

1 **The neuroendocrine transition in prostate cancer is dynamic and dependent on**
2 **ASCL1**

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1 **ABSTRACT:**

2 Lineage plasticity is a recognized hallmark of cancer progression that can shape therapy
3 outcomes. The underlying cellular and molecular mechanisms mediating lineage
4 plasticity remain poorly understood. Here, we describe a versatile *in vivo* platform to
5 identify and interrogate the molecular determinants of neuroendocrine lineage
6 transformation at different stages of prostate cancer progression. Adenocarcinomas
7 reliably develop following orthotopic transplantation of primary mouse prostate organoids
8 acutely engineered with human-relevant driver alterations (e.g., *Rb1*^{-/-}; *Trp53*^{-/-}; *cMyc*⁺ or
9 *Pten*^{-/-}; *Trp53*^{-/-}; *cMyc*⁺), but only those with *Rb1* deletion progress to ASCL1+
10 neuroendocrine prostate cancer (NEPC), a highly aggressive, androgen receptor
11 signaling inhibitor (ARSI)-resistant tumor. Importantly, we show this lineage transition
12 requires a native *in vivo* microenvironment not replicated by conventional organoid
13 culture. By integrating multiplexed immunofluorescence, spatial transcriptomics and
14 PrismSpot to identify cell type-specific spatial gene modules, we reveal that ASCL1+ cells
15 arise from KRT8+ luminal epithelial cells that progressively acquire transcriptional
16 heterogeneity, producing large ASCL1⁺;KRT8⁻ NEPC clusters. *Ascl1* loss in established
17 NEPC results in transient tumor regression followed by recurrence; however, *Ascl1*
18 deletion prior to transplantation completely abrogates lineage plasticity, yielding
19 adenocarcinomas with elevated AR expression and marked sensitivity to castration. The
20 dynamic feature of this model reveals the importance of timing of therapies focused on
21 lineage plasticity and offers a platform for identification of additional lineage plasticity
22 drivers.

23

1 INTRODUCTION:

2 Prostate cancer is the leading cause of cancer death globally in men¹. Survival has
3 improved through development of next generation ARSIs; however, patients eventually
4 progress to castration-resistant prostate cancer². Although men receiving ARSIs are living
5 longer, an increasing fraction display features of lineage plasticity at relapse,
6 characterized by reduced or absent expression of luminal lineage markers such as AR
7 and the downstream target gene prostate specific antigen^{3,4}. In its most extreme form,
8 lineage plasticity manifests as a transition to neuroendocrine (NE) histology called NEPC,
9 with expression of synaptophysin (SYP) and chromogranin⁴. NEPC histology is more
10 commonly seen in patients with metastasis to soft tissue (e.g., liver) rather than bone,
11 raising a potential role of the tumor microenvironment (TME) in this transition^{5,6}. Similar
12 lineage transitions are observed in other tumor types treated with targeted therapies, such
13 as *EGFR*-, *ALK*-, and *KRAS*^{G12C}-mutant lung adenocarcinoma, underscoring the broad
14 relevance of lineage plasticity in tumor progression and therapy resistance⁷⁻¹¹.

15
16 The molecular details underlying these lineage transitions are poorly understood, largely
17 owing to a shortage of tractable model systems that accurately and dynamically replicate
18 plasticity-associated transitions observed in patients. Autochthonous models of prostate
19 cancer have contributed substantially to our understanding of prostate tumor progression,
20 but few capture the transition at all stages or are amenable to intervention in a timely and
21 cost-effective manner¹²⁻¹⁷. Conversely, studies using prostate tumor cell line transplant
22 models can be completed more quickly, but the number of models is limited, and they fail
23 to replicate all stages of the lineage transition that occurs in patients. To gain a better

1 understanding of NEPC and to develop intervention strategies that curtail lineage
2 plasticity, model systems that accurately reproduce the molecular and morphologic
3 features of these lineage transitions over time are needed.

4
5 Organoid technology has greatly expanded our ability to model epithelial biology,
6 including prostate cancer initiation and progression^{18,19}. Previously, we described a
7 strategy to assess putative genetic drivers of prostate adenocarcinoma (PRAD), as well
8 as tumor cells of origin using mouse prostate organoids coupled with orthotopic
9 transplantation²⁰ (OT). Here, we optimize this approach into a robust platform that
10 enables rapid, side-by-side assessment of cancer initiation and progression phenotypes
11 using multiple combinations of human-relevant cancer drivers *in vivo*. Using multiplexed
12 spatial techniques, we detect isolated NE cells emerging from luminal epithelial cells,
13 which subsequently evolve to fully penetrant NEPC, together with temporal changes
14 within the TME, and perform functional perturbations that dramatically impact the lineage
15 plasticity program.

16

17 **RESULTS:**

18 Rapid tumor phenotyping across an allelic series of prostate cancer drivers

19 We sought to develop a platform to interrogate prostate cancer drivers rapidly and
20 comprehensively at larger scale compared to traditional genetically-engineered mouse
21 models (GEMMs), focusing particularly on the unmet need to dynamically model the
22 PRAD-to-NEPC transition observed in patients. Using multiplexed editing approaches²⁰
23 and lentiviral oncogene delivery, we established organoids with six relevant combinations

1 of cancer drivers selected based on their enrichment and co-occurrence in human
2 prostate cancer (**Fig. 1a, Extended Data Fig. 1a-c, and Supplementary Table 1**;
3 hereafter: $Pten^{-/-}; Trp53^{-/-}$ = PtP, $Rb1^{-/-}; Trp53^{-/-}$ = RP, $Pten^{-/-}; Rb1^{-/-}$ = PtR, $Pten^{-/-}; Trp53^{-/-}; cMyc^{+}$ = PtPM, $Rb1^{-/-}; Trp53^{-/-}; cMyc^{+}$ = RPM, $Pten^{-/-}; Rb1^{-/-}; cMyc^{+}$ = PtRM). In line with
4 previous work, histological assessment of edited mouse organoids grown in 3D culture
5 conditions revealed a mixture of KRT5+ basal and KRT8+ luminal cells, with both
6 populations staining for nuclear AR (ref. 18; **Extended Data Fig. 1d-e**). All cultured
7 organoids lacked expression of the NE transcription factors achaete-scute family bHLH
8 transcription factor 1 (ASCL1) and Neuronal Differentiation 1 (NEUROD1), critical
9 regulators of the neuronal and NE lineages in mammalian development, despite
10 prolonged *in vitro* culture (**Extended Data Fig. 1d-e**).

12
13 Having generated this allelic series, we next evaluated tumorigenicity following OT (**Fig.**
14 **1a**). Because expansion of organoids grown in 3D culture is labor intensive (requiring
15 serial propagation of single cell suspensions embedded in matrigel), we compared 3D
16 expansion to short term (five day) monolayer expansion as a simpler alternative
17 (**Extended Data Fig. 2a-c**). Although monolayer expansion was fast and yielded highly
18 penetrant tumor growth for most genotypes (PtP, RP, PtPM, RPM), pathologic evaluation
19 revealed a high frequency of sarcomatoid-like histology that is not seen in typical human
20 prostate cancers²¹ (**Extended Data Fig. 2d-g**). In contrast, tumors arising from organoids
21 expanded exclusively in 3D culture consistently and reliably established histologic
22 phenotypes and lineage marker expression that closely mirror the human disease,
23 particularly for the PtPM and RPM genotypes as detailed below (**Extended Data Fig. 2d-**

1 **h)**. Phenotypes of each of the six combinations of genetic drivers, expanded using 3D or
2 monolayer culture, are summarized in **Supplementary Table 2**. Due to the sarcomatoid-
3 like histology seen following monolayer culture, all subsequent experiments were
4 performed using 3D expansion only.

5

6 *Rb1* loss is a critical gatekeeper event for NEPC transformation

7 Based on the rapid, highly penetrant development of PRAD using PtPM and RPM
8 organoids, we comprehensively evaluated disease progression across both models
9 (hereafter called PtPM and RPM mice). In these models, we consistently observed PRAD
10 with moderate to poorly differentiated histology during the first 2-3 weeks post
11 transplantation (**Fig. 1b-c**); however, RPM tumors also contained pockets of small cell-
12 like tumors with “salt-and-pepper” chromatin and a mixture of trabecular or diffuse
13 architecture suggestive of NEPC (**Fig. 1c**). The mitotic index in RPM tumors, particularly
14 in large areas of NEPC that emerged late (8-10 weeks), was greater than PtPM tumors,
15 consistent with the rapid disease progression seen clinically in patients with NEPC
16 transformation (**Fig. 1d**). Despite this difference in proliferation rate, the overall survival
17 of PtPM mice was shorter, likely due to higher tumor engraftment potential of PtPM
18 organoids, since a 250-fold reduction in the number of cells injected results in comparable
19 survival to RPM mice (**Fig. 1e and Extended Data Fig. 2i**).

20

21 Consistent with the moderately differentiated luminal histology, early RPM tumors
22 displayed significantly more KRT8+ cells compared to KRT5+ cells, markers of luminal
23 and basal identity respectively (**Supplementary Fig. 1a**). Moreover, ASCL1 expression

1 was observed as early as 4 weeks post-engraftment, with a significant increase in the
2 proportion of ASCL1+ cells by 8-10 weeks (**Supplementary Fig. 1b**). These late stage
3 NEPC regions also expressed canonical NE markers such as FOXA2, DLL-3, SYP,
4 NCAM-1, and rarely NEUROD1^{4,5,22} (**Fig. 2a-b**). In contrast, tumors in PtPM mice rarely
5 contained ASCL1+ cells and never progressed to NEPC (**Fig. 2a and Supplementary**
6 **Fig. 1c-d**). We therefore conclude that functional *Rb1* loss is a critical gatekeeper event
7 required for NEPC transformation, consistent with preclinical and clinical datasets
8 demonstrating enrichment of *RB1* pathway mutations in small cell lung cancer (SCLC)
9 and NEPC^{22,23}.

10
11 PtPM and RPM mice both developed regional metastases in the draining iliac lymph
12 nodes, but RPM mice also established distant metastases (primarily liver and lung; **Fig.**
13 **1b-c, f**). Metastases in RPM mice mostly retained the same NEPC profile seen in primary
14 tumors, except for rare ASCL1-negative patches that were also negative for SYP, NCAM-
15 1, and NEUROD1 but occasionally positive for vimentin (VIM), a marker of mesenchymal-
16 like cells (**Extended Data Fig. 3a-d**). Whether these ASCL1-negative regions reflect
17 ongoing lineage plasticity after metastasis of ASCL1+ cells, or independent metastatic
18 events prior to NEPC transformation, requires further investigation. Interestingly, lung
19 metastases in RPM mice contained a higher proportion of ASCL1+/KRT8+ (double-
20 positive) cells compared to liver metastases (mostly ASCL1 single-positive), and AR
21 expression was absent in tumor cells at both metastatic sites (**Extended Data Fig. 3e-f**).

22

1 To further benchmark the PtPM and RPM models relative to autochthonous prostate
2 cancer models and human samples, we performed bulk RNA-sequencing of tumors
3 harvested early (PtPM \leq 4 weeks, RPM \leq 6 weeks) and late (PtPM = 5 weeks, RPM = 10
4 weeks). Consistent with the immunohistochemical findings, we observed progressive
5 upregulation of genes involved in neuronal differentiation in RPM compared to PtPM
6 tumors, including *Ascl1*, *Foxa2*, *Sox1*, *Chga*, and *Olig3*, several NOTCH pathway
7 ligands^{4,24} (e.g., *Dll1*, *Dll3*, *Hes5*), as well as downregulation of AR and several AR-target
8 genes (e.g., *Tmprss2*, *Pmepa1*, *Folh1*; **Fig. 2c-d**, **Extended Data Fig. 4a-b**, and
9 **Supplementary Table 3**). Critically, RPM tumors were significantly enriched for
10 transcriptional signatures derived from prostate GEMMs that undergo NEPC
11 transformation and from human NEPC specimens, demonstrating that RPM transplants
12 rapidly establish and recapitulate key molecular features observed in gold-standard
13 preclinical models and clinical samples¹³ (**Fig. 2e**, **Extended Data Fig. 4c**, and
14 **Supplementary Table 4**). Further highlighting the critical role of the *in vivo* TME in
15 initiating NEPC transformation, *Ascl1* transcript levels were ~2000-fold higher in RPM
16 tumors compared to long-term cultured RPM organoids. Moreover, the *in vivo* TME is
17 required for maintenance of the NEPC state as *Ascl1* expression progressively declined
18 in RPM tumor-derived organoids (tumoroids; **Extended Data Fig. 4d**).

19

20 Dynamic tumor microenvironment changes during adenocarcinoma to NEPC transition

21 Because the *in vivo* setting is required to trigger lineage plasticity in the RPM model, we
22 were particularly interested in surveying changes in the TME. Toward that end, we
23 developed a 20-plex immunofluorescence panel to visualize prostate tumor cells (PRAD

1 and NEPC) in the context of adjacent immune populations, vasculature, and stroma (**Fig.**
2 **3a-g, and Supplementary Tables 5 and 6**). We focused our analysis on the later stages
3 of tumor progression within the RPM model to identify changes to the TME within large
4 patches of NEPC histology (**Fig. 3b**). We used GFP expression to define tumor cells,
5 together with co-expression of either KRT8 and AR or ASCL1 to distinguish PRAD from
6 NEPC (**Extended Data Fig. 5a**). We selected co-expression of EGFP+/ASCL1- and
7 EGFP+/ASCL1+ as the principal metric to score PRAD and NEPC (**see Methods**). After
8 mapping these respective regions across multiple tissue sections from RPM tumors
9 containing patches of NEPC differentiation, we then looked for selective changes in cell
10 type composition within the TME.

11
12 Focusing initially on stroma, we noted that mesenchymal cells were abundant in regions
13 of PRAD but depleted in regions of NEPC. We observed a similar trend for LYVE1+
14 lymphatics although this did not reach statistical significance. However, there were no
15 obvious differences in CD31+ endothelial populations which localized primarily to the
16 boundaries of NEPC and PRAD (**Supplementary Fig. 2a-c**).

17
18 We next turned our attention to immune cells and noted striking depletion of CD8+ and
19 FOXP3+;CD4+ regulatory T cells (Treg) as well as F4/80+ macrophages across all NEPC
20 regions, consistent with reports showing similar absence of immune cells within human
21 NE cancers^{23,25,26}. Conversely, FOXP3-;CD4+ T cells were equally distributed within
22 PRAD and NEPC, with a high fraction located at PRAD boundaries, suggestive of
23 differential recruitment and retention of T cell subsets between histologies (**Fig. 3d-e, h-i**

1 **and Extended Data Fig. 5b-d**). Of the CD8⁺ T cells within PRAD regions, the vast
2 majority (~96%) were TCF1-negative, consistent with prior work demonstrating
3 downregulation of TCF1 and upregulation of an effector program in tumor infiltrating
4 compared to draining lymph node resident CD8 T cells²⁷ (**Extended Data Fig. 5e-f**).

5
6 We identified five distinct myeloid populations which we labeled Mac1 (CD11b⁺;F4/80⁻),
7 Mac2 (CD11b^{lo};CD11c⁺;F4/80⁺), Mac3 (CD11b⁺;F4/80⁺), neutrophil
8 (CD11b⁺;Ly6G⁺;S100A9⁺) and DC (CD11c⁺;F4/80⁻; **Extended Data Fig. 6a and**
9 **Supplementary Table 6**). Neutrophil infiltration was low and confined to the outer
10 boundary of PRAD regions (**Fig. 3f and Extended Data Fig. 6a-b**). Mac1 and Mac3
11 populations were largely absent from the NEPC TME; however, Mac2, which harbors
12 similar marker expression as alveolar and wound-healing macrophages was present
13 within NEPC²⁸ (**Fig. 3f-g,j and Extended Data Fig. 6c-e**). We were surprised to also see
14 substantial numbers of CD11c⁺;F4/80⁻ cells within NEPC regions of primary tumors,
15 raising the possibility of dendritic cell infiltration (**Fig. 3f and Extended Data Fig. 6a-b**).

16
17 To determine if the differences in PRAD versus NEPC immune infiltrates in RPM mice
18 are seen in human prostate cancer, we examined a recently published human single cell
19 RNA-sequencing dataset that includes PRAD and NEPC samples¹³. Both histologies had
20 evidence of myeloid infiltration, but NEPC harbored fewer tumor associated macrophages
21 (hereafter abbreviated TAM) relative to PRAD (**Extended Data Fig. 6f; see Methods**).
22 However, CD11c (*ITGAX*) expression was evident across TAM populations within both
23 PRAD and NEPC, and highest in *IL1B*⁺ TAMs (**Extended Data Fig. 6f-h**). We also

1 observed decreased immune infiltration in the NEPC regions of a human prostatectomy
2 specimen from a patient with mixed PRAD/NEPC histology but confirmed the presence
3 of CD11c+;CD68+ macrophage populations within ASCL1+ tumor regions (**Extended**
4 **Data Fig. 6i-j**). Whether these CD11c+ myeloid populations correspond to professional
5 antigen presenting cells remains uncertain and will require further phenotypic (e.g., MHC-
6 II, CD103, BATF3 expression) and functional characterization. Nonetheless, the evidence
7 of early CD8+ T cell infiltration in PRAD and persistence of potential dendritic cells in late
8 stage NEPC in this model suggest that deeper analysis may be informative in addressing
9 the disappointing clinical results to date using conventional immune checkpoint blockade
10 therapy in prostate cancer.

11
12 We next profiled the TME of RPM metastases, a clinically relevant site of NEPC histology
13 (**Fig. 4a-c**). Turning first to RPM lymph node metastases, there was a striking absence of
14 CD45+ cells within ASCL1+ tumor nests, thus highlighting the capacity of NE tumors to
15 promote immune exclusion within lymphocyte-dense microenvironments (**Fig. 4a,d**).
16 Within distant metastases (liver and lung), we also observed exclusion of Treg, CD4+ and
17 CD8+ T cell subsets but retention of IBA-1+ macrophages that co-stain with markers
18 consistent with the Mac1, Mac2, or Mac3 identities seen in the primary tumors, with
19 findings confirmed by neighborhood composition analysis (**Fig. 4b-c, e-i, see Methods**).
20 Taken together, spatial profiling of primary tumors and metastases demonstrates
21 exclusion of nearly all T cell populations within NEPC regions but not PRAD. However,
22 subsets of myeloid cells such as Mac2 and those with DC-like cell surface marker
23 expression (CD11c+ F4/80-) are retained in NEPC. Critically, our syngeneic models are

1 readily suited for studies using model antigens to evaluate strategies to overcome the
2 immunosuppressive prostate tumor microenvironment.

3

4 Origin and progression of neuroendocrine cells within prostate adenocarcinoma

5 In addition to tracking changes in the TME, the dynamic nature of the RPM model allows
6 a careful examination of the earliest stages of NEPC transformation. ASCL1, a marker of
7 emerging NE cells, was first detected at 4-6 weeks with the appearance of
8 EGFP+;KRT8+;ASCL1+ tumor cell clusters (**Fig. 5a, Extended Data Fig. 5a, and**
9 **Supplementary Fig. 1a-b**). By 10 weeks, larger homogeneous clusters of
10 ASCL1+;KRT8- tumor cells with small cell NEPC histology were easily visible. The
11 observation that the earliest detectable ASCL1+ cells also co-express KRT8 suggests
12 that NE cells may arise from KRT8+ luminal cells. Indeed, KRT8+;ASCL1+ cells were 4-
13 to 5-fold more abundant than KRT5+;ASCL1+ cells at intermediate timepoints (6-weeks,
14 $p = 0.025$ two-tailed t -test, **Fig. 5b**). At later time points (8-10 weeks) primary and
15 metastatic tumor cells were mostly AR-negative and ASCL1-positive with heterogenous
16 expression of KRT8 and E-cadherin (**Fig. 3b, Fig. 5b, and Extended Data Fig. 3e-f and**
17 **5a**).

18

19 The appearance of histologically homogeneous, spatially separate clusters of highly
20 proliferative NE cells within weeks of detecting isolated ASCL1+;KRT8+ cells is consistent
21 with a clonal expansion model. To further characterize the level of transcriptomic
22 heterogeneity, we performed spatial transcriptomics (st; 10X Visium) using tissue
23 sections containing both PRAD and NEPC, coupled with single cell nuclear RNA

1 sequencing (snRNA-seq) from 10-week RPM tumors (**Fig. 5c and Supplementary Fig.**
2 **3a**). We observed distinct NE tumor cell clusters from snRNA-seq with variable KRT8
3 expression (**Supplementary Fig. 3a-b**), consistent with the evidence of heterogeneity
4 within NEPC seen by multiplexed immunofluorescence.

5
6 Given the mixture of multiple cell types within individual tissue spots used for spatial
7 transcriptomic sequencing, we applied BayesPrism^{29,30} to deconvolve tumor cell from
8 non-tumor cell transcripts using the snRNA-seq data as the reference (**Fig. 5d**).
9 BayesPrism integrates a single cell genomics reference with spatial transcriptomics data
10 to deconvolve each spot into the cell type fractions present and provide a cell type specific
11 count matrix for each spot, while accounting for differences between Visium and single
12 cell reference. This method has superior performance in deconvolving spatial
13 transcriptomics data using ground truth datasets^{29,30}. Prior to deploying BayesPrism for
14 further downstream analysis, we assessed the robustness of the inferred deconvolution
15 by comparing BayesPrism on technical replicates profiled from adjacent tissues and
16 found strong correspondence of inferred cell type fraction (**Supplementary Fig. 4a-b**).
17 Specifically, the tumor cell type fraction inferred by BayesPrism recapitulates the
18 distribution of NEPC observed by histology (**Fig. 5c**).

19
20 We next investigated the expression of TFs within regions with NEPC histology as well
21 as those containing a high content of NEPC as inferred by BayesPrism. Consistent with
22 its role regulating neuronal expression programs, all NEPC regions showed *Ascl1*
23 expression with minimal *Neurod1* and *Pou2f3* expression (**Supplementary Fig. 5**).

1 Conversely, other TFs previously implicated in NEPC (e.g. *Mycn*, *Onecut2*, *Pou3f2*,
2 *Pou3f4*) and cerebellar development (*Olig3*) were expressed only within subsets of the
3 NEPC regions examined^{16,31–34} (**Supplementary Fig. 5**). The spatial heterogeneity in
4 expression of these selected TFs, as well as similar TF heterogeneity reported in SCLC
5 (a tumor of NE origin)^{35–39}, led us to examine the structure underlying this heterogeneity
6 using Hotspot⁴⁰, which identifies spatially-varying genes. However, the limited resolution
7 of Visium technology makes identification of gene modules specifically associated with a
8 single cell type of interest challenging because direct application of Hotspot would detect
9 co-localization of genes expressed within multiple cell types or between a pair of
10 colocalized cell types, resulting in false positives when studying cell type-specific gene
11 modules. To overcome this, we leveraged a powerful feature of BayesPrism: inference of
12 cell-type specific count matrices, thereby associating each transcript with its respective
13 cell type (**see Methods**). Therefore, as input to Hotspot, we used the deconvolved tumor
14 count matrices, a strategy we have termed "PrismSpot" resulting from a combination of
15 BayesPrism and Hotspot (**Fig. 5d**). Compared to directly applying Hotspot on un-
16 deconvolved Visium data, the spatial auto- and pairwise-correlation computed by
17 PrismSpot showed significantly stronger signal-to-noise ratio for tumor-specific gene
18 modules (**Extended Data Fig. 7a-g and Supplementary Fig. 6a-c**). Application of
19 PrismSpot identified five distinct spatial modules (**Supplementary Table 7**). To ensure
20 robustness of the clustering of gene modules, we selected genes with the highest co-
21 occurrence within each gene module upon iterative subsampling of the Visium data (**see**
22 **Methods**), which narrowed down our gene list to 71 TFs spanning three of the original
23 five modules (**Supplementary Table 7**).

1
2 We label these three final (robust) TF modules defining two NEPC states (stNE-1, stNE-
3 2) and a single PRAD state (stNonNE; **Fig. 5e-f and Supplementary Table 7**). stNE-1,
4 whose leading genes include coordinated expression of *Ascl1* and other TFs implicated
5 in neuronal biology⁴¹ (*Hes6*, *Ascl1*, *Prox1*, *Insm1*), was enriched across all NEPC regions.
6 The stNE-1 regions correspond to those with a high density of *Mycn* and *Olig3* expression
7 (by spatial transcriptomics) and KRT8+;ASCL1+ (double-positive) tumor cells (by
8 multiplexed IF; **Fig. 3b and Supplementary Fig. 5**). stNE-2, defined primarily by *Nfatc2*
9 (a regulator of *Tox* expression within lymphocytes^{42–44}) but also includes the epithelial-to-
10 mesenchymal (EMT) TF *Snai3* was enriched in some but not all NEPC regions (**Fig. 5f**
11 **and Extended Data Fig. 7h**). Of note, *Nfatc2* expression has been linked with an EMT-
12 like state in melanoma⁴⁵. As further validation of these spatially derived signatures, both
13 stNE modules are selectively enriched in the NEPC signature derived from previously
14 reported scRNA-seq data of prostate GEMMs¹³ (**Extended Data Fig. 7i**) as well as
15 human NEPC samples previously characterized using RNAseq⁴⁶ (stNE-1 $p = 1.17E-7$,
16 stNE-2 $p = 5.50E-4$, stNonNE $p = 0.742$, two-sided Wilcoxon test; **Fig. 5g and**
17 **Supplementary Table 8**). Collectively, multiplexed IF and spatial transcriptomics
18 combined with PrismSpot analysis suggest that NE differentiation arises from KRT8+
19 luminal epithelial cells which progressively evolve into spatially distinct ASCL1+
20 subpopulations with heterogeneous expression of other NE-associated TFs in various
21 combinations.

22

23 *Ascl1* is essential for NEPC transformation

1 In addition to its role as a master TF in neural lineage specification^{47,48}, several human
2 SCLC cell lines and at least one human NEPC xenograft model are dependent on ASCL1
3 for proliferation^{39,49,50}. Whether ASCL1 upregulation is required during the transition from
4 PRAD to NEPC progression is unknown. The reliable kinetics of disease progression in
5 the RPM model, coupled with the flexibility to perform multiplexed genome editing, allow
6 us to rapidly address this question through CRISPR editing of the *Ascl1* locus in RPM
7 organoids (hereafter called *Ascl1*^{KO}; **Supplementary Table 9 and Supplementary Fig.**
8 **7a-b**). To assess the requirement of *Ascl1* for NEPC transformation, we compared the
9 growth and histologic features of *Ascl1*^{wt} versus *Ascl1*^{KO} RPM tumors following either OT
10 or subcutaneous (SQ) transplantation (**Fig. 6a-d**). As expected, *Ascl1*^{wt} RPM mice
11 developed PRAD initially that, over 6-10 weeks, progressed to NEPC. Of note, we also
12 observed a reproducible NE-lineage transition, with similar kinetics, following SQ
13 injection, indicating that the *in vivo* signal that triggers lineage plasticity is not restricted
14 to the prostate microenvironment. Multiplexed IF revealed that the TME of these SQ
15 tumors shared many of the features seen in the OT tumors (**Supplementary Fig. 8a-c**).
16 In stark contrast, all *Ascl1*^{KO} RPM tumors (OT and SQ) developed PRAD with moderate
17 to well-differentiated glandular histology, slower growth kinetics than *Ascl1*^{wt} RPM tumors
18 and, importantly, no evidence of NE transformation (**Fig. 6b-f and Extended Data Fig.**
19 **8a-f**). Furthermore, no metastases were detected in *Ascl1*^{KO} RPM mice after OT,
20 compared to 50% incidence in *Ascl1*^{wt} RPM mice, despite comparable end-stage tumor
21 weights at the primary OT site in either intact or castrated hosts (**Fig. 6g and Extended**
22 **Data Fig. 8g**). Thus, *Ascl1* is obligately required for transition to NEPC and for metastasis
23 in the RPM model.

1
2 We and others previously found that perturbations preventing lineage plasticity may
3 restore sensitivity to androgen deprivation therapy in prostate cancer^{13,50}. To address if
4 this is also true in the context of *Ascl1* loss, we compared the tumorigenicity and histologic
5 features of *Ascl1*^{wt} and *Ascl1*^{KO} RPM tumors following OT or SQ injection into intact
6 versus castrated mice. Of note, *Ascl1*^{KO} tumors grew significantly slower in castrated
7 versus intact hosts in both the OT and SQ settings, an intriguing result given that loss of
8 *RB1* and *TP53* are strongly linked to castration-resistance in multiple prostate models and
9 in patients (**Fig. 6b and Extended Data Fig. 8a-f**). To distinguish between effects of
10 castration on tumor engraftment versus tumor maintenance, we initiated chemical
11 castration therapy (degarelix) in established SQ tumors ($\geq 150\text{mm}^3$). Degarelix treatment
12 completely abrogated the growth of *Ascl1*^{KO} RPM tumors and significantly extended
13 survival whereas progression of *Ascl1*^{WT} RPM tumors was only marginally impacted (**Fig.**
14 **6c, Extended Data Fig. 8h-i, and Supplementary Fig. 9**). Interestingly, one castrated
15 mouse injected with *Ascl1*^{KO} RPM organoids developed a tumor with chondrocyte-like
16 histology, reminiscent of a similar phenotype reported in RPM-driven; *Ascl1*^{KO} SCLC
17 mouse models⁴⁹ (**Extended Data Fig. 8j**).

18
19 To investigate why tumors with *Rb1* and *Trp53* loss display increased androgen
20 dependence in the context of *Ascl1* loss, we examined the expression of AR as well as
21 luminal (KRT8) and basal (KRT5) cytokeratins. Consistent with their well-differentiated
22 glandular morphology, RPM-*Ascl1*^{KO} tumors were dominated by AR+;KRT8+ tumor cells
23 (**Fig. 7a and Extended Data Fig. 9a-c**). Furthermore, the intensity of nuclear AR staining

1 was significantly elevated relative to RPM-*Ascl1*^{WT} tumors (**Fig. 7b-d and Extended Data**
2 **Fig. 9d-f**). Notably, degarelix treated RPM SQ tumors harbored an increase in NEPC
3 tumor area as measured by histological evaluation (**see Methods**), with an increase in
4 the fraction of ASCL1+, but decreased AR+ tumor cells relative to vehicle treated RPM
5 SQ tumors (**Extended Data. Fig. 8k**). Taken together, these data suggest that *Ascl1*^{KO}
6 tumors are phenotypically and transcriptionally bottlenecked into a luminal AR-dependent
7 state.

8

9 *Loss of Ascl1 in established NEPC promotes tumor heterogeneity*

10 Given the exquisite dependence on *Ascl1* for the transition to NEPC, we next asked if
11 *Ascl1* is also required for the maintenance of established RPM-NEPC tumors. To address
12 this question, we introduced a doxycycline (Dox) regulatable *Ascl1* cDNA (with a cis-
13 linked *mScarlet* reporter allele) into RPM-*Ascl1*^{KO} organoids and performed OT
14 experiments in mice receiving Dox (**Fig. 8a and Extended Data Fig. 10a**). As expected,
15 mScarlet-positive OT primary tumors developed quickly (within 5 weeks) in mice
16 transplanted with RPM-*Ascl1*^{KO} organoids harboring the Dox-*Ascl1* allele (hereafter
17 *Ascl1*^{ON}) whereas tumors in mice transplanted with RPM-*Ascl1*^{KO} organoids containing
18 the Dox-*mScarlet* allele alone were delayed (*Ctrl*^{ON}) (**Fig. 8b and Extended Data Fig.**
19 **10b-c**). *Ascl1*^{ON} mice also developed metastases whereas *Ctrl*^{ON} mice did not (**Extended**
20 **Data Fig. 10d**), thus fully recapitulating the findings reported earlier (**Fig. 6g**).

21

22 Having established the fidelity of the Dox-*Ascl1* rescue allele, we asked if ASCL1 is
23 required for the sustained growth of *Ascl1*^{ON} tumors in a second cohort of *Ascl1*^{ON} mice

1 and *Ctrl*^{ON} mice that received Dox until tumors were established ($\geq 100 \text{ mm}^3$) followed by
2 withdrawal (hereafter *Ascl1*^{OFF} and *Ctrl*^{OFF}; **Fig. 8a and Extended Data Fig. 10b**).
3 Consistent with prior evidence that *ASCL1*-knockdown delays the growth of human NEPC
4 xenografts, most *Ascl1*^{OFF} tumors regressed within one week of Dox withdrawal but
5 resumed growth within 2-3 weeks. Although short lived, Dox withdrawal resulted in a
6 statistically significant (albeit modest) survival benefit (log-rank Mantel-Cox test, $p <$
7 0.0001 ; **Fig. 8b and Extended Data Fig. 10e-f**).

8
9 To gain insight into the mechanism of relapse after Dox withdrawal, we examined the
10 histologic features and lineage of relapsed *Ascl1*^{OFF} tumors. To avoid the confounding
11 issue of PRAD cells within RPM primary transplants (recall that RPM tumors retain mixed
12 PRAD and NEPC histology), we focused our analysis solely on NEPC cells by isolating a
13 pure population of RPM-NEPC from primary *Ascl1*^{ON} OT tumors then retransplanting
14 these cells SQ into secondary recipients (**Fig. 8c and Supplementary Fig. 10; see**
15 **Methods**). As expected, the SQ transplants mirrored the results seen by OT in that
16 *Ascl1*^{ON} tumors grew rapidly, whereas *Ascl1*^{OFF} tumors had slower growth with a modest
17 but significant extension in survival (log-rank Mantel-Cox test, $p = 0.0007$; **Fig. 8d and**
18 **Extended Data Fig. 10g**). As expected, *Ascl1*^{OFF} tumors lacked nuclear *ASCL1*
19 expression, confirming the fidelity of the Dox^{ON}/Dox^{OFF} platform (**Fig. 8e**). While loss of
20 *ASCL1* expression resulted in the reacquisition of some histologic features of PRAD (with
21 pockets of moderate- to well-differentiated adenocarcinoma harboring KRT8 and AR
22 expression), the predominant histologic phenotypes were high-grade *ASCL1*- NEPC and
23 regions of sarcomatoid-like differentiation (**Fig. 8e-g, Extended Data Fig. 10h, and**

1 **Supplementary Fig. 11a-c).** In contrast to the RPM tumors discussed earlier where we
2 found no evidence of NEUROD1 expression (**Fig. 2b**), we now observed several regions
3 of NEUROD1+ NEPC (**Fig. 8f-g and Extended Data Fig. 10i**). In summary, and in
4 contrast to clear dependency on *Ascl1* for the initiation of NE plasticity, *Ascl1*-dependency
5 in established NEPC tumors is rapidly circumvented, revealing unique pathologies and
6 marker profiles not seen previously in RPM or RPM-*Ascl1*^{KO} tumors. Moreover, these
7 results provide evidence of selective pressure to maintain the NE state through
8 upregulation of NEUROD1 and perhaps other TF programs that remain to be identified.

9

10 **DISCUSSION:**

11 Because lineage plasticity in cancer is a dynamic process that evolves over time, a
12 precise understanding of the underlying molecular events requires a model amenable not
13 only to repetitive interrogation but also rapid perturbation and reconstitution of the full
14 repertoire of cells found within the TME. Through application of organoid techniques,
15 genome engineering and *in vivo* transplantation assays, we have generated a scalable,
16 flexible, and robust platform that captures the evolution from PRAD to NEPC with highly
17 reproducible kinetics in a manner that closely resembles human disease. As with human
18 NEPC, the mouse NEPC transition is accelerated by castration, although it is worth noting
19 that plasticity also occurs in hormonally intact mice. Using this platform, we have identified
20 at least two steps that are required for plasticity to develop. The first is *Rb1* loss which we
21 postulate creates a cell state poised for lineage transformation. This is followed by a
22 second “trigger” derived from the TME that initiates and cooperates with lineage-defining
23 TFs such as *Ascl1* to complete the transition from an epithelial to NE lineage. Detailed

1 characterization of the chromatin state of tumor cells in this model prior to and during the
2 lineage transition, coupled with side-by-side analysis of signaling crosstalk with the TME
3 in *Rb1*^{-/-} versus *Rb1*^{+/+} backgrounds (e.g., RPM versus PtPM) and cross referenced with
4 published *Rb1* chromatin residence data should shed light on the underlying molecular
5 events⁵¹.

6
7 Application of spatial methods to this model allowed us to gain additional insight into the
8 origin of NEPC and its subsequent evolution as well as changes to the local TME. For
9 example, the earliest detectable ASCL1+ cells nearly always co-express KRT8 or are
10 adjacent to KRT8+ epithelial cells. In addition to implicating luminal cells as a likely cell of
11 origin, this may provide an important clue as to the source of the TME trigger. Our spatial
12 analysis also allowed us to track the expansion of ASCL1+ cells following the initial
13 lineage transformation event, where we see further evolution into transcriptionally distinct
14 NE clusters that continue to express ASCL1 but now gain expression of other TFs
15 associated with neural lineage development. This NEPC evolution is also associated with
16 substantial changes in the TME, such as near complete loss of mesenchymal cells and
17 loss of infiltrating CD8+ T cells and CD4+ Tregs.

18
19 In addition to the unique capability of capturing critical aspects of lineage plasticity that
20 are not recapitulated *in vitro*, the platform is well positioned for rapid throughput functional
21 studies. For example, application of multiplexed gene editing at the time of tumor initiation
22 established the critical role of ASCL1 in NE transformation in a matter of months (versus
23 1-2 years required for multigenic crosses using GEMMs). The role of ASCL1 in the

1 development and maintenance of NE cancers has been previously addressed in the
2 context of SCLC but, importantly, that dependency is a consequence of tumor initiation in
3 pre-existing ASCL1+ NE cells^{39,49}. By contrast, ASCL1 is not expressed in prostate
4 cancer except during the epithelial to NE lineage transition. While prior work has shown
5 delayed growth of ASCL1-expressing human xenograft models following *ASCL1*
6 knockdown⁵⁰, the dynamic nature of our platform allowed us to document an essential
7 role of *Ascl1* in initiating the transformation of PRAD to NEPC. Deletion of *Ascl1* prior to
8 histological transformation resulted in homogeneous well-differentiated adenocarcinomas
9 with no evidence of escape to another lineage despite *Rb1/Trp53* loss and *c-Myc*
10 overexpression.

11
12 In addition to evaluating the importance of genes such as *Ascl1* in initiating the lineage
13 plasticity program, the model is also well positioned, through use of a Dox-inducible
14 rescue alleles, to address dependencies on such genes once NEPC is fully established.
15 In contrast to the absolute dependence on *Ascl1* for the NEPC transition, termination of
16 *Ascl1* expression within established NEPC resulted in transient tumor regressions.
17 Notably, the relapsed tumors contained small foci of AR+, KRT8+ PRAD (indicative of
18 some lineage reversion) but are dominated by sarcomatoid features and regions of
19 NEUROD1+ NEPC that rapidly progress. In addition to revealing additional layers of
20 phenotypic plasticity, this result underscores the advantage of early pharmacologic
21 intervention to prevent plasticity rather than intervening after plasticity is fully established.
22 Whether such an approach is clinically feasible with ASCL1 remains to be determined as
23 we are unaware of any drug development efforts that have succeeded in directly targeting

1 ASCL1. However, clinical benefit has been reported using a bi-specific T cell engager
2 targeting the downstream cell surface protein DLL3 in SCLC, and other DLL3-directed
3 radio-ligand and cell-based therapies are also in development^{52–54}.

4
5 The establishment of this model in a fully immunocompetent setting provides an
6 opportunity to address several unresolved topics regarding the immunobiology of prostate
7 cancer. In contrast to cell lines derived from tumors that have escaped immune
8 suppression (and are commonly used to evaluate novel immunotherapies), the immune
9 evasive mechanisms in the current model develop without any pre-transplantation
10 immune-mediated selective pressure. This scenario allows deeper analysis of the earliest
11 steps in immune escape and may shed light on novel strategies to buttress immunity
12 before tumors become depleted of T cells. Indeed, our spatial analysis shows that CD8 T
13 cells are present early in PRAD but absent in NEPC. We hope to unravel these details
14 using prostate tumors expressing model antigens, combined with tetramer-based
15 monitoring of T cell responses and selective depletion of specific myeloid and Treg
16 subpopulations.

17
18 Although the work reported here is exclusively based on prostate cancer models, the
19 platform is, in principle, adaptable to other epithelial lineages in which short-term organoid
20 culture and orthotopic transplantation methods have been developed. One disease that
21 closely approximates the lineage transitions observed in prostate cancer is *EGFR*- or
22 *ALK*-mutant lung adenocarcinoma where epithelial to NE transition is seen as a
23 mechanism of escape from EGFR or ALK inhibition, particularly in patients with co-

1 occurring loss of function mutations in *TP53* and *RB1*, and recently demonstrated in an
2 EGFR-driven GEMM^{7,8,11,55,56}. *KRAS*^{G12C}-mutant lung adenocarcinoma is a second
3 example where transition to squamous histology is a resistance mechanism for RAS
4 inhibitor therapy^{10,57}. Other applications in bladder, pancreas, breast, and gastrointestinal
5 cancer can also be easily envisioned. In closing, we report a robust, scalable platform to
6 study lineage plasticity in a format amenable to deep molecular interrogation and
7 perturbation and identify *Ascl1* as a critical gatekeeper of NE transformation and tumor
8 heterogeneity in prostate cancer.

9

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10

11 **AUTHOR CONTRIBUTIONS:**

12 R.R. and C.L.S. designed the study and wrote the manuscript with comments from all
13 authors. R.R. designed, analyzed, and oversaw all experiments. R.R., P.S., K.E.L., H.K.,
14 and O.G. performed experiments. R.R., P.S., and K.E.L. performed mouse work. T.C.,
15 T.J.G.R, and Y.X. performed computational analysis. T.C. developed PrismSpot,
16 analyzed snRNA-seq, scRNA-seq and spatial transcriptomics data. T.J.G.R. performed
17 bulk RNA-sequencing and immunofluorescence spatial analysis. Y.X. analyzed COMET
18 spatial immunofluorescence datasets. H.Z. and E.D. performed or oversaw orthotopic
19 surgeries, respectively. S.Y. and R.R. optimized Lunaphore COMET multiplexed
20 immunofluorescence. C.M., W.K., and M.V.P. performed 7-plex immunofluorescence.
21 R.R. performed immunohistochemical staining and confocal microscopy. A.G. assessed
22 and cross-validated histopathology and grade of tumors. S.Z., K.Y., and J.C. performed
23 macrophage subset scRNAseq analyses across human prostate tumor samples. N.F.

1 performed tissue embedding and preparation for 10X Visium spatial transcriptomics.
2 W.R.K. was involved in optimization of improved organoid culture methods. K.V.R.
3 oversaw bulk RNA sequencing analysis. P.M.K.W. was involved in optimization of
4 organoid transplantation assays. D.P. oversaw all snRNA-seq, spatial transcriptomics,
5 and multiplexed immunofluorescence analyses. D.P. and C.L.S. oversaw the project.

6

7 **COMPETING INTERESTS:**

8 C.L.S. is on the board of directors of Novartis, is a cofounder of ORIC Pharmaceuticals,
9 and is a co-inventor of the prostate cancer drugs enzalutamide and apalutamide, covered
10 by US patents 7,709,517; 8,183,274; 9,126,941; 8,445,507; 8,802,689; and 9,388,159
11 filed by the University of California. C.L.S. is on the scientific advisory boards for the
12 following biotechnology companies: Beigene, Blueprint Medicines, Column Group,
13 Foghorn, Housey Pharma, Nextech, PMV Pharma and ORIC. D.P. is on the scientific
14 advisory board of Insitro.

15

16 **MAIN FIGURES 1-8**

17 **EXTENDED DATA FIGURES 1-10**

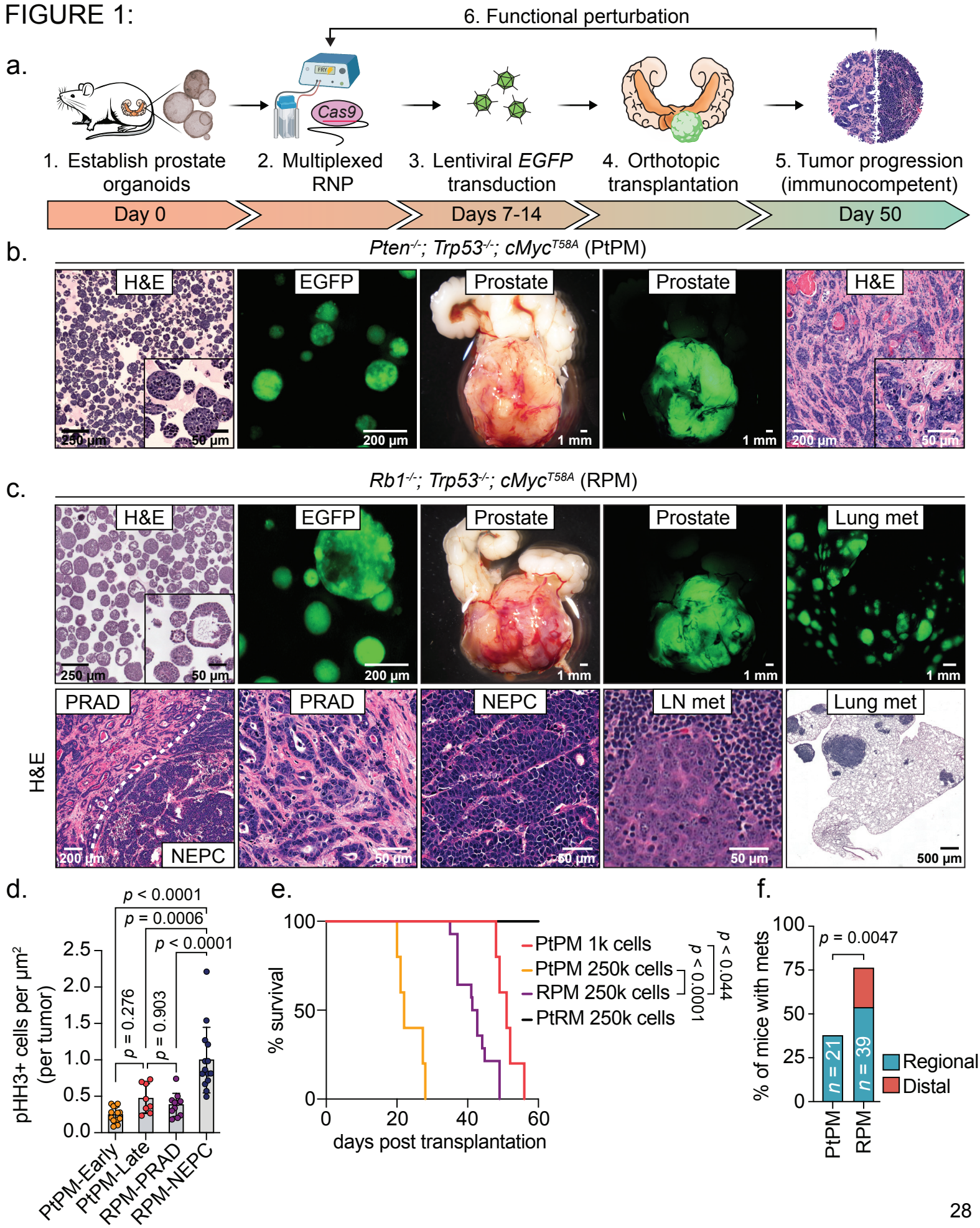
18 **SUPPLEMENTARY FIGURES 1-11**

19 **Supplementary Table 1:** Mutational co-occurrence analyses in prostate cancer patients
20 for all genetic combinations modeled in mouse prostate organoids.

21 **Supplementary Table 2:** Summary table of organoid genotypes and in vivo
22 transplantation efficiency and histology.

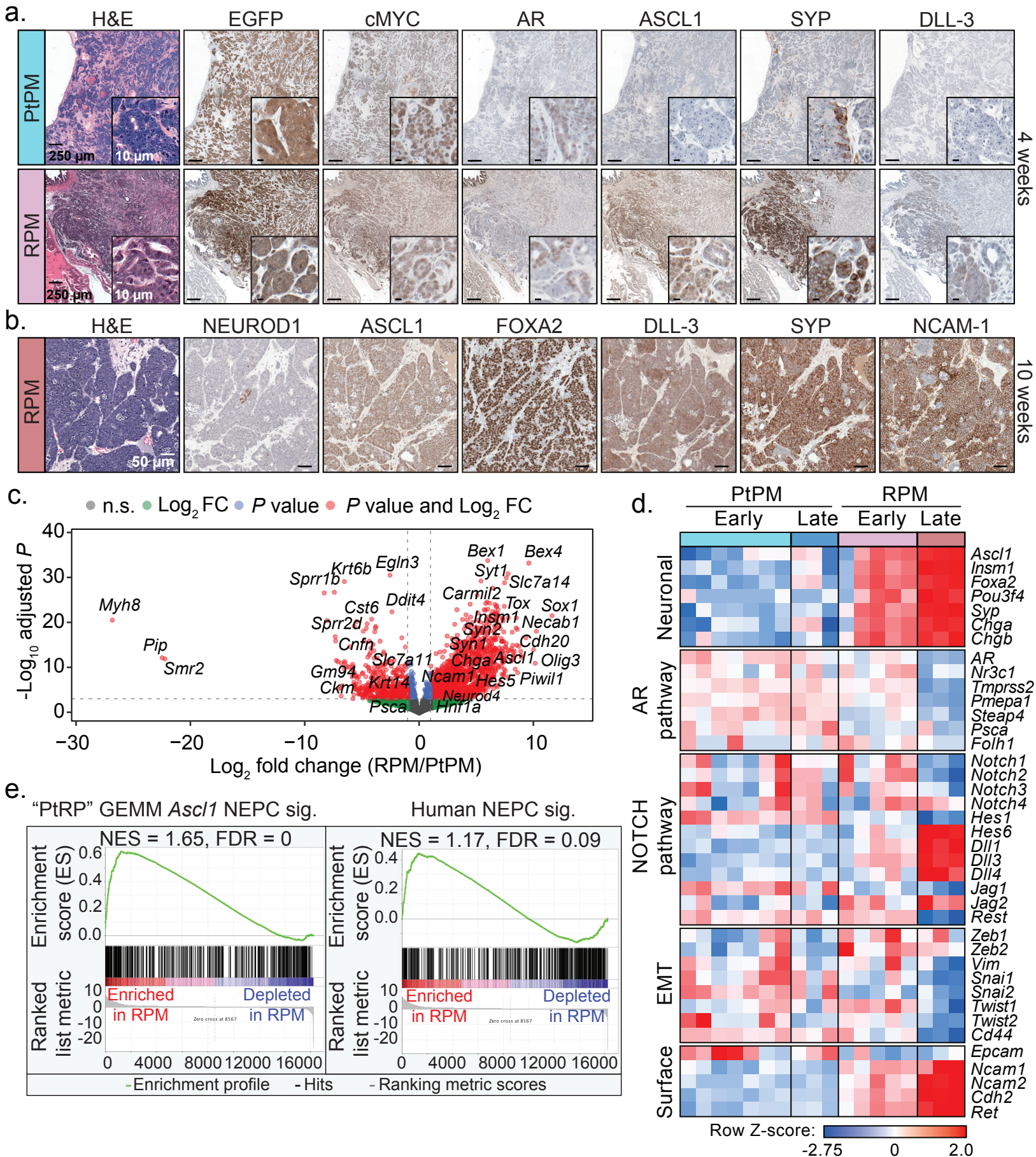
- 1 **Supplementary Table 3:** PtPM and RPM tumor bulk RNAseq normalized ($\log_2(X+1)$)
- 2 read counts. Related to Fig. 2c-d and Extended Data Fig. 5e.
- 3 **Supplementary Table 4:** Gene set enrichment analysis (REACTOME) results from RPM
- 4 vs PtPM tumors processed for bulk RNAseq. Related to Supplementary Table 3.
- 5 **Supplementary Table 5:** Antibodies used for multiplexed immunofluorescence
- 6 (Lunaphore COMET).
- 7 **Supplementary Table 6:** Marker co-expression used for cell typing COMET datasets.
- 8 **Supplementary Table 7:** PrismSpot module analysis. Related to Fig. 5e.
- 9 **Supplementary Table 8:** PrismSpot consensus TF differential gene expression in human
- 10 NEPC versus PRAD.
- 11 **Supplementary Table 9:** *Ascl1* edited alleles in RPM-*Ascl1*^{KO} pooled clones.
- 12 **Supplementary Table 10:** Antibodies and concentrations used for IHC, multiplexed IF,
- 13 western blot, and flow cytometry.
- 14 **Supplementary Table 11:** Supplementary sequences.
- 15 **Source Data Figure 1:** Unedited western blot scans (related to Extended Data Fig. 2c).

FIGURE 1:



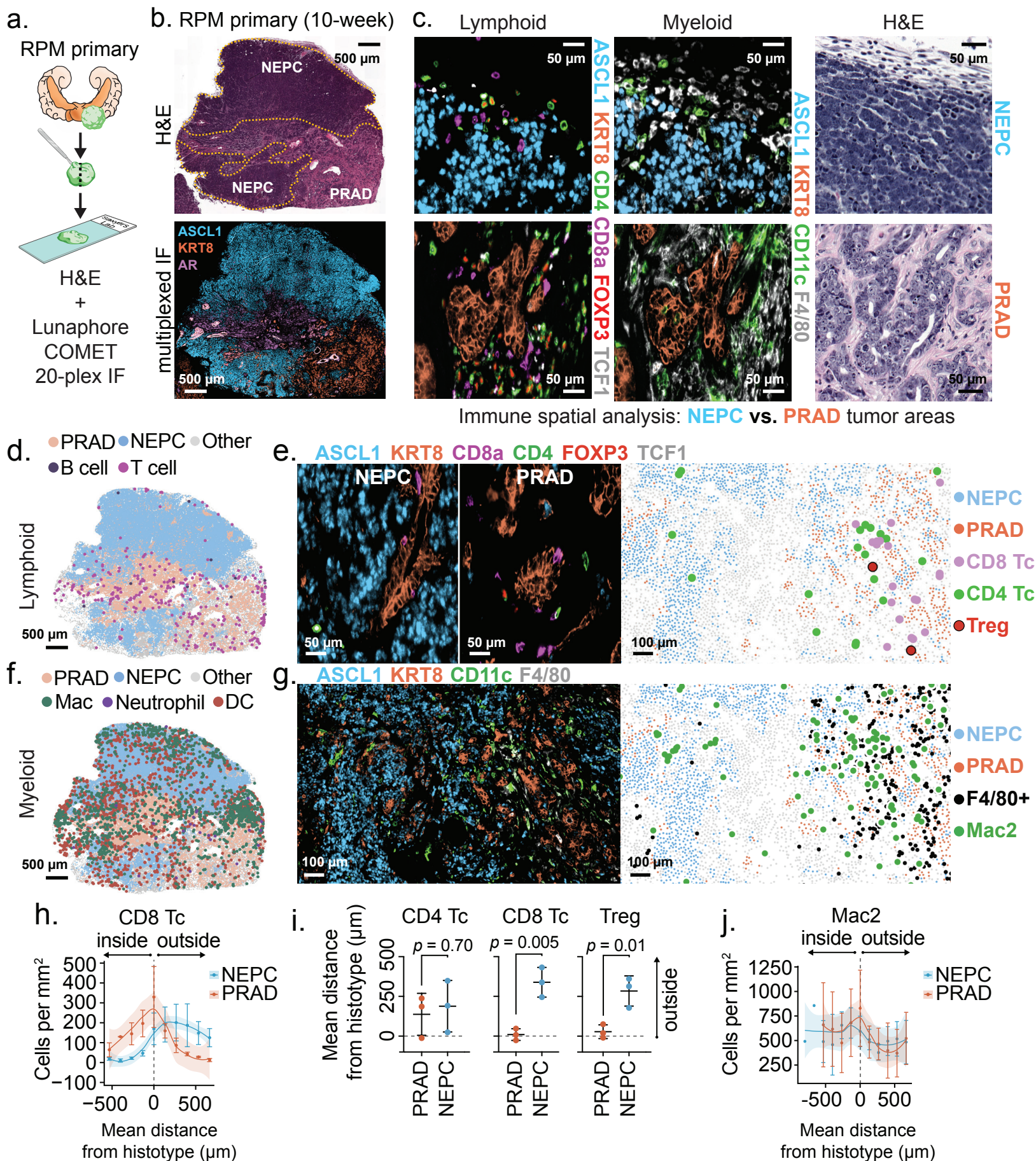
1 **Figure 1: Rapid establishment of genetically-defined prostate cancer with prostate**
2 **organoids transplanted into immunocompetent syngeneic hosts.**
3 **a.** Schematic of timeline required to establish, propagate, edit, and select for
4 organoids harboring mutations in cancer-associated genes prior to transplantation into
5 immunocompetent hosts for tumor establishment. **b.** Representative microscopy of *Pten*^{-/-};
6 *Trp53*^{-/-}; *cMyc*^{T58A} (PtPM) organoids, and stereoscopic and fluorescence images of
7 orthotopic (OT) prostate tumors with prostate adenocarcinoma (PRAD) histology. Tumor
8 images are representative of *n*=22 independent mice. H&E, hematoxylin and eosin. **c.**
9 Top: Representative microscopy of *Rb1*^{-/-}; *Trp53*^{-/-}; *cMyc*^{T58A} (RPM) organoids, and
10 stereoscopic and fluorescence images of OT prostate tumors and lung metastases.
11 Bottom: Representative histological assessment of RPM-PRAD and RPM-
12 neuroendocrine prostate cancer (NEPC) primary tumor or metastases histology at varying
13 magnifications. Primary and metastatic histology are representative of *n*=25 independent
14 mice. LN, lymph node (iliac). **d.** Phospho-Histone H3 (Ser10; pHH3) positive tumor cells
15 per total tumor area (μm^2). Each data point represents the average number of pHH3+
16 cells per individual tumor subset by tumor histology (PRAD vs NEPC) and experimental
17 end point. PtPM-Early (<4 weeks), *n*=14; PtPM-Late (>6 weeks), *n*=8; RPM-PRAD, *n*=11;
18 RPM-NEPC, *n*=14. Statistics derived using one-way ANOVA with Tukey's multiple
19 comparisons correction. Error bars denote mean and standard deviation. **e.** Survival of
20 mice transplanted with the indicated cell numbers of PtPM, RPM, and *Pten*^{-/-}; *Rb1*^{-/-};
21 *cMyc*^{T58A} (PtRM) *ex-vivo* edited organoids. PtPM 1k, *n*=5; PtPM 250k, *n*=5; RPM 250k,
22 *n*=14; PtRM 250k, *n*=8. Statistics derived from the Log-rank (Mantel-Cox) test for each
23 pair-wise comparison. **f.** Metastatic disease penetrance of the indicated organoid
24 transplants. Regional metastases include dissemination into the iliac lymph nodes. Distal
25 metastases include dissemination to kidney, pancreas, liver, or lungs. Statistics derived
26 from two-sided Fisher's exact test. Number of biological replicates indicated within the
27 figure. Scale bars indicated within each figure panel.

FIGURE 2:



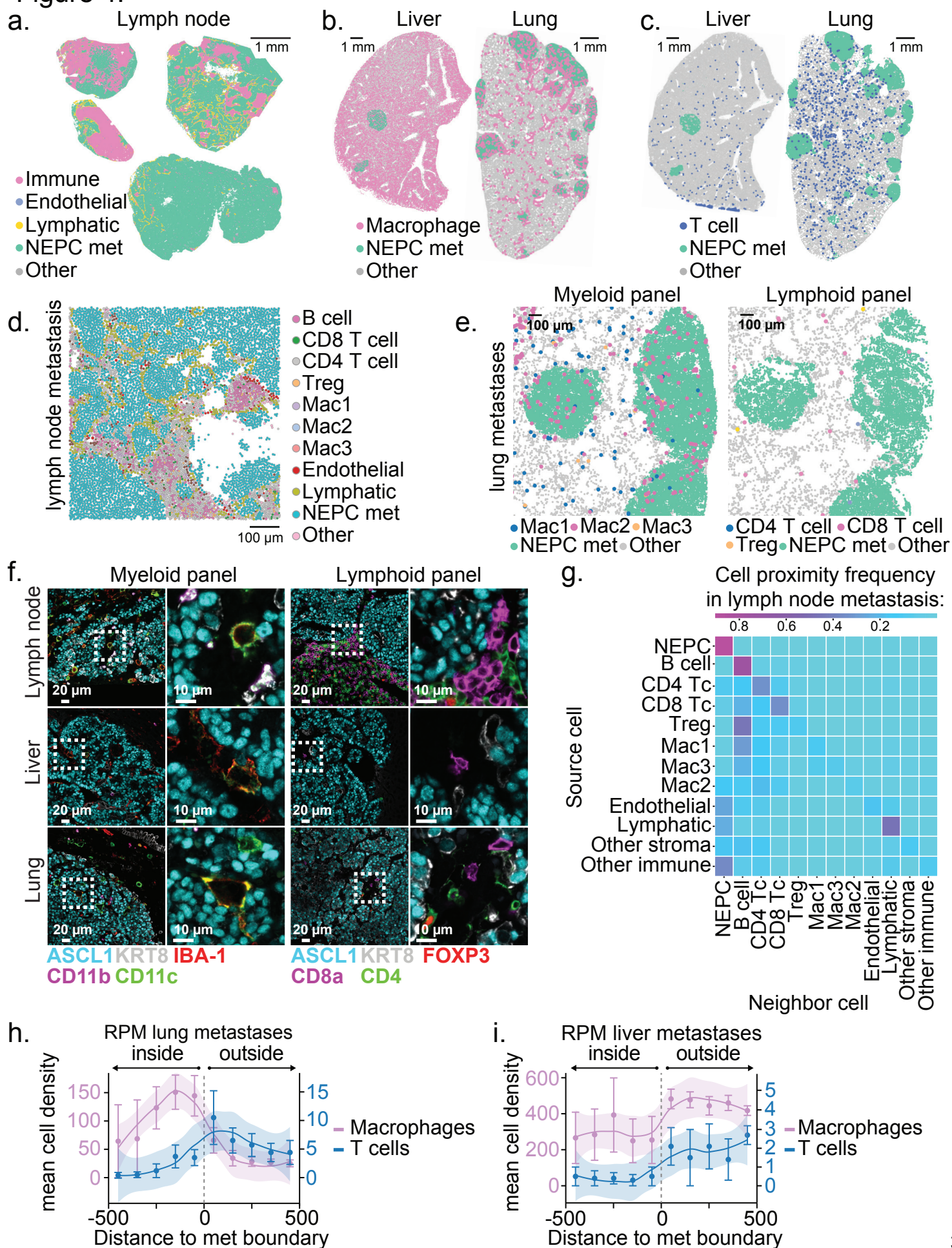
1 **Figure 2: Molecular characterization of PtPM and RPM primary prostate tumor**
2 **transplants demonstrates emergence of neuroendocrine carcinoma marker**
3 **expression.**
4 **a.** Representative histological analysis of PtPM (top) and RPM (bottom) tumors
5 isolated at 4-weeks post-transplantation. Serial sections depict immunohistochemical
6 staining of the indicated markers. Data are representative of $n=22$ independently
7 transplanted tumors. **b.** Representative histological analysis of RPM tumors isolated at
8 10-weeks post-transplantation. Serial sections depict immunohistochemical staining of
9 the indicated markers. Data are representative of $n=25$ independently transplanted
10 tumors. **c.** Volcano plot depiction of the \log_2 fold change in RNA expression of primary
11 (OT) RPM tumors relative to primary (OT) PtPM tumors. Genes that meet or surpass the
12 indicated thresholds of significance and fold change in expression are color coded as
13 depicted in the figure legend. Data derived from the comparison of PtPM ($n=10$) and RPM
14 ($n=8$) independent prostate tumors. **d.** Heatmap depicting the Z-score normalized
15 differential expression of select genes in PtPM versus RPM tumors. Time points of
16 isolation are color coded in the figure as they are in Fig. 2a. Genes are grouped by the
17 listed class or pathway. Early PtPM ≤ 4 weeks, early RPM ≤ 6 weeks. Late PtPM =5 weeks,
18 late RPM =10 weeks. Data related to samples used in Fig. 2f. **e.** Enrichment plots (GSEA)
19 of established expression signatures of (left) genetically engineered mouse model
20 (GEMM) of NEPC harboring conditional deletion of *Pten*, *Rb1*, and *Trp53* (PtRP), and
21 (right) histologically verified human NEPC within RPM primary tumors. FDR and NES
22 indicated in the figure. Analysis derived from the transcriptional profiles of multiple
23 independent RPM tumors ($n=8$) relative to PtPM tumors ($n=10$). Data related to samples
24 used in Fig. 2d. All scale bars noted in each panel and are of equivalent magnification
25 across each marker.

FIGURE 3:



1 **Figure 3: Multiplexed immunofluorescence identifies unique spatial distribution of**
2 **immune cells within RPM prostate tumors, with local depletion of immune cell**
3 **types in NEPC areas.**
4 **a.** Schematic representation of the methods used to process RPM tumors for 20-plex
5 cyclic immunofluorescence. **b.** (Top) Representative H&E and (bottom) serial section
6 depicting a 3-marker pseudo-colored 10-week RPM tumor. Histological regions (PRAD
7 vs. NEPC) are denoted in the H&E and demarcated by dotted yellow line. **c.**
8 Representative enhanced magnification of lymphoid (left) and myeloid cell markers
9 (middle), and serially sectioned H&E (right). **d.** Representative segmented field of view
10 (FoV) for the indicated general lymphoid cell types in a 10-week RPM tumor. **e.**
11 Representative immunofluorescence of the indicated pseudo-colored lymphocyte
12 markers within NEPC (left) or PRAD (middle). (Right) Data presented as a segmented
13 FoV indicating the localization of each lymphoid and tumor cell type in space. **f.**
14 Representative segmented field of view (FoV) for the indicated general myeloid cell types
15 in a 10-week RPM tumor. **g.** Representative immunofluorescence of the indicated
16 pseudo-colored myeloid and tumor histotype markers. (Right) Segmented FoV indicating
17 the localization of each myeloid and tumor cell type in space. **h.** Frequency distribution of
18 CD8⁺ T cells within binned distance outside or inside the defined interface region (NEPC
19 or PRAD). Scale bar represents mean and standard error of the mean of the cell counts
20 per bin. **i.** Mean distance of the indicated cell types to the nearest histotype boundary.
21 Error bars denote mean and standard deviation. **j.** Frequency distribution of Mac2 cells
22 (CD11b^{lo}; CD11c⁺; F4/80⁺) within each binned distance outside or inside of the defined
23 interface region (NEPC or PRAD). Scale bar represents mean and standard error of the
24 mean of the cell counts per bin. Data calculated as in h. Shaded regions in panels h and
25 j approximated through Loess method. Dotted line in h-j represents the boundary of the
26 tumor histotype or tumor edge. All scale bars denoted within each panel. Data derived
27 from $n=3$ independent tumor samples. Infiltration analyses representative of $n>3$ distinct
28 NEPC and PRAD boundaries.

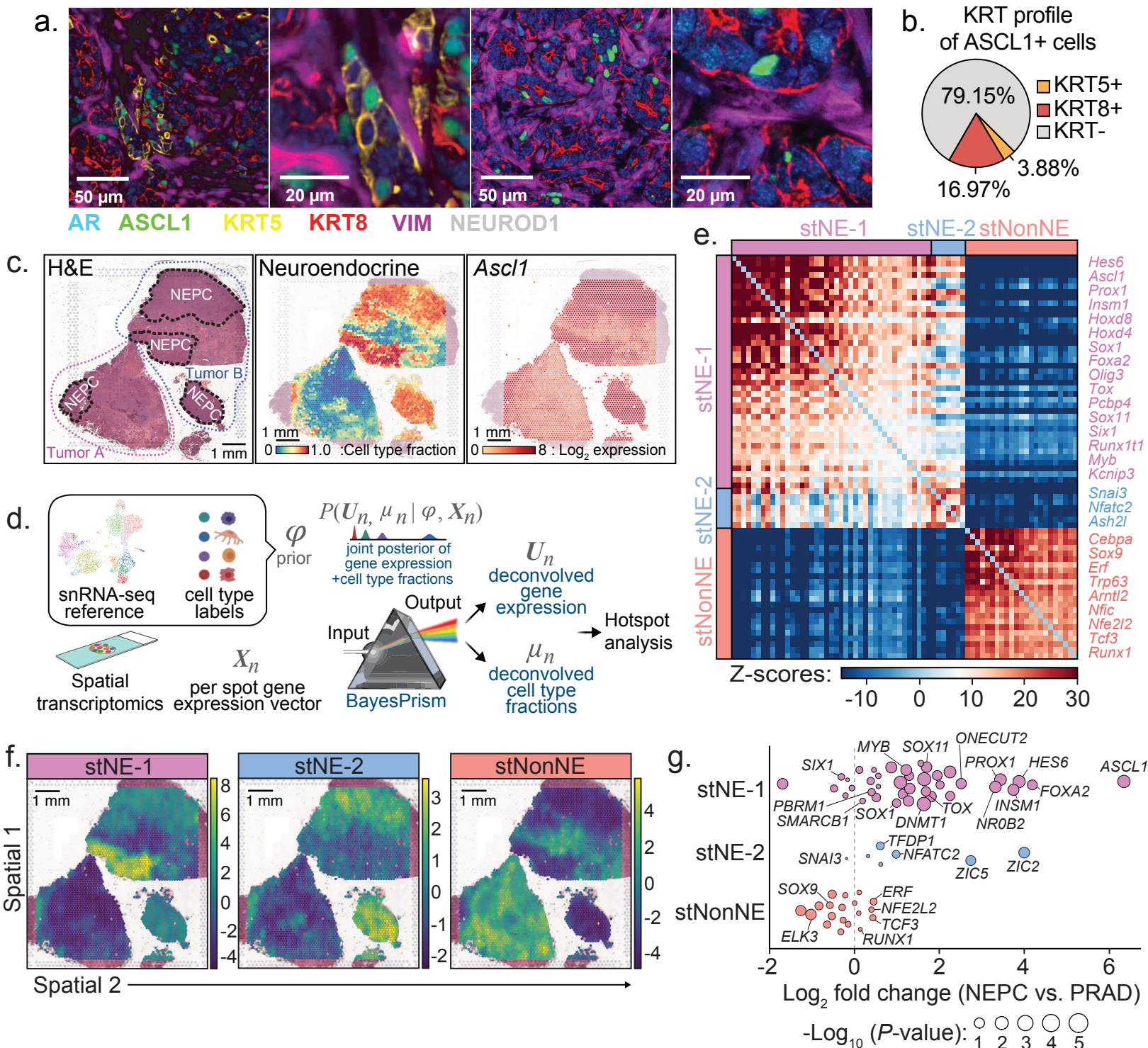
Figure 4:



1 **Figure 4: NEPC metastatic lesions are T cell excluded but retain macrophage**
2 **infiltrates.**

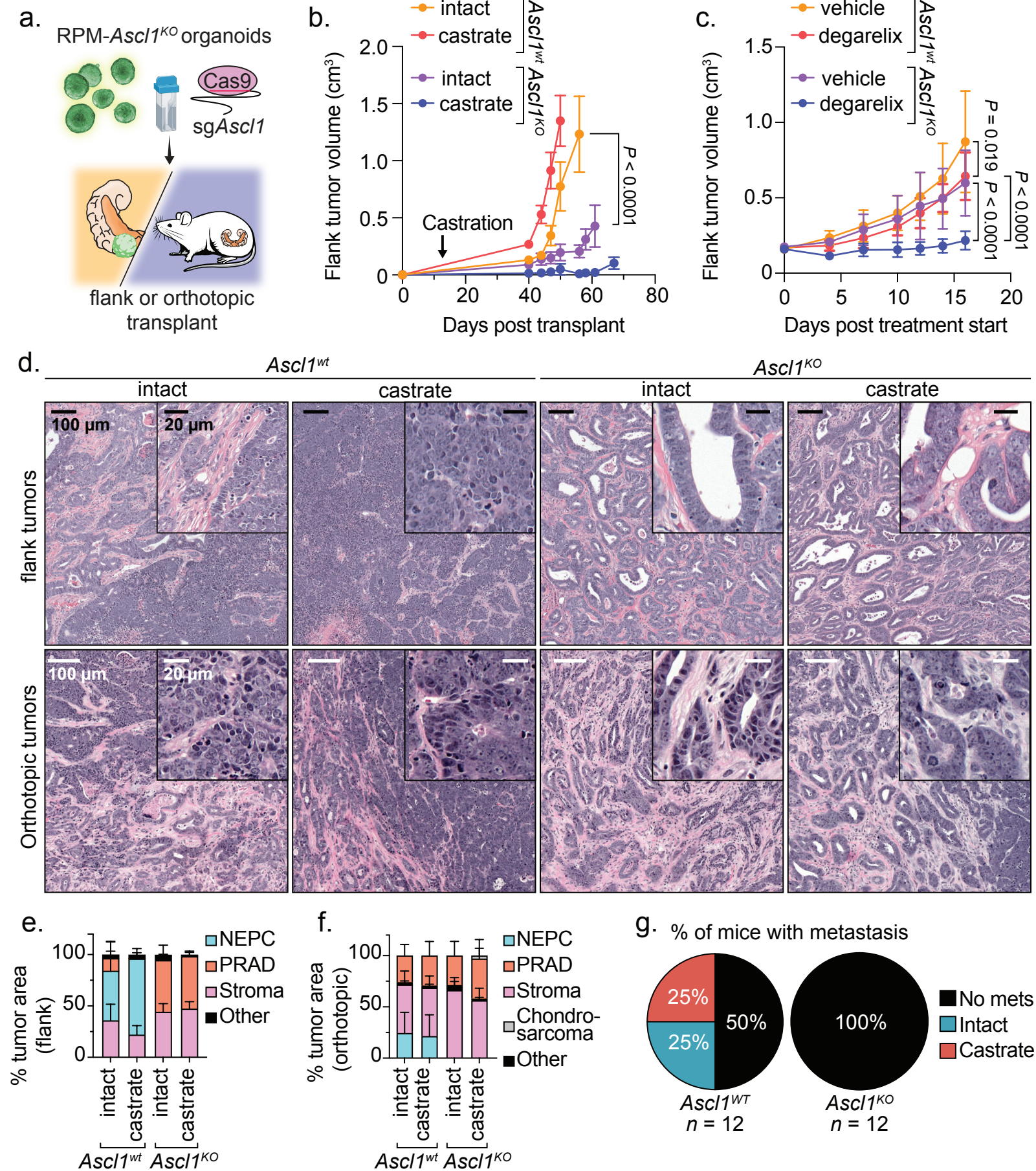
3 **a.** Representative segmented field of view (FoV) for the indicated cell types within 4
4 independent draining lymph node metastases derived from $n=2$ mice transplanted OT
5 with RPM organoids. **b.** Representative segmented FoV of macrophages (IBA-1+) within
6 liver or lung sections obtained from mice transplanted OT with RPM organoids. Note,
7 liver-resident macrophages (Kupffer cells) are IBA-1+. **c.** Representative segmented FoV
8 of T cells (CD4+ or CD8+) within liver or lung sections obtained from mice transplanted
9 OT with RPM organoids. **d.** Representative zoomed in segmented FoV for all cell types
10 listed within a draining lymph node metastasis. Scale denotes relative cell size. **e.**
11 Representative zoomed in segmented FoV across serial lung sections obtained from mice
12 transplanted OT with RPM organoids, identifying NEPC metastatic nodules infiltrated with
13 (left) macrophage subsets or (right) T cell subsets. **f.** Representative multiplexed
14 immunofluorescence of the indicated cell type markers across distinct metastatic sites
15 obtained from mice OT transplanted with RPM organoids. **g.** Neighborhood composition
16 heatmap of cell types found within RPM draining lymph node metastases demonstrating
17 the proximity of the source cell relative to a neighboring cell (20-pixel distance). Data are
18 derived from $n=4$ independent metastatic lymph node samples isolated from $n=2$ mice. **h.**
19 Frequency distribution for Macrophages (IBA1+) or T cells (CD4+ or CD8+) within each
20 binned distance outside or inside of RPM lung metastatic samples. **i.** Frequency
21 distribution for Macrophages (IBA1+) or T cells (CD4+ or CD8+) within each binned
22 distance outside or inside of RPM liver metastatic samples quantified as in Fig. 4h.
23 Shaded region in h-i approximated through Loess method. Scale bar in h-i represents
24 mean and standard error of the mean of the cell counts per bin. Dotted line in h-i
25 represents the boundary of a tumor histotype or tumor edge. All metastatic tumors per
26 section within an individual mouse were combined for infiltration analysis and
27 subsequently averaged between replicates ($n=3$ independent mice).

FIGURE 5:



1 **Figure 5: PrismSpot reveals spatial transcriptomic heterogeneity within NEPC**
2 **marked by *Ascl1* co-expressed with distinct NE-related TFs.**
3 **a.** Representative confocal images of 7-plex IF of the indicated markers. Second and
4 fourth images are digitally magnified versions of the first and third panel from the left.
5 Data are representative of $n=29$ individual RPM tumors at varying time points post OT
6 transplantation. **b.** Percentage of all ASCL1+ cells co-expressing KRT5, KRT8, or KRT-
7 negative within an individual RPM OT tumor. Data is derived from the average percentage
8 of cells within each tumor across $n=10$ independent tumors 6-weeks post OT
9 transplantation. **c.** (Left) H&E stains of two independent 10-week RPM tumors. Tumors A
10 and B are outlined in red and blue dotted lines, respectively. NEPC regions are highlighted
11 in black dotted lines. (Middle) BayesPrism inferred cell type fraction for NEPC. (Right)
12 Log_2 fold expression of *Ascl1* overlaid on the tumor histology. **d.** Workflow of PrismSpot
13 method. BayesPrism infers the posterior of cell type-specific gene expression, U , and cell
14 type fraction, μ , of each spot. The expression profile of the cell type of interest (NEPC)
15 was selected as the input for Hotspot analysis. **e.** Heatmap shows PrismSpot output of
16 the pairwise local correlation Z-scores of 71 TFs of high consensus scores (>0.8) and
17 significant spatial autocorrelation ($\text{FDR}<0.01$). TFs are clustered into 3 modules based
18 on pairwise local correlations between all TFs of significant spatial autocorrelation. **f.**
19 Spatial expression patterns of TFs within each module are visualized using smoothed
20 summary module scores. **g.** Beeswarm plot shows the log_2 fold change in expression of
21 TFs in each module between bulk RNA-seq of human NEPC and PRAD samples. Dot
22 size shows the two-sided p -values based on Wilcoxon test. All scale bars indicated within
23 each figure panel.

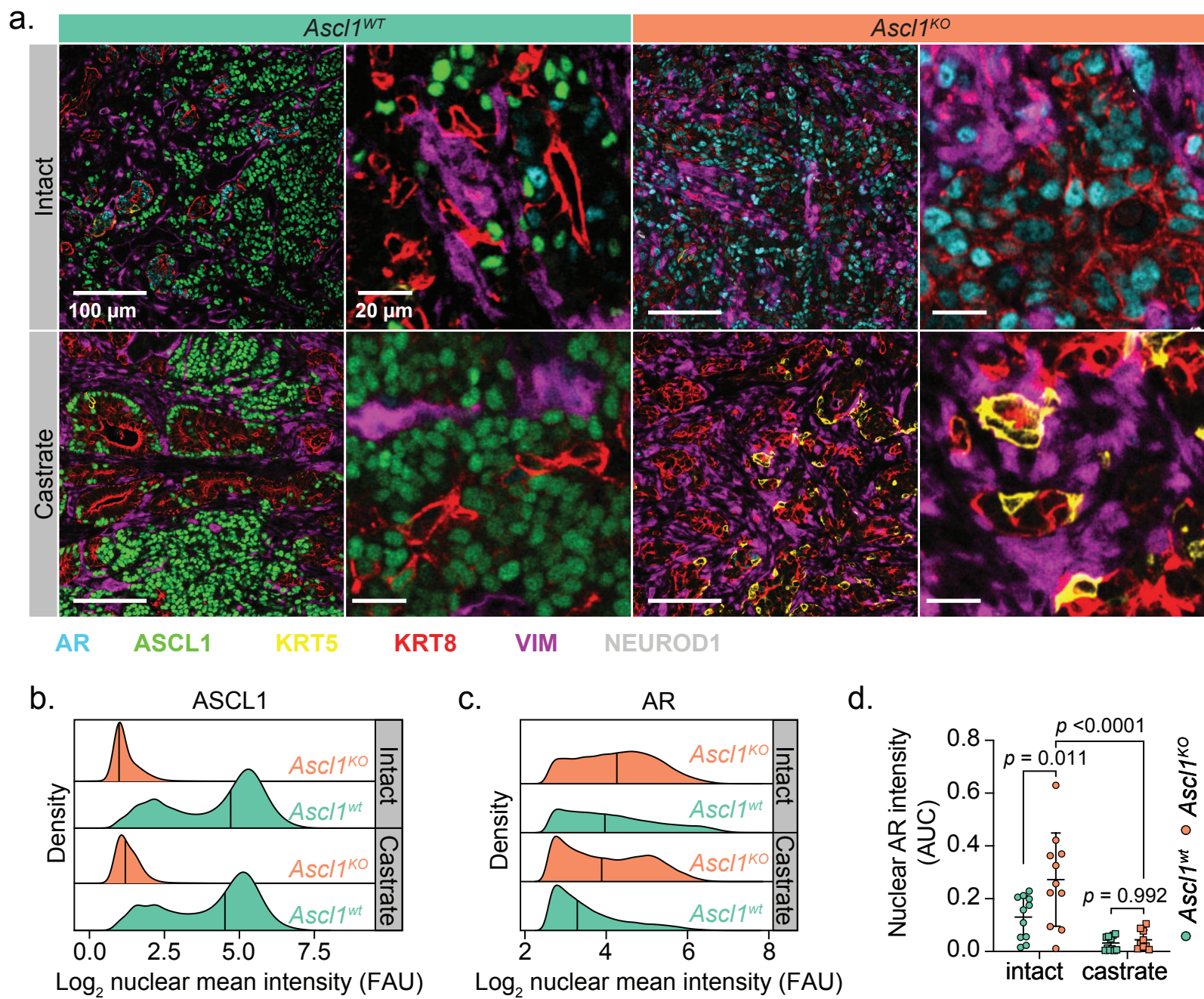
FIGURE 6:



1 **Figure 6: Loss of *Ascl1* results in abrogated NEPC establishment and castration-**
2 **sensitivity.**

3 **a.** Schematic for the generation of RPM-*Ascl1*^{WT} and RPM-*Ascl1*^{KO} tumors
4 transplanted into the flanks or prostates of immunocompetent C57BL6/J hosts. **b.**
5 Longitudinal subcutaneous (SQ) tumor volumes of the indicated tumor genotypes and
6 host backgrounds. Statistics derived using two-way ANOVA with Tukey's multiple
7 comparisons correction for data collected between days 0-56 to ensure equal sample size
8 comparisons. Error bars denote mean and standard error of the mean. *n*=6 independent
9 tumors across each group. Castration or sham surgery performed 14 days post SQ
10 transplantation. **c.** Longitudinal SQ tumor volumes of the indicated tumor genotypes and
11 host backgrounds. Statistics derived using two-way ANOVA with Tukey's multiple
12 comparisons correction for data collected between 0-16 days post treatment start to
13 ensure equal sample size comparisons. Error bars denote mean and standard deviation.
14 *Ascl1*^{WT}-vehicle, *Ascl1*^{WT}-vehicle, and *Ascl1*^{KO}-degarelix, *n*=8; *Ascl1*^{KO}-vehicle, *n*=9
15 independent tumors. Vehicle or degarelix treatment was initiated upon tumor
16 establishment (≥ 150 mm³). **d.** Representative H&E of SQ (top) and OT (bottom) tumors
17 isolated at endpoint. Genotype and treatment groups listed within the figure panel. Data
18 related to mice in Fig. 5b-c. Scale bars denoted within the figure panel. Data are
19 representative of 4-6 independent tumors per experimental group. **e.** Stacked bar charts
20 representing percentage of OT tumor area composed of the histological categories
21 depicted in the figure legend. Data are quantified histology of tumors generated in Fig. 5b
22 and represent average tumor area. **f.** Stacked bar charts representing percentage of SQ
23 tumor area composed of the histological categories depicted in the figure legend. Data
24 are quantified histology of tumors generated in Fig. 5c and represent the average tumor
25 area. **g.** Pie charts representing percentage of mice with metastatic disease (regional and
26 distal) in intact or castrated hosts of the indicated genotypes. Statistics derived from two-
27 sided Fisher's exact test, *p*=0.0137. Number of biological replicates indicated in the figure
28 panel. All scale bars denoted in the figure panels.

FIGURE 7:

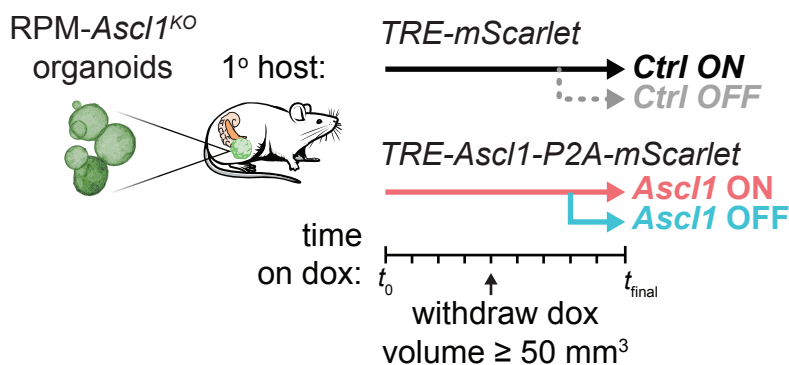


1 **Figure 7: Loss of *Ascl1* results in enhanced AR expression and proportion of**
2 **KRT8+ tumor cells.**

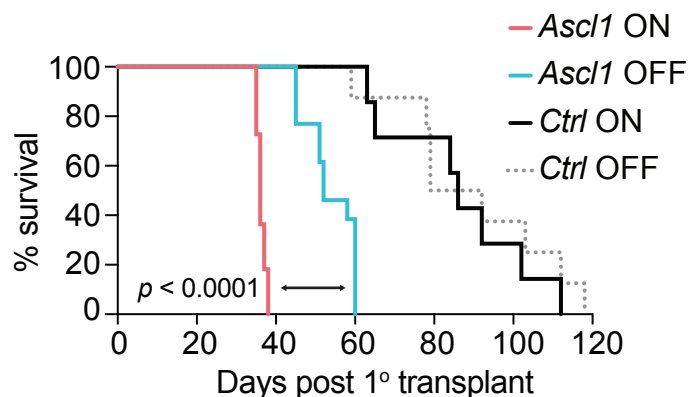
3 **a.** Representative confocal images of the tumors isolated from mice in Fig. 5b-c.
4 Scale bars and pseudocolor legend indicated within the figure. **b.** Density plots of the
5 $\log_2(x+1)$ transformed ASCL1 mean fluorescence intensity from all (OT and SQ) tumor
6 cells. Tumor cells subset by all cells staining negatively for VIMENTIN. Tumor genotype
7 and treatment indicated in the figure panel. Data derived from $n < 10$ independent RPM
8 tumors per group. **c.** Density plots of the $\log_2(X+1)$ transformed AR mean fluorescence
9 intensity from all OT tumor cells within the indicated genotypes and treatment groups.
10 Tumor cells subset by all cells staining negatively for VIMENTIN and positively for KRT8
11 and AR. **d.** Area under the curve for all KRT8+:AR+ tumor cells (VIMENTIN-) across both
12 SQ and OT tumor transplants, containing a \log_2 transformed nuclear AR intensity score
13 ≥ 3 . Statistics derived using two-way ANOVA with Tukey's multiple comparisons
14 correction. Error bars indicate mean and standard deviation. Combined OT and SQ tumor
15 sample sizes for all quantification and analysis performed in Fig. 7: $n=11$ (*Ascl1*^{wt} and
16 *Ascl1*^{KO} intact groups), $n=12$ (*Ascl1*^{wt} castrate group), $n=9$ (*Ascl1*^{KO} castrate group).
17 FAU=fluorescence arbitrary units.

FIGURE 8:

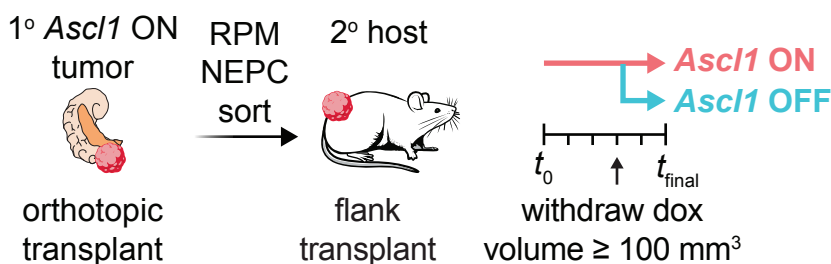
a.



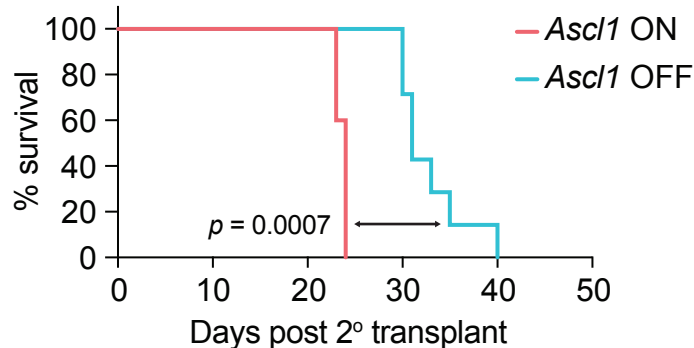
b.



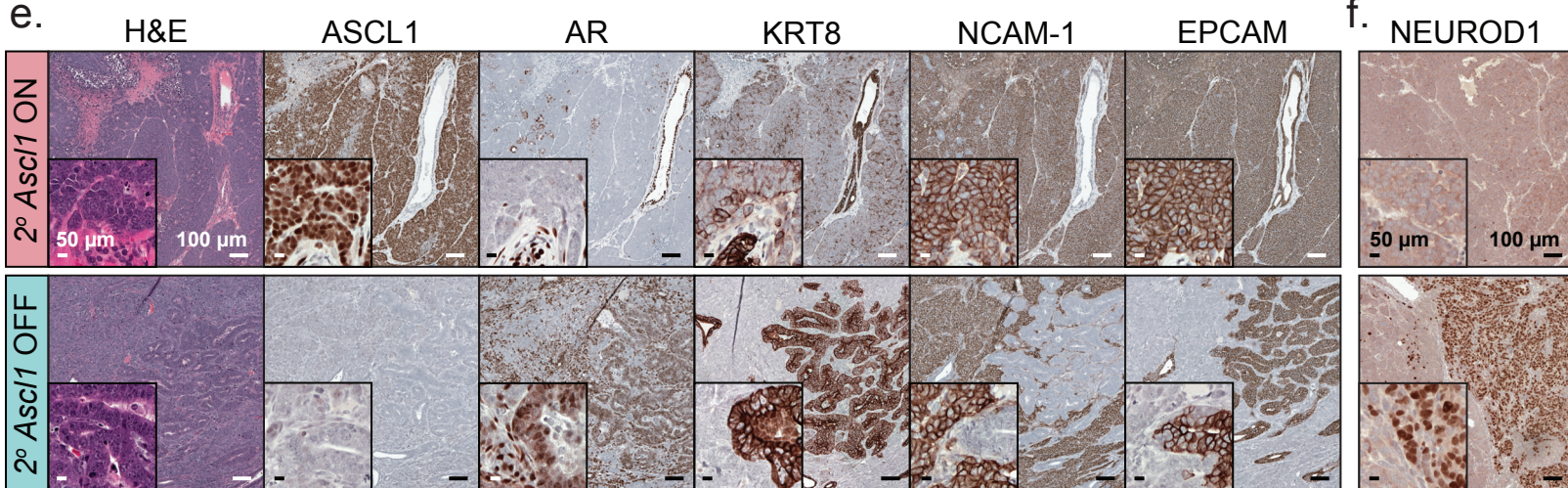
c.



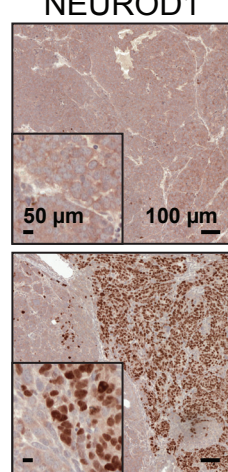
d.



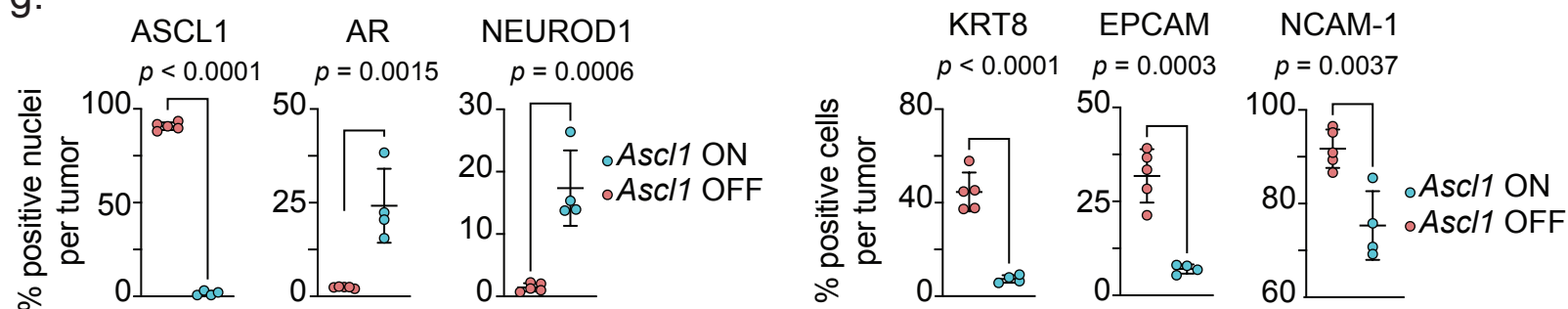
e.



f.



g.



1 **Figure 8: Loss of *Ascl1* in established NEPC results in modest tumor control and**
2 **increased tumor heterogeneity.**

3 **a.** Schematic of *Ascl1* doxycycline (dox)-inducible *in vivo* platform. RPM-*Ascl1*^{KO}
4 organoids infected with inducible *mScarlet* (*Ctrl*) or *Ascl1-P2A-mScarlet* (*Ascl1*) vectors
5 were transplanted OT into mice fed dox-chow (primary recipient host, 1^o). Tumor volume
6 was monitored by ultrasound. Upon primary tumor establishment, mice were randomized
7 into dox ON (maintained) or dox OFF (withdrawal) groups. **b.** Survival curves of *Ctrl* or
8 *Ascl1* induced OT tumors following dox-maintenance (ON groups) or withdrawal groups
9 (OFF groups). Statistics derived from log-rank (Mantel-Cox) test comparing primary *Ascl1*
10 ON to primary *Ascl1* OFF groups. *Ctrl* ON *n*=7, *Ctrl* OFF *n*=8, *Ascl1* ON *n*=11, *Ascl1* OFF
11 *n*=13 independent mice. **c.** Schematic of SQ *Ascl1* doxycycline (dox)-inducible *in vivo*
12 platform (secondary recipient host, 2^o). *Ascl1* ON primary tumors were dissociated for
13 flow cytometry to enrich for RPM-NEPC cells used for transplantation assays into the
14 flanks of secondary recipient mice fed dox-chow. Tumor volume was monitored by
15 caliper. Upon tumor establishment, mice were randomized into dox ON (maintained) or
16 dox OFF (withdrawal) groups. **d.** Survival curves of *Ctrl* or *Ascl1* induced secondary
17 tumors following dox-maintenance (ON groups) or withdrawal groups (OFF). Stats
18 derived from Log-rank (Mantel-Cox) test. *Ascl1* ON *n*=5, *Ascl1* OFF *n*=7 independent
19 mice per group. **e.** Serial sections from secondary transplanted mice (SQ) stained for the
20 indicated markers by H&E and IHC. **f.** Representative NEUROD1 IHC within a secondary
21 transplant containing mostly NEPC histology. Data representative of *n*=5 independent
22 tumors. **g.** (Left) Average percent marker positive nuclei or (right) cells across biologically
23 independent secondary SQ *Ascl1* ON (*n*=5) or OFF (*n*=4) tumors. Statistics derived from
24 two-sided *t*-test. Error bars indicate mean and standard deviation. All scale bars depicted
25 in the figure panels.

1 **METHODS:**

2 **Mice**

3 Animal studies were carried out in full compliance with Research Animal Resource Center
4 guidelines and the MSKCC Institutional Animal Care and Use Committee under protocol
5 #06-07-012). Only male mice were used for transplantation studies. All mice used for
6 transplantation harbored conditional EGFP alleles to tolerize against EGFP-derived
7 antigens expressed within organoids (Jax, #026179). All studies employed ≥ 3 animals
8 per genotype per experimental cohort. Mice were maintained on a pure C57BL/6J genetic
9 background. At established experimental end-point, mice were euthanized by CO₂
10 asphyxiation followed by cervical dislocation.

11
12 *Orthotopic prostatic organoid transplantation:* For tumor transplant studies, 8-12-week-
13 old animals with appropriate genotypes were randomized for surgical implantation of ex-
14 vivo manipulated organoids as described previously²⁰. Briefly, the ventral abdomen was
15 depilated (using clippers) the day prior to surgery. Animals were anesthetized with
16 isoflurane and the surgical area was disinfected three times with alternating
17 Betadine/Isopropanol. Eye lubrication was used to maintain eye health. Sterile tools were
18 used for all procedures. A 0.5 cm midline incision was made along the lower abdominal
19 midline and peritoneal wall to allow for exteriorization of the bladder, prostate, and seminal
20 vesicles. Local analgesia was used at the incision site (bupivacaine). Using straight
21 forceps, the bladder was gently pressed down caudally, exposing the dorsal prostate
22 lobes. A 30-gauge needle was inserted into the right dorsal prostatic parenchyma and 20
23 μ L (containing single cell suspensions of organoids in 50% PBS + 50% matrigel) was

1 injected. Organoid/matrigel mix was kept on ice throughout the entire procedure.
2 Successful injection was visualized by local expansion in volume of the right dorsal lobe
3 without leakage. The prostate, bladder, and seminal vesicles were gently internalized,
4 and the peritoneal layer was sutured using 5-0 vicryl sutures. The outer skin layer was
5 closed with 3 to 4 wound clips. All mice received pre- and post-operative analgesia with
6 buprenorphine and meloxicam and were followed post-operatively for any signs of
7 discomfort or distress. Tumor-containing area was measured on hematoxylin and eosin
8 (H&E) slides. Histological assessment was performed in consultation with clinical
9 pathologist Dr. Anuradha Gopalan.

10
11 *Subcutaneous prostatic organoid transplantation:* For allograft experiments, 250k single
12 cell suspension of organoids in 100 μ L 50% + 50% matrigel were injected into the
13 depilated flanks (clippers) of isoflurane anesthetized C57BL/6J mice. Mice were followed
14 for tumor measurement and signs of discomfort or distress. Subcutaneous tumor volumes
15 (mm^3) were calculated using the formula: $(a^2*b)*(\pi/6)$, where a and b are the smaller and
16 larger dimensions, respectively.

17
18 *Castration surgery:* Mice harboring orthotopic prostate tumors were randomized into
19 castration or sham surgeries 2 weeks post orthotopic surgery. Mice were anesthetized
20 with isoflurane. Eye lubrication was used to maintain eye health. The perineal region was
21 cleaned three times with alternating Betadine/Isopropanol. Sterile tools were used for the
22 procedure. A 0.5 cm incision was made in the scrotal sack. Forceps were used to locate
23 and exteriorize the testes. Using a cauterizing iron, the testes were amputated via the

1 seminal tubules. The scrotum was closed shut with 2-3 wound clips. A local triple antibiotic
2 was applied over the region to facilitate healing. All mice received pre- and post-operative
3 analgesia with meloxicam and were followed post-operatively for any signs of discomfort
4 or distress.

5
6 *Chemical castration studies (GnRH antagonist):* 1E6 RPM-*Ascl1*^{WT} or RPM-*Ascl1*^{KO}
7 organoids were injected into the flanks (one tumor per mouse) of immunocompetent mice
8 as described above. Mice were randomized into vehicle (5% D-Mannitol, Sigma M4125-
9 500G) or Degarelix (15 mg/kg, Sigma SML2856-25MG) groups once tumors measured \geq
10 150 mm³. Treatment was performed by subcutaneous delivery once every 14-days in a
11 total of 100 μ L. Tumor volumes were obtained and calculated as above. At sacrifice, blood
12 was collected from mice and coagulated for 1hr on ice, followed by centrifugation at 1000
13 g for 30 min to isolate serum. Successful serum testosterone depletion was assessed by
14 ELISA according to manufacturer recommendations (Abcam, ab285350).

15
16 *Transplantation of dox inducible Ascl1 organoids:* 100E5 RPM-*Ascl1*^{KO} organoids
17 harboring dox-inducible *Ascl1-P2A-mScarlet* or *mScarlet* alone were injected (50%
18 matrigel) into the prostates of C57BL/6J *Prkcd*^{KO} mice (Jax, #001913) to avoid rtTA-
19 mediated rejection. Dox chow (Inotiv, 0.625 g/kg) was started 1 week prior to
20 transplantation ensure immediate induction of transgene expression. Mice were
21 randomized into *Ascl1*^{ON} (maintained on dox) or *Ascl1*^{OFF} (withdrawn from dox) cohorts
22 when tumor volumes reached \geq 50mm³, as measured by small animal ultrasound. For
23 secondary transplantation assays of pure RPM-NEPC tumor cells, 5-week *Ascl1*^{ON}

1 tumors were harvested as described for primary prostate organoid single cell
2 suspensions, and sorted (Sony MA900, Sony Biotechnology, 100- μ m sorting chip (Sony
3 Biotechnology, #LE-C3210) for DAPI-, EGFP+, mScarlet+ (*Ascl1* reporter), EPCAM+,
4 NCAM-1+ cells (gating strategy **Supplementary Fig. 10**). Antibodies used for flow
5 cytometry listed in **Supplementary Table 10**. Post sort, 100E5 cells were immediately
6 injected (50% matrigel) into the flanks of secondary C57BL/6J *Prkcd*^{KO} mice pre-fed dox
7 diet for 1 week pre-transplantation. Tumor volumes were assessed by caliper
8 measurements as above. Mice were randomly separated into *Ascl1*^{ON} or *Ascl1*^{OFF} groups
9 when flank tumors reached $\geq 50\text{mm}^3$. At experimental end-point, secondary tumors from
10 *Ascl1*^{ON} or *Ascl1*^{OFF} groups as well as RPM organoids and primary *Ctrl* ON tumors
11 (mScarlet-induced expression alone) were harvested for FFPE and processed for flow-
12 cytometry for the markers listed above (**Supplementary Fig. 11a-c**).

13
14 *Small animal ultrasound:* Animals were anesthetized using isoflurane and the ventral
15 abdominal areas were depilated with Nair. Eye lubrication was used to maintain eye
16 health. Animals were imaged using the Vevo2100 ultrasound and photoacoustic imaging
17 system (Fujifilm-Visualsonics). Animals were placed on the imaging platform in the supine
18 position and a layer of ultrasound gel was applied over the entirety of the abdominal area.
19 The ultrasound transducer was placed on the abdomen orthogonal to the plane of the
20 imaging platform. The bladder and urethra were used as landmark organs to define the
21 area of the prostate. The transducer was set at the scanning midpoint of the normal
22 prostate or prostatic tumor and a 3D image of 10-20 mm, depending on tumor size, at a
23 Z-slice thickness of 0.04mm. 3D images were uploaded to the Vevo Lab Software and

1 volumetric analysis function was used to determine the tumor border at various Z-slices
2 through the entirety of the tumor and derive the final calculated tumor volume.

3

4 **Human Specimens:**

5 Informed consent was obtained for all patients and approved by MSKCC's Institutional
6 Review Board (IRB) #12-245 (NCT: 01775072) and #06-107. The human prostate tumor
7 specimen was collected from a 62-year-old male with localized PRAD undergoing XRT
8 followed by salvage prostatectomy post ADT and docetaxel. Tumor in the bladder arose
9 by extension of a prostate tumor recurrence in the surgical bed. Pathological evaluation
10 showed small cell carcinoma arising from PRAD. Tumor cells were focally positive for
11 SYP, CHGA (patchy), PSA (focal), and PSMA (focal weak). Tumor sample was sectioned
12 and processed for COMET-based multiplexed IF according to the antibodies listed in
13 **Supplementary Table 10.**

14

15 **Immunohistochemistry and Immunofluorescence**

16 *Immunohistochemistry:* Prostate tumors were cleaned under a stereomicroscope, fixed
17 overnight in zinc-formalin, washed in PBS, transferred to 70% ethanol, and embedded in
18 paraffin. Sections were cut to six micrometers and stained with H&E. Chromogenic
19 immunohistochemistry (IHC) was performed on fresh cut sections. Briefly, slides were
20 heated for 30 min at 58°C and deparaffinized. Antigen retrieval was performed in freshly
21 prepared citrate buffer (pH 6.0) followed by Tris-EDTA (pH 9.0, Abcam #ab93684) in a
22 decloaking chamber and subsequently slowly cooled on ice. Slides were washed in PBS
23 + 0.1% Tween (PBST) followed by an endogenous peroxidase block (Bloxall, Vector

1 Labs, SP-6000-100). Slides were subsequently blocked in 2.5% normal horse serum and
2 stained overnight in primary antibodies at 4°C in PBS + 0.01% Tween-20. The following
3 day, slides were washed in PBST and stained with the secondary-HRP conjugated
4 antibodies, washed in PBST, and developed with 3,3'-diaminobenzidine (DAB, Vector
5 Labs, SK-4100). For mouse-IgG primary, a M.O.M. kit was used after the peroxidase
6 block (Vector Labs, MP-2400). Antibodies used for IHC are listed in **Supplementary**
7 **Table 10**. Slides were scanned on a Panoramic Scanner (3DHitech) with a 20X/0.8NA
8 objective and visualized in ImageJ or QuPath (v0.4.2).

9
10 *Multiplexed immunofluorescence (Leica Bond RX)*: Samples were pretreated with EDTA-
11 based epitope retrieval ER2 solution (Leica, AR9640) for 20 minutes at 95°C. The 6-plex
12 antibody staining and detection were conducted sequentially. Antibodies used for
13 multiplexed IF are listed within **Supplementary Table 10**. After 1 hr incubation, Leica
14 Bond Polymer anti-rabbit HRP was applied followed by Alexa Fluor tyramide conjugate
15 488 and 647 (Life Technologies, B40953, B40958), or CF® dye tyramide conjugate 430,
16 543, 594, and 750 (Biotium, 96053, 92172, 92174, 96052) for signal amplification-based
17 detection. At each round, epitope retrieval was performed for denaturation of primary and
18 secondary antibodies before the following primary antibody was applied. Slides were
19 washed in PBS and incubated in 5 µg/ml 4',6-diamidino-2-phenylindole (DAPI; Sigma
20 Aldrich) in PBS for 5 min, rinsed in PBS, and mounted in Mowiol 4–88 (Calbiochem).
21 Slides were scanned on a Panoramic Scanner (3DHitech) with a 20X/0.8NA objective
22 and visualized in ImageJ or QuPath. Confocal microscopy was performed on a Leica
23 Stellaris 8.

1
2 *Multiplexed immunofluorescence (Lunaphore COMET)*: Tissue was cut at 4 mm onto
3 positively charged glass slides. Slides were baked at 64°C for 1 hr. Dewaxing and antigen
4 retrieval was performed on the Leica Bond RX with 30 min retrieval EDTA-based epitope
5 retrieval ER2 solution. Before loading onto the COMET, slides were washed 3X for 1 min
6 in DI water. 20-plex antibody panel and dilutions can be found in **Supplementary Table**
7 **5**.

8 9 **Immunoblotting**

10 Single cell organoid suspensions or monolayer cells were lysed in 125-250 µL ice-cold
11 RIPA buffer (Pierce, #89900) supplemented with 1x Complete Mini inhibitor mixture
12 (Roche, #11836153001) and mixed on a rotator at 4°C for 30 minutes. Protein
13 concentration was quantified using the Bio-Rad DC Protein Assay (Catalog #500-0114).
14 10–20 µg of total protein was separated on 4–12% Bis-Tris gradient gels (Bio-Rad) by
15 SDS-PAGE and then transferred to nitrocellulose membranes. Antibodies used for
16 western blots are listed in **Supplementary Table 10**. Blots were developed in Amersham
17 ECL western detection reagent (Cytiva, RPN2236) and imaged on a Cytiva Amersham
18 ImageQuant 800.

19 20 **Lentiviral production**

21 Lentiviruses were produced by co-transfection of 293T cells (Takara, #632180) with
22 lentiviral backbone constructs and packaging vectors (psPAX2 and pMD2.G; Addgene
23 #12260 and #12259) using TransIT-LT1 (Mirus Bio #MR 2306). Virus was concentrated

1 through ultracentrifugation (Beckman, Optima L-100 XP) at 25,000 RPM for 2hrs. The
2 viral pellet was resuspended in OptiMEM (Thermo, 31985062), aliquoted, frozen at -80°C,
3 and titered through serial dilution assays.

4

5 **Organoid culture**

6 Murine prostate organoids were established and maintained as previously described^{18,19}.
7 Full prostate organoid media composed of: 1X ADMEM, 10 mM HEPES, 1X Glutamax,
8 0.5X Pen/Strep, 1X B27 (Fisher Scientific, #17504-044), 1.25 mM N-acetylcysteine
9 (Sigma, A9165-100G), 10 mM Nicotinamide (Sigma, N0636-500G), 500 nM A83-01
10 (Tocris, #2939), 5 ng/mL recombinant murine EGF (Peprotech, #315-09), 10 ng/mL
11 recombinant NRG1 (Peprotech, #100-03), 1 nM Dihydrotestosterone (Selleck Chemicals,
12 S4757) 10% NOGGIN conditioned media, 5% RSPO-I conditioned media. Single cell
13 embedded prostate organoids were supplemented with 10 μM Y-27632 (Fisher Scientific,
14 #50-863-7) for the first 2-3 days in culture prior to change with fresh organoid media
15 lacking Y-27632. *Ex-vivo* transformed organoids were seeded at 3E3 cells per 20 μL
16 matrigel dome and passaged every 3-4 days. Wild-type organoids were seeded at 10E3
17 cells per 20 μL matrigel dome and passaged every 5-7 days. For monolayer adaptation,
18 western blot validated knockout organoids were dissociated using methods described
19 above and seeded at 1E5 cells/mL in full organoid media containing 10 μM Y-27632 on a
20 collagen I coated 10 cm plate (Fisher Scientific, #08-772-75). After 5 days adaptation and
21 expansion, cells were processed for protein lysates, or dissociated for orthotopic
22 transplantation.

23

1 **Organoid Cas9 ribonucleoprotein electroporation**

2 Organoids were electroporated as previously described²⁰. Briefly, organoids were
3 electroporated with Cas9-RNP complexes (IDT) and recovered in organoid conditions for
4 3-5 days. *Trp53* loss was selected by supplementing media with 5 μ M Nutlin-3a (Tocris,
5 6075). *Rb1* loss was selected for by supplementing media with 2.5 μ M Palbociclib (Tocris,
6 4786). *Pten* loss can be optionally selected for by growing EGF and NRG1 knockout
7 organoid media. Sequences for sgRNAs can be found in **Supplementary Table 11**.

8

9 **Organoid lentiviral infection**

10 RNP-edited and selected single cell organoid suspensions were infected with
11 concentrated lentiviral supernatants at predetermined titers as previously described¹⁸. 3-
12 5 days post lentiviral infection, organoids were dissociated with TrypLE (see above) and
13 resuspended in 0.1% BSA in PBS + 1 mM EDTA and supplemented with 10 μ M Y-27632.
14 Single cell suspensions were passed through a sterile 5 mL polypropylene tube with cell
15 strainer (Corning, #352235) and enriched by sorting for DAPI- EGFP+ cells using a Sony
16 MA900 (Sony Biotechnology) with a 130- μ m sorting chip (Sony Biotechnology, #LE-
17 C3213). Sorted organoids were expanded for 5-6 days before transplantation.

18

19 **Molecular Cloning**

20 Lentiviral vector (LVt-UBC-cMYC-P2A-EGFP) was generated using Gibson assembly.
21 Briefly, PCR fragments were amplified containing UBC promoter, *cMyc*^{T58A} codon
22 optimized cDNA, and a P2A-EGFP sequence, mixed within a Gibson master mix reaction
23 and transformed into Stbl3 chemically competent *E. coli* (Thermo, #C737303). All

1 plasmids were purified (Qiagen, #12943) and sequence validated through long-read
2 sequencing (SNPsaurus). Lentiviral construct UT4GEPIR (Addgene #186712) was used
3 for cloning Dox-inducible *Ascl1* and *mScarlet* constructs. Briefly, UT4GEPIR was digested
4 with BamHI and I-SceI and a geneblock (IDT) encoding mouse codon-optimized *Ascl1*-
5 *T2A-mScarlet* or *mScarlet* sequences with compatible overhangs was cloned by ligation
6 and transformation as above.

7

8 **Isolation and validation of *Ascl1* knockout organoid clones**

9 *Ascl1* sgRNA targeted RPM organoids with Cas9 RNP were expanded for 5 days post
10 electroporation and gently triturated in 0.5% BSA in PBS. Intact spheres were
11 subsequently diluted 1:10 in PBS and placed in a 6 well plate (Fisher Scientific, #07-000-
12 646). Using a standard tissue culture microscope and a 20 uL pipet tip, individual intact
13 spheres with a healthy morphology were isolated and individually dissociated in 100 μ L
14 TrypLE and quenched in 1 mL of 0.5% BSA in PBS. Organoid clones were centrifuged at
15 600g for 3 min in protein low-bind microcentrifuge tubes and resuspended in 50 μ L of
16 matrigel and plated into 24-well suspension plates. Full organoid media supplemented
17 with 10 μ M Y-27632 was added after 10 min. Individual organoid clones were expanded
18 in parallel and genomic DNA was isolated using a DNeasy Blood & Tissue kit (Qiagen,
19 69506). 35 cycle PCR reactions with an input of 100 ng of gDNA and an annealing
20 temperature of 58°C were performed using primers flanking the sg*Ascl1* edit site and
21 KAPA mouse genotyping kit following manufacturer protocols (Fisher Scientific, #50-196-
22 5243; **Supplementary Table 11**). A PCR product of 170 bp was purified using the
23 QIAquick Gel Extraction Kit (Qiagen, 28706) and submitted for library preparation and

1 next generation sequencing at the Integrated Genomics Operation (IGO) at MSKCC. A
2 total of 6 sequence-validated bi-allelic RPM-*Asc1* KO clones were subsequently pooled
3 and expanded for transplantation experiments.

4

5 **CRISPR sequencing**

6 Sequencing libraries were prepared from amplicons with an average size of 200-280bp
7 (see **Supplementary Table 11** for PCR primer sequences). The reported concentration
8 of 500 ng was used as input for the KAPA Hyper Library Preparation Kit (Kapa Biosystems
9 KK8504) according to the manufacturer's instructions with 8 cycles of PCR. Barcoded
10 libraries were pooled at equal volumes and run on NovaSeq 6000 in a PE150 run, using
11 the NovaSeq 6000 S4 Reagent Kit (300 Cycles) (Illumina). The average number of read
12 pairs per sample was 1.3M. Alignment and modification quantification was done with
13 CRISPResso2 (<http://crispresso.pinelloab.org/>) using default parameters.

14

15 **RNA isolation from organoid cultures and bulk tumors**

16 Tumors were isolated from euthanized mice and validated for EGFP fluorescence under
17 fluorescence stereomicroscope. Tumors were quickly placed within 250-500 μ L of RLT
18 buffer supplemented with B-mercaptoethanol into 2mL tube with ceramic beads (MP,
19 6910500). Tumor samples were lysed on a Fisher Bead Mill 24 using manufacturer
20 recommended settings. Lysates were passed through a Qias shredder (Qiagen, 79656)
21 and RNA isolated using the Qiagen RNeasy kit (Qiagen, 74106) with manufacturer
22 recommended protocols. Organoids were dissociated as above and resuspended in 300
23 μ L of RLT buffer supplemented with B-mercaptoethanol and spun through a Qias shredder.

1 Qiagen RNeasy kit was performed to isolate RNA using manufacturer recommended
2 protocols. For qPCR, purified RNA was reverse transcribed (Thermo, 4368814) and
3 quantified (Applied Biosystems, Quantstudio 6) with SYBR green reagent (Thermo,
4 A46110). See **Supplementary Table 11** for primer sequences used for qPCR.

5

6 **Bulk Transcriptome sequencing and gene set enrichment analysis**

7 After RiboGreen quantification and quality control by Agilent BioAnalyzer, 500 ng of total
8 RNA with RIN values of 8.3-10 underwent polyA selection and TruSeq library preparation
9 according to instructions provided by Illumina (TruSeq Stranded mRNA LT Kit, catalog #
10 RS-122-2102), with 8 cycles of PCR. Samples were barcoded and run on a NovaSeq
11 6000 in a PE100 run, using the NovaSeq 6000 SX Reagent Kit (Illumina). An average of
12 24 million paired reads was generated per sample. Ribosomal reads represented 0.4-
13 1.5% of the total reads generated and the percent of mRNA bases averaged 86%.
14 Analysis of bulk RNA sequencing was performed at NYULMC HPC UltraViolet (formerly
15 BigPurple) cluster using software provided in the Seq-N-Slide pipeline
16 (<https://github.com/igordot/sns>), through the *rna-star* followed by *rna-star-groups-dge*
17 routes. Briefly, after quality control assessment with MultiQC⁵⁸ (python/cpu/v2.7.15) and
18 sequencing adaptor trimming with Trimmomatic⁵⁹ (v0.36), reads were aligned to the
19 mm10/GRCm38 mouse reference genome with a splice-aware⁶⁰ (STAR v2.7.3a)
20 alignment, followed by featureCounts⁶¹ (subread/v1.6.3) to generate the RNA counts
21 table. Counts were normalized for gene length and sequencing depth and tested for
22 differential mRNA expression between groups using negative binomial generalized linear
23 models implemented by the DESeq2⁶² 1.40.1 R package (r/v4.1.2). Differential

1 expression was assessed by principal component analysis (prcomp function from the
2 stats v4.3.1 R package) or unsupervised hierarchical clustering (pheatmap v1.0.12 and
3 ComplexHeatmap v2.16.0) and visualized by a volcano plot (EnhancedVolcano v1.18.0)
4 and TPM expression heatmap illustrating genes of interest. Differentially expressed
5 genes (DEGs) identified were further analyzed for gene set enrichment analysis (GSEA)
6 and pathway analysis with R packages: fgsea v1.26.0 and msigdb v7.5.1. Moreover,
7 DEGs were analyzed for enrichment of previously curated neuroendocrine signatures¹³.
8 For this, we used the java GSEA Desktop Application (v4.3.2) with the GSEA Preranked
9 module using the variance-stabilized log fold changes as metric to rank the DEG list in a
10 descending order.

11

12 **snRNA sequencing**

13 Briefly, a single 10-week RPM tumor was extracted from the mouse prostate, and EGFP
14 signal assisted in tumor microdissection (Nikon, SMZ18). Tumor sample was sliced into
15 ~5 mm x 5 mm pieces, dabbed on a kimwipe to remove moisture, and flash frozen in
16 liquid nitrogen. Tumor piece was loaded onto a Singulator 100 (S2 Genomics) cartridge
17 supplemented with 3.5 μ L of 1 M DTT (Sigma, 43816-10mL) and 87.5 units of Protector
18 RNase inhibitor (Sigma, 3335402001). Nuclei were isolated with standard-nuclei isolation
19 protocol according to manufacturer recommendations. Nuclei suspension was cleaned
20 with sucrose density gradient (Sigma, NUC201-1KT) in protein low-bind microcentrifuge
21 tubes (Eppendorf, 0030108442) at 500g for 5 min. Nuclei were subsequently
22 resuspended in nuclei wash buffer: 10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 1
23 mM DTT, 1% BSA, 1 U/ μ L Protector RNase inhibitor, strained through blue-capped 35

1 μ m FACS tube, and flow sorted for 7-AAD+ population to obtain single nuclei suspension.
2 Sorted nuclei were submitted to the Single Cell Analytics Innovation Lab (SAIL) at
3 MSKCC. Nuclei were validated for integrity under phase-contrast microscopy, and
4 processed on Chromium instrument (10X Genomics) following user guide manual for 3'
5 v3.1 snRNAseq. Nuclei were captured in droplets, emulsions were broken, and cDNA
6 purified using Dynabeads (Thermo, 37012D) followed by PCR amplification per manual
7 instruction. ~10,000 cells were targeted for each sample. Final libraries were sequenced
8 on Illumina NovaSeq S4 platform (R1 - 28 cycles, i7 - 8 cycles, R2 - 90 cycles) at the
9 Integrated Genomics Operation (MSKCC). FASTQ files were processed using the 10X
10 Cell Ranger 6.1.2. Cell Ranger count was used to align reads to the GRCm38 (mm10)
11 reference genome for snRNAseq samples and a modified version of GRCm38 (mm10)
12 that includes the *Myc-P2A-EGFP* transgene sequences, given that organoids were
13 infected with lentiviruses harboring its expression (**Extended Data Fig. 1b**). We set
14 "include introns=TRUE" to accommodate the higher rate of intronic reads in snRNA.
15 Finally, Cell Ranger count was used to generate feature-barcode matrices for subsequent
16 bioinformatic analyses.

17

18 **Spatial transcriptomics by 10X Genomics Visium**

19 We generated Visium data from two adjacent sections as technical replicates, with each
20 slide containing 10-week RPM tumor tissues from two individual mice. Visium Spatial
21 Gene Expression slides were prepared with FFPE sections by the Molecular Cytology
22 Core at MSKCC. Tumor samples with a target RIN value > 0.5 were processed for spatial
23 transcriptomics. Probe pairs targeting the whole transcriptome were added to slide

1 capture areas and allowed to hybridize overnight at 50°C. Bound pairs were ligated to
2 one another, then released from the tissue by RNase treatment and permeabilization and
3 captured by oligos bound to the slide. Probe extension and library preparation proceeded
4 using the Visium Spatial for FFPE Gene Expression Kit, Mouse Transcriptome (10X
5 Genomics, 1000337) according to the manufacturer's protocol. After evaluation by real-
6 time PCR, sequencing libraries were prepared with 11-14 cycles of PCR. Indexed libraries
7 were pooled equimolar and sequenced on a NovaSeq 6000 in a PE28/88 run using the
8 NovaSeq 6000 SP Reagent Kit (100 cycles) (Illumina). FASTQ files from sequencing
9 (NovaSeq) were processed via spaceranger count (version 2.0.0) to align reads to the
10 GRCm38 (mm10) reference genome and generate count matrices for subsequent
11 bioinformatic analyses. An average of 74 million paired reads was generated per sample,
12 corresponding to 37,000 reads per spot.

13

14 **Pathology annotation and spatial immunofluorescence analysis**

15 Sections processed for H&E and multiplexed IF were reviewed by a board-certified
16 genitourinary pathologist (A.G.). Graded histological areas were used to identify regions
17 of PRAD and NEPC on serially sectioned samples processed for 10X Visium and
18 multiplexed IF.

19

1 Cell segmentation: We utilized Mesmer⁶³, a deep-learning algorithm designed for cell
2 segmentation, to identify cell boundaries in COMET images. The input to Mesmer is a
3 single nuclear image and single membrane or cytoplasm image. We used DAPI as a
4 nuclear marker. To demarcate various cell types, we merged images from multiple cell-
5 type-specific markers of membranes or cytoplasm by applying min-max normalization to
6 each channel before summing them. The min-max scaling was performed using the
7 *MinMaxScaler* function in *sklearn.preprocessing* package using default parameters. For
8 COMET, we combined CD45, CD20, CD4, CD8, CD11b, CD11c, Ly6G (immune cells),
9 CD31 (endothelial cell), GFP, KRT8 (tumor epithelial cells), VIM, α -SMA (stromal cells).
10 The markers were chosen because they cover the cell types of our interests. We ran
11 Mesmer on these images with standard parameters to predict cell boundaries (modified
12 slightly to exclude cells smaller than 36 pixels), and calculated the cell size, eccentricity,
13 and centroid of each cell boundary. We preprocessed COMET images to half their size
14 (to 0.46 μ m per pixel) to accommodate system memory constraints (128 GB).

15

1 *Normalization:* Initially, we quantified raw per-cell marker expressions by aggregating
2 pixel brightness within each cell boundary. To neutralize cell size variance in our
3 analysis, expressions per channel were normalized against cell boundary area. We
4 found bimodal distributions of cell size and DAPI expression in our dataset. The lower
5 mode of DAPI contained primarily empty regions and the upper mode of cell size
6 contained cell segmentation errors. We then filtered all cells with DAPI value less than
7 2096 (estimated from the distribution) and cell size larger than 2500 (estimated from the
8 distribution). The marker intensity signals then underwent logarithmic transformation.
9
10 *Cell type identification:* For tumor cell identification within our dataset, we used K-
11 nearest neighbor analysis (k=30) and clustered normalized marker expressions using
12 the Leiden algorithm⁶⁴, yielding 27 distinct clusters. These clusters were labeled as
13 stromal, tumor (notably marked by GFP, ASCL1, KRT8), or artifacts (characterized by
14 low marker expression), with artifact cells excluded from further analysis. Cell types
15 were then classified based on marker intensity distributions: lymphatic endothelial cells
16 (LYVE1>7.5), blood vessel endothelial (CD31>8), and immune (CD45>8). The
17 distribution patterns of this coarse classification is reported in **Figure 4a**. With the same
18 approach, we identified sub population in immune cell one by one: CD4 T cell (CD4>7),
19 B cell (CD20>8), CD8 T cell (CD8>8.3), Treg (FoxP3>8), CD11b F4/80+ myeloid (Mac3;
20 CD11b>7 & F4/80>7), CD11b F4/80- myeloid (Mac1; CD11b>7 & F4/80<7), CD11c
21 F4/80+ myeloid (Mac2; CD11c>8.5 & F4/80>7).

22

1 *Spatial analysis*: To examine cellular organization within lymph node (LN) tissue,
2 particularly dense with immune cells, we constructed a spatial neighborhood graph by
3 linking cells within a 20-pixel radius, approximately 2.5 times the median cell radius.
4 This analysis indicated an average of 4.5 neighbors per cell. A neighborhood
5 enrichment matrix was generated to map interactions between cell types, with axis
6 labeling indicating source and neighboring cell types and matrix values representing
7 total neighboring counts. Normalizing these counts by the total number of neighbors per
8 cell type produced a cell proximity frequency matrix (illustrated in **Figure 4g**),
9 showcasing the likelihood of each cell type neighboring another. The spatial
10 neighborhood graph and the neighborhood enrichment matrix were implemented
11 utilizing Squidpy⁶⁵.

12
13 For infiltration analyses, we employed HALO and HALO A.I. module v.3.6.4134 (Indica
14 Labs). HALO A.I. module was used to obtain nuclear and cytoplasmic segmentation
15 masks from a set of training slides. Cell nuclei were segmented using the DAPI channel.
16 The trained module was then applied to each experimental slide. Annotations of nuclear,
17 cytoplasmic, and background staining were reviewed manually for each slide.
18 Thresholding parameters for each marker were kept consistent across all slides. Single
19 cells were filtered to exclude errors in cell segmentation through DAPI-based criteria,
20 including: 1) nuclear DAPI measurements above a user-input threshold, and 2) the
21 nuclear/cytoplasmic ratio DAPI measurement was above a user-input threshold. Cell
22 types were classified based on a hierarchy for positive or negative stains. Each cell is
23 evaluated based on predetermined marker co-expression and then assigned as cell types

1 in a layered fashion (**Supplementary Table 6**). PRAD and NEPC regions were manually
2 annotated as above. Exported data files were used for image analysis. For distance
3 infiltration metrics, each individual primary tumor region was separated into 15 bins
4 spanning a total of 1000 μm inside and outside the region of interest and within the
5 confines of the tumor boundary. For smaller metastatic nodules in the lung and liver, 10
6 bins spanning a total of 500 μm inside and outside and within the confines of the tissue
7 were used for cell infiltration analysis. All tumors within an individual metastatic tissue
8 sample were pooled together for infiltration analysis. Cells residing within each bin were
9 quantified and normalized to the bin area. Subsequently, the data mean cell density per
10 bin was averaged across independent mouse samples. Biological replicate samples ($n \geq$
11 3) were used to calculate standard error. Data visualized in R using the ggplot2 package.
12 Loess function was implemented to visualize smoothed error curves across the defined
13 tissue region. For histological characterization and quantification of tumor area, a
14 Random Forest tissue classifier was created on the HALO platform and iteratively refined
15 using a training set of $n=10$ primary and metastatic RPM tumors containing examples of
16 NEPC, PRAD, and stroma. A single primary RPM tumor derived from a castrated host
17 was used as training input for chondrosarcoma histology. All tissue classification was
18 subsequently cross-referenced with G.U. pathologist, Dr. Gopalan.

19

20 **snRNA-seq analysis**

21 *Preprocessing:* We first used CellBender⁶⁶ to remove ambient RNA molecules from the
22 raw count matrices with the parameter `--expected-cells 5000` and `--total-droplets-included`
23 `20000`. These parameters were chosen by inspecting the barcode rank plot generated by

1 Cell Ranger, following author recommendations. Defaults were used for all other
2 parameters. All downstream processing of snRNA-seq data and scRNA-seq data,
3 discussed in the section “Reclustering of the public scRNA-seq dataset”, were performed
4 in Scanpy⁶⁷. We next removed low quality cells, unexpressed genes, and potential
5 doublets from the CellBender output. We filtered to exclude i) genes detected in fewer
6 than 3 cells, ii) cells with under 200 genes or 1000 UMIs, and iii) cells with a mitochondrial
7 fraction above 10%. Mitochondrial and protein-coding ribosomal protein coding genes
8 were also omitted from downstream analyses, as these are often a large source of
9 spurious variance that can dominate clustering and confound deconvolution²⁹. We used
10 Scrublet⁶⁸ to remove doublets. After Scrublet, 4,872 cells were retained for downstream
11 analyses, with a median of 7055.5 UMIs per cell. Raw counts were normalized by log₂
12 (X+1) transformation, where X = library size of each cell / 10⁴.

13
14 *Dimension reduction, clustering and cell type annotation:* We selected the top 3000 highly
15 variable genes in each dataset using the Scanpy function `pp.highly_variable_genes` with
16 raw counts as input, `n_top_genes = 3000`, `flavor = 'seurat_v3'` and `span = 1`. We applied
17 PCA to these genes using the `scanpy.tl.pca` function with the `arpack` solver. We selected
18 30 PCs, which explain 40% of variance. For clustering, we used Phenograph⁶⁹ with `k =`
19 `30` and `clustering_algo = 'leiden'`, which generated 19 clusters. Cell typing was performed
20 using marker genes as shown in **Supplementary Fig. 4**. To distinguish malignant cells
21 from non-malignant cells, we performed copy number analysis inference using inferCNV⁷⁰
22 (Trinity CTAT Project, <https://github.com/broadinstitute/inferCNV>). We used myeloid
23 and endothelial cells determined based on marker expression as the normal cell

1 reference. inferCNV showed a major CNV group of low CNV score containing two
2 additional phenograph clusters, which were labeled as normal epithelial and
3 mesenchymal cells. All other clusters show significant CNV changes, and were labeled
4 as NE (neuroendocrine), EMT (epithelial-to-mesenchymal transition) and Tumor
5 Luminal/Basal according to expression of marker genes, with each subtype containing 6,
6 4 and 5 cell states respectively.

7

8 **Reclustering of the public scRNA-seq dataset**

9 To support BayesPrism and PrismSpot, we used an independent scRNA-seq data from
10 prostates derived from *Pten^{fl/fl}*; *Rb1^{fl/fl}*; *Trp53^{fl/fl}*; *Probasin-Cre* (PtRP) GEMMs¹³. We
11 chose to use this dataset as 1) to demonstrate generality, as the dataset was generated
12 from an independent experiment using a mouse model that similarly transitions from an
13 adenocarcinoma to neuroendocrine prostate cancer, 2) it contains increased cell type
14 diversity due to the mechanical and enzymatic digestion that skews towards non-tumor
15 cells (total cells $n = 67,622$). To improve resolution for our BayesPrism deconvolution
16 and PrismSpot analysis, we re-clustered the data to improve the granularity of
17 mesenchymal cells. Specifically, as mesenchymal cells were not the focus of the original
18 paper, authors only clustered it into two populations: endothelium and mesenchymal.

19

20 However, given the observed heterogeneity in this population, we subsetted GFP-
21 negative mesenchymal cells from this dataset, and reclustered this population. We
22 selected the top 3000 highly variable genes in each dataset using the Scanpy function
23 `pp.highly_variable_genes` with raw counts as input, `n_top_genes = 3000`, `flavor =`

1 'seurat_v3' and span = 1. Raw counts were normalized by $\log_2(X+1)$ transformation,
2 where X = library size of each cell / 10^4 . We applied PCA to these genes using the
3 scanpy.tl.pca function with the arpack solver and selected 30 PCs. For clustering, we
4 used Phenograph with $k = 30$ and clustering_algo = 'leiden', which generated 20 clusters.
5 Clusters were annotated by expression of marker genes curated from Niec et al.³⁰, guided
6 by hierarchical clustering over the PC space. Endothelial, lymphatic, glial cell,
7 pericytes/myofibroblast were clearly distinguishable based on the marker genes
8 (**Supplementary Fig. 6a**). The rest of the clusters fall into two major groups as shown by
9 hierarchical clustering and were annotated as Mes-1 and Mes-2 (Mesenchymal,
10 **Supplementary Fig. 6a and b**). The original mesenchymal cell type label was replaced
11 by this higher granular cell type annotation (**Supplementary Fig. 6c**).

12

13 **Analysis of Visium dataset**

14 *Cell type deconvolution using BayesPrism:* We deconvolved Visium data from two
15 replicates using the snRNA-seq data collected from the 10-week RPM tumor. We used
16 BayesPrism to perform statistical deconvolution as it has been previously shown to yield
17 accurate results for Visium data and outperformed other methods in multiple settings^{30,71–}
18 ⁷⁵. More importantly, BayesPrism jointly imputes the posterior of cell type fraction and the
19 cell type-specific gene expression profile in each Visium spot, enabling the cell type-
20 specific gene count matrices by PrismSpot, as described below.

21

22 To increase signal to noise ratio for deconvolution, we selected marker genes that are
23 differentially upregulated in each of the 19 cell types, which was defined as the 19

1 phenograph clusters in the snRNA-seq data. We then took the union of these marker
2 genes and deconvolved over these genes. Specifically, we performed a pairwise *t*-test
3 using the findMarker function from the scran package⁷⁶, provided in the wrapper function
4 get.exp.stat from the BayesPrism package. As each cell type is compared against every
5 other cell type, we need to summarize N-1 *p*-values and log₂ fold change statistics for
6 each cell type (N denotes number of total cell types). For each non-tumor cell types (4 in
7 total), i.e., mesenchymal, myeloid, normal L/B (luminal/basal) and endothelial cells, we
8 required both the maximum *p* value to be less than 0.01, and the minimum log₂ fold
9 change to be above 0.1, where the max and min were taken over every other cell type.
10 For each tumor cell type (15 in total), i.e., NE, EMT and tumor L/B, we required the same
11 threshold for *p*-value and log₂ fold change, but the max and min were taken over every
12 other non-tumor cell type. As a result, we avoided the comparison between tumor cell
13 types when taking the max and min to retain the maximum number of tumor-specific
14 genes for PrismSpot analysis. This was achieved by defining a coarse level cell type label,
15 where 15 tumor cell types were grouped into a single tumor cell type and using it as the
16 cell.type.labels argument, while setting the original 19 cell types as cell.state.labels in
17 BayesPrism's built-in function get.exp.stat. To speed up deconvolution we further
18 excluded genes expressed in less than or equal to 3 spots. This yielded 5,125 genes in
19 total used as the input for BayesPrism. We defined each of the 19 cell states as individual
20 cell types when constructing reference for deconvolution. We set pseudo.min to 0, the
21 numerical lower bound for genes with zero count in a given cell type, to maximize the
22 contrast between different cell types in the reference. All other parameters of BayesPrism

1 were used as default. We performed the updated Gibbs sampling for a more robust
2 correction of batch effects between the snRNA-seq and the FFPE Visium.

3
4 *Benchmarking of BayesPrism:* We benchmarked both the accuracy and robustness of the
5 cell type fraction deconvolved by BayesPrism. A big challenge in the deconvolution field
6 is the lack of ground truth for benchmarking. To estimate some ground truth of the cell
7 type fractions from the Visium data we used 1) histology with H&E, and 2) a marker
8 genes-based approach. The comparison with histologically defined NEPC regions
9 showed strong concordance with the fraction of NEPC cells inferred by BayesPrism (**Fig.**
10 **5c**). Additionally, we benchmarked the robustness of cell type fraction inferred by
11 BayesPrism by assessing the concordance across 5 histologically defined regions
12 between two technical replicates, which includes 3 neuroendocrine regions and 2 non-
13 neuroendocrine regions. As tissues are slightly shifted between technical replicates, we
14 manually adjusted the selected regions to achieve better spatial concordance on the x-y
15 plane. For each region, we compute the average fractions for each cell type. We then
16 computed both cell type-level and region-level Pearson's correlation coefficients, which
17 was shown in **Supplementary Fig. 4a-b**.

18

19 **PrismSpot analysis**

20 BayesPrism's deconvolution infers cell type-specific gene expression matrices for each
21 spot. For the Hotspot analysis, we use spot specific tumor gene expression as the input.
22 Specifically, to generate tumor-specific gene expression profiles, we summed up the
23 posterior mean of cell type-specific gene expression, Z , across all tumor clusters ($n = 15$)

1 outputted by BayesPrism. As Hotspot models the raw count data using negative binomial
2 distribution, we rounded up the posterior mean of Z . An important step in Hotspot analysis
3 is to define neighborhood structure over which the spatial co-expression is defined. In our
4 Visium data, we have two adjacent slides, referred to as technical replicates. Each
5 technical replicate contains tumor samples from two different mice, referred to as
6 biological replicates. To leverage the full set of Visium data points to yield higher statistical
7 power while accurately reflecting the neighborhood structure, we performed a single
8 Hotspot analysis for all Visium spots while restricting neighborhoods to be only within
9 each mouse and each technical replicate. We achieved this by shifting the coordinates of
10 Visium data points for each tumor sample in each replicate by a large numeric number,
11 e.g., 1000, such that coordinates have no overlap within the K th ($K = 6$, discussed below)
12 nearest neighbor for Visium spots from different tumor samples. We excluded i) genes
13 with zero count in all spots, and ii) spots containing fewer than 1000 genes or 1000 UMIs
14 from downstream Hotspot analysis. We define `n_neighbors=6` in the `create_knn_graph`
15 in Hotspot to include only Visium spots that are adjacent to each other and disabled
16 `weighted_graph` arguments to treat all adjacent spots and the self-spot equally. For the
17 analysis in **Figure 5**, we selected genes with spatial autocorrelation FDR less than 0.01
18 and transcription factors within the top 500 autocorrelation Z -score, followed by re-
19 computing the pairwise local correlation between the top 500 transcription factors. To
20 cluster transcription factors into modules, we used the “`create_modules`” function
21 provided by the Hotspot package, with parameters `min_gene_threshold=15`,
22 `core_only=True`, `fdr_threshold=0.05`, which performs a bottom-up clustering procedure
23 by iteratively merging two genes/modules with the highest pairwise Z -score.

1
2 To enhance the robustness of the identified gene modules against sampling noise, we
3 implemented a subsampling strategy. Specifically, we subsampled 60% of the reads from
4 the Visium data (tumor-specific gene expression deconvolved by BayesPrism) for each
5 mouse tissue on each Visium slide using multinomial distribution. This process was
6 repeated 100 times. Subsequently, we re-evaluated the clustering using the Hotspot
7 method, which reclusters the genes into modules using the recalculated pairwise local
8 correlations among genes. For each gene pair initially grouped together in a module using
9 the full dataset, we assessed their co-occurrence in the same module across the
10 subsampled datasets. This frequency of co-occurrence was defined as the consensus
11 score. We then calculated the average consensus score for each gene with all other
12 genes within the same module. A higher average consensus score indicates that a gene
13 consistently remains within its module across different subsampling simulations, and
14 hence stability within that cluster. Finally, we identified representative genes for each
15 module by selecting those with an average consensus score ≥ 0.8 (**Fig. 5e and**
16 **Supplementary Table 7**). In total, 71/181 genes from three modules were chosen as
17 their representative genes.

18
19 *Benchmarking of PrismSpot:* We compared the Hotspot results between PrismSpot and
20 “standard” Hotspot, i.e., only un-deconvolved raw count was used as input. We compared
21 the autocorrelation and pairwise local correlation coefficients over marker genes of
22 different cell types. We analyzed the Hotspot local correlation statistics over a more
23 comprehensive gene set without pre-filtering genes based on autocorrelation to

1 demonstrate the behavior of the statistics of different types of markers. We derived
2 marker genes from the GEMM scRNA-seq dataset as mentioned above and grouped the
3 cell types such that the granularity matches that of snRNA-seq reference. Specifically, we
4 grouped Mes-1 and Mes-2 as stromal; *Tff3*, mutant L1, mutant L2, mutant B1, NEPC-
5 *Pou2f3* and NEPC as tumor; macrophages, neutrophils, and DC as myeloid. We then
6 performed differential expression using the pairwise *t*-test between tumor, stromal,
7 myeloid, and endothelial cells, like the strategy described above. Genes with maximum *p*
8 value less than 0.01, and the minimum log₂ fold change above 0.1 were used as markers
9 of each cell type for benchmarking PrismSpot.

10

11 We performed one-sided paired *t*-tests to compare auto-correlation Z-scores between
12 PrismSpot and Hotspot on un-deconvolved raw counts, hereafter referred to as Hotspot.
13 To ensure the statistical power was similar in the comparison of autocorrelation scores
14 for each cell type, we selected the top 100 genes that pass the threshold mentioned above
15 based on log₂ fold change. For non-tumor cell types, i.e., endothelial, myeloid, and
16 mesenchymal cells, we define null hypothesis H₀: PrismSpot Z > Hotspot Z, while for
17 tumor cells we define H₀: PrismSpot Z < Hotspot Z. The *p*-values were 4.0×10⁻³, 2.1×10⁻
18 ⁶, 6.0×10⁻⁹, and 3.8×10⁻³ for endothelial, myeloid, mesenchymal and tumor cells,
19 respectively (**Extended Data Fig. 7c**).

20

21 Likewise, we performed one-sided paired *t*-tests to compare local pairwise correlation Z-
22 scores between PrismSpot and Hotspot across three categories, 1) between a pair of
23 tumor marker genes, 2) between a tumor marker gene and a marker gene for non-tumor

1 cell types, and 3) between a pair of marker gene for non-tumor cell types. As pairwise
2 local correlation can be both positive and negative, we performed statistical tests on the
3 absolute value of Z-scores. For tumor vs. non-tumor and non-tumor vs. non-tumor
4 categories, we define null hypothesis $H_0: |\text{PrismSpot}| > |\text{Hotspot}|$, while for tumor vs.
5 tumor category, we define $H_0: |\text{PrismSpot}| < |\text{Hotspot}|$. The p -value of tumor vs tumor
6 category was 3.3×10^{-5} . For tumor vs. non-tumor and non-tumor vs. non-tumor
7 categories, p values were less than the numeric limit 2.2×10^{-16} (**Extended Data Fig. 7g**).

9 **Analysis of human scRNAseq PRAD and NEPC myeloid subsets**

10 FASTQ files from a previously published single cell dataset of 12 prostate cancer
11 patients¹³ (histologically verified CRPC-PRAD ($n=9$) and NEPC ($n=3$)) was mapped to
12 human reference genome GRCh38 using cellranger-7.0.1 to generate count matrices
13 (transcripts/features x cells). Downstream analyses were performed using the Seurat R
14 package (version 4.4.0). Cells were removed if features were not detected in at least 10
15 cells. Cells were filtered based on the following criteria: 1) less than 500 features; 2) \geq
16 30% mitochondrial counts and \leq 500 UMI counts. Putative doublets were removed
17 using scDbIFinder⁷⁷. Combining samples from all CRPC-PRAD and NEPC sampled
18 yielded 63,834 cells x 30,519 features. To normalize the data, the “LogNormalize”
19 method was used with a pseudocount of 1 and a scale factor of 10,000. The top 2,000
20 highly variable genes were identified using the FindVariableFeatures() function. As
21 patient tended to cluster by sample instead of by cell type, fastMNN was utilized across
22 all cell types to perform batch correction (using all 30,519 features). Clustering was
23 performed using FindNeighbors() and FindClusters() functions with a resolution of 0.3

1 on the batch-corrected count matrix. The resolution value was determined to be 0.3 as
2 it best matched the expression patterns of all lineage markers. The clustered cells were
3 visualized using RunUMAP() with the first 30 dimensions from the dimensional
4 reduction 'MNN', and the clusters expressing myeloid lineage markers (*CD14*, *LYZ* and
5 *IL1B*) were identified with these cells being subsetted for downstream analysis (N=7,004
6 myeloid cells). Re-clustering with a resolution of 1 was then conducted these putative
7 myeloid cells using the batch-corrected matrix (from the upstream correction) yielded 17
8 clusters. Differentially expressed genes (DEGs) for each cluster were identified using
9 FindMarkers() with MAST algorithm (version 1.24.1) and thresholds of Bonferroni
10 adjusted P value < 0.05 and $\log_2FC > 0.5$. Of note, 4 clusters (N=1,282 cells) had low
11 UMI counts, 1 cluster (N=382 cells) showed top DEGs possibly indicative of doublet cell
12 types expressing markers for both epithelial cells and myeloid cells (*KLK3* and *CD14*)
13 and 1 cluster (N=42 cells) expressed high levels of proliferation related genes (*MKI67*,
14 *TOP2A* and *STMN1*) and therefore were removed. This yielded a total of 5,298 myeloid
15 cells (4,348 CRPC-PRAD and 950 NEPC cells). To identify the subtypes of tumor-
16 associated macrophages (TAMs), module scores from pre-defined gene sets for each
17 TAM⁷⁸ were used and scores were calculated using AddModuleScore(). Cells were
18 labeled based on the median and maximum signature scores per cluster.

19

20 **Analysis of human prostate SU2C dataset**

21 The FPKM-normalized RNA-seq from Abida et al., was downloaded from
22 https://github.com/cBioPortal/datahub/tree/master/public/prad_su2c_2019 (ref. 45). We
23 selected patient samples sequenced by the poly-A enrichment protocol, as it contains

1 more samples with histologically verified NEPC. In total there were 9 patients with
2 neuroendocrine features, and 50 patients with non-neuroendocrine features. To
3 compute the \log_2 fold change between neuroendocrine and non-neuroendocrine
4 samples, we computed the \log_2 ((mean expression of neuroendocrine samples +1) /
5 mean expression of non-neuroendocrine samples +1)) . The statistical significance was
6 computed using a two-sided Wilcoxon test.

7

8 **Statistics and reproducibility**

9 We used GraphPad Prism software v.9.5.1 for statistical analyses or in-house scripts in
10 R v.4.3.1 which are available from the corresponding author upon reasonable request.
11 Variance was similar between compared groups and p -values were determined by two-
12 tailed Student's t -test for all measurements comparing untreated to treated samples of
13 single time points. One-way analysis of variance (ANOVA) with Sidak's or Tukey's
14 multiple comparisons correction listed in the figure legends for comparisons across more
15 than two groups. For analysis between groups over multiple time measurements (growth
16 curves), two-way ANOVA was used with appropriate multiple comparisons testing listed
17 in the figure legends. Figure legends denominate statistical analysis used. No statistical
18 method was used to predetermine the required sample size. No data were excluded from
19 this study. Investigators were not blinded to allocation during experiments and outcome
20 assessment, except for mouse specific study analyses.

21

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