writing-review and editing: VG, JCM, DM, GL, LK, FR, ASVA, AK

Data and materials availability

All data needed to evaluate the conclusions of this study are presented in the main text and the Supplementary Materials. Computational analyses were based on already published pipelines. The detailed code is available upon request. Gene expression data used in this study were deposited in the Gene Expression Omnibus repository (accession number: GSE273079).

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