

1 **Extensive patient-to-patient single nuclei transcriptome heterogeneity in**
2 **pheochromocytomas and paragangliomas**

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16 **ABSTRACT**

17 Pheochromocytomas (PC) and paragangliomas (PG) are rare neuroendocrine tumors
18 of varied genetic makeup, associated with high cardiovascular morbidity and a
19 variable risk of malignancy. The source of the transcriptional heterogeneity of the
20 disease and the underlying biological processes determining the outcome in PCPG
21 remains largely unclear. We focused on PCPG tumors with germline SDHB and RET
22 mutations, representing distinct prognostic groups with worse or better prognoses,
23 respectively. We applied single-nuclei RNA sequencing (snRNA-seq) on tissue
24 samples from 11 patients and found high patient-to-patient transcriptome
25 heterogeneity of the neuroendocrine tumor cells. The tumor microenvironment also
26 showed heterogeneous profiles mainly contributed by macrophages of the immune
27 cell clusters and Schwann cells of the stroma. Performing non-negative matrix
28 factorization we identified common transcriptional programs active in RET and SDHB
29 as well as distinct modules? including neuronal development, hormone synthesis and
30 secretion, and DNA replication. Comparison of the SDHB and RET transcriptomes
31 with that of developmental stages of adrenal gland formation suggests different
32 developmental stages at which PC and PG tumors appear to be arrested.

33

34 **KEYWORDS**

35 Pheochromocytoma, neuroendocrine tumor, single cell RNA-sequencing,
36 transcriptome, heterogeneity, SDHB, RET

37 **INTRODUCTION**

38 Pheochromocytomas (PC) and sympathetic paragangliomas (PG) are rare
39 neuroendocrine tumors, originating from chromaffin cell-related populations located
40 inside or outside the adrenal glands, respectively. PCPG is associated with
41 significant morbidity and mortality [1]. The current therapy of choice is surgical
42 resection; however, the disease can be associated with a lifelong risk of tumor
43 persistence or recurrence [2].

44 A plethora of genes has been reported to be responsible for a diverse hereditary
45 background in up to 40% of PCPG [3, 4]. Based on the bulk transcriptional and
46 genomic profiles, PCPG has been divided into two major classes. Tumors in class 1
47 are predominantly extra-adrenal and display germline mutations in the succinate
48 dehydrogenase complex (SDHB, SDHC, SDHD collectively referred to as SDHx), the
49 most common form of PCPG. SDHx tumors have the worst prognosis with a 30–70%
50 risk of metastasis or recurrence [5]. Class 2 PCPG detected in 5% of hereditary
51 PCPGs comprise amongst others germline and/or somatic mutations of the RET
52 proto-oncogene and have a better prognosis.

53 In this study, we exploited recent advances in single-nuclei RNA-seq to compare the
54 gene expression landscapes of PCPG with SDHB and RET germline mutations and
55 explore the transcriptional heterogeneity and to gain insight into the molecular basis
56 of their different prognosis.

57 MATERIALS AND METHODS

58

59 Preparation of Single-Nuclei Suspensions

60 Previously selected tissue blocks were transferred for the RadboudUMC biobank and
61 stored at -80°C. Nuclei were prepared from frozen tissue under RNase-free
62 conditions. Briefly, samples were cut to ~7 mm pieces, while kept on dry-ice. The
63 pieces were minced in a pre-cooled douncer in 500uL ice-cold Nuclei EZ Lysis buffer
64 5x with pestle-A and 10x with pestle-B. The suspension was passed through a 70 µm
65 cell strainer and washed with 1.5 mL cold Nuclei EZ Lysis and incubated on ice for 5'.
66 The lysate was washed in Nuclei wash/resuspension buffer (1xPBS completed with
67 1% BSA and 0.2U/ul RNAsin Plus (Promega, #N2611) and passed through a 40 µm
68 cell strainer. Nuclei were stained with DAPI. To exclude doublets and debris from the
69 final mix and to precisely determine the number of loaded nuclei, we applied FACS.
70 15000 nuclei were sorted into a pre-cooled tube containing the RT-mix (RT-reagent +
71 TSO + Reducing agent B), right before loading the mix to one lane of the Chromium
72 chip, 8.3 ul RT-enzyme was added to the mix, according to the standard protocol of
73 the Chromium Single Cell 3' kit (v2). All the following steps for the library preparation
74 were performed according to the manufacturer's protocol. Paired-end sequencing
75 was used to sequence the prepared libraries using an Illumina NextSeq sequencer.

76

77 Single-Cell RNA-seq Data Processing and Quality Control (QC)

78 Raw sequencing data were converted to FASTQ files with bcl2fastq. Reads were
79 aligned to the human genome reference sequence (GRCH38) and counted with
80 STAR. The CellRanger (10X Genomics) analysis pipeline was used to sample
81 demultiplexing and single cell gene counting to generate the gene-cell expression
82 matrix for each library. The gene expression matrix was then processed and
83 analyzed by *Seurat* package in R. To filter out low-quality cells, we first removed cells
84 (nuclei) for which less than 300 or more than 4000 genes were detected. The cell
85 count and gene count information for single cell datasets of the PCPG samples are
86 listed in Table1.

87

88 Dimensionality Reduction, Clustering and Visualization

89 Data were normalized by sequencing depth, scaled to 10000 counts, log-
90 transformed, and regressed against the UMI-counts and percentage using the
91 ScaleData function of the Seurat package. Principal components analysis was
92 performed on the scaled data with the 4000 most variable genes. Using 15 first
93 principal components, we calculated a UMAP representation of the data for
94 visualization and calculated clusters using the *FindNeighbors* and *FindClusters*
95 functions with the resolution parameter set to 0.3. Marker genes differentiating
96 between the clusters were identified with the *FindAllMarkers* function. Before running
97 a second round of clustering after sub-setting the original dataset to a cell type of
98 interest, we applied the *DietSeurat* function to collect the unmodified expression
99 matrix of the subset of cells, without any transformations.

100 To identify cell types, we used sets of well-established marker and annotated each
101 cell type based on their average expression. Cluster (or clusters) marker genes were
102 determined with the *FindAllMarkers* function and required to be expressed in at least
103 25% of the cells in a cluster with a minimal log expression difference of 0.25 between
104 clusters.

105 **Inferred CNV Analysis from snRNA-seq**

106 Large-scale copy number variations (CNVs) were inferred from single-nuclei gene
107 expression profiles using the *inferCNV* package [6] using the *i3* HMM parameter, a
108 window size of 101 genes and the “cluster_by_groups” parameter is true. To identify
109 the distinct chromosomal gene expression pattern of neuroendocrine cells, all other
110 cell were set as the “reference” cells. CNVs in the reference cells would still be
111 detectable.

112

113 **Expression Programs of Intra-tumoral Heterogeneity**

114 We applied non-negative matrix factorization (NMF) via the *RunNMF* function of the
115 *swne* [7] package to extract transcriptional programs of malignant cells of each
116 sample. We set the number of factors to 28 for each sample. For each of the
117 resulting factors, we considered the top 50 genes with the highest NMF scores as
118 characteristics of that given factor. We used the *AddModuleScore* function in the
119 Seurat package to evaluate the degree to which individual cells express a certain
120 pre-defined expression program, and thus determine the scores. All tumor cells were
121 then scored according to the 280 NMF programs. Hierarchical clustering of the
122 scores for each program using Pearson correlation coefficients as the distance metric
123 and Ward's linkage revealed 10 correlated sets of metaprograms. The gene list of 10
124 meta-programs can be found in TableS5.

125

126 **Logistic Regression for Similarity Calculation**

127 To measure the similarity of a target single-cell transcriptome to a reference single-
128 cell dataset, we used the logistic regression method described in [8]. Briefly, we
129 trained a logistic regression model with elastic net regularization ($\alpha = 0.6$) on the
130 reference training set. We then used this trained model to infer a similarity score for
131 each cell in the query dataset for each cell type in the reference data. Predicted logits
132 were averaged within each cluster or sample group in the query dataset.

133 RESULTS

134 We performed single-nuclei transcriptomic profiling (snRNA-seq) on resected tumor
135 tissues from 11 treatment-naïve patients to generate a comprehensive PCPG atlas.
136 Molecular diagnoses revealed germline RET and SDHB mutations in 5 and 6
137 patients, respectively (**Table S1**). All RET-PCPG samples were retrieved from the
138 adrenal gland, while the SDHB-PCPG tumors are from various locations, including
139 the bladder, the adrenal gland, the retroperitoneal- and the mediastinal area.

140

141 Cell Type Composition of PCPG Tissue

142 Stringent quality filtering yielded 50,868 nuclei with an average of 1,800 genes
143 detected per nuclei (**Methods, Table S1**). The merged expression profiles were
144 compressed into a 2D-coordinate system using Uniform Manifold Approximation and
145 Projection for Dimension Reduction (UMAP). The cells were grouped into 20 clusters
146 and were annotated by their location, mutation group as well as patient ID (**Fig1A,**
147 **Table S2**).

148 Based on canonical marker genes, we identified three major groups of cell types:
149 neuroendocrine (NEs (markers TH, DBH, CHGB)), immune (PTPRC, CD163,
150 CD247), and stromal (COL4A1, COL1A2) cells (**Fig1B**). The analysis of cluster 7
151 revealed that it originated almost exclusively from one donor (P370) and was
152 hallmarked by elevated expression of typical adrenocortical rather than
153 adrenomedullar marker genes, such as CYP11A1 and CYP11B1 (**Fig. 1B-C, Fig.**
154 **S1B-C**). Hence, cells from donor P370 were considered non-representative and
155 excluded from downstream analysis. Neuroendocrine cells (NEs) represented the
156 largest cell fraction (63%, Clusters 0, 1, 2, 3, 4, 6, 10, 11, 16, 19), followed by the
157 stromal (16%, Clusters 9, 12, 13, 15, 17, 18) and the immune cells (16%, Clusters 5,
158 8, 14, 16) (**Fig. 1C**). Most NE clusters consisted largely of cells from single patients
159 (**Fig. S1A-C**). Cells of the tumor microenvironment (TME), however, occupied shared
160 UMAP territories (**Fig. 1D**). Based on these observations we decided not to apply
161 batch correction in subsequent analyses to maintain the biological heterogeneity.

162 To obtain a more detailed insight into the cellular complexity of the TME, the immune
163 and stromal cells were sub-selected separately for deeper analyses. Annotation of
164 the immune cells (**Fig. S2A**) resulted in the assignment of macrophages being the
165 major component of the immune TME [9] (expressing CD163, CDSF1R, TGFBI),
166 followed by T-cells (CD247, IL7R, TCF7) and B-cells (MS4A1, BLK, BANK1) (**Fig.**
167 **S2B**). The macrophages were the most heterogenous immune cells which could be
168 related to tissue-specific transcriptional programs as macrophages are widely known
169 to exert context specific functions [10, 11] (**Fig. S2A, arrows**). However, the adrenal
170 macrophages (colored green) that are derived from the same location but from
171 different tumor samples are very different. (**Fig. S2A, blue and red arrows**). This
172 suggests that the macrophage transcriptome not only has a strong locational but also
173 a tumor type-specific component. The T- and B-cells originating from different
174 locations and mutation groups appear rather similar as they clustered together.
175 Finally, annotation of the stromal group (**Fig. S2C-D**) revealed Schwann cells
176 (expressing SOX6, CDH19, NRXN1), endothelial cells (FLT1, PECAM1, PTPRB) and
177 fibroblasts (TAGLN, ACTA2, COL1A1).

178 The numbers of individual immune and stromal cell populations were deemed too
179 small for in-depth analysis and were not further investigated.

180 **RET and SDHB Tumor Cells Display Chromosomal Aberrations**

181 We explored inferred Copy Number Variation (iCNV) to determine large-scale
182 somatic chromosomal changes (**Fig. 2A**). Immune- and stromal cells served as
183 'reference' in the assumption that large CNVs do not occur in the non-malignant. In
184 agreement with published whole-genome sequencing profiles of PCPG tissue [12-
185 15], segmental loss in the p-arm of chromosome 1 (1p) was present in all examined
186 tumors regardless of the mutation type. Loss of 1p was not found in the TME cells
187 confirming the assignment of the neuroendocrine cells as PCPG tumor cells. In
188 addition, we observed widespread loss in other chromosomes for example the 3q
189 and 6p arms as well as patient-specific aberrations such as loss in chr21 and gain in
190 1q, 3q, 13q and 14q (**Fig. 2A**). Apart from a few exceptions (RET-PCPG P66) we
191 found different iCNV patterns in chr13 and chr15 in a subset of the tumor cells; in
192 P227 (SDHB-PCPG) we identified small variations in chr3 and chr17 but observed
193 few intra-individual heterogeneities.

194 In contrast to the extensive inter-individual and tumor-specific genomic aberrations,
195 the inferred genomic profiles of tumor cells within each tumor population were largely
196 homogeneous, suggesting that the genome remained largely stable following an
197 initial catastrophic event, the genome remained largely stable.

198

199 **Transcription Programs Separate RET- and SDHB PCPG Tumor Cells**

200 To assess the inter-tumoral heterogeneity between RET and SDHB PCPG tumor
201 cells, we selected and re-clustered the tumor cells. With this finer grained resolution,
202 we identified UMAP-clusters that consisted of cells mostly from one patient. This
203 impinged on both the UMAP-plots annotated by patient IDs (**Fig. 3A**) and the
204 heatmap annotation of the hierarchical clustering of top20 cluster markers (**Fig. 3B**,
205 **Table S3**), reinforcing the strong inter-individual heterogeneity observed in the iCNV
206 analysis. Selecting the tumor cells gave us the opportunity to determine the genes
207 that are differentially expressed between the mutation groups (**Fig. 3C**, **Table S4**).
208 The newly identified markers were associated with either overlapping KEGG
209 pathways ('nervous system development') or with gene ontology terms related to the
210 secretory function of chromaffin cells ('ion channel activities', data not shown)[16].

211 We wished to determine the transcriptional programs that are active across the tumor
212 cells and then identify the programs that are differentially enriched between RET and
213 SDHB tumors. We applied non-negative matrix factorization (NMF)[7] over the sub-
214 selected tumor cells to determine the full transcriptional spectrum behind the
215 intratumoral heterogeneity and to extract the most representative biological
216 processes in the tumor cells. Firstly, we identified 28 active transcriptional programs
217 in PCPG tumor cells of each sample, based on their transcriptional profiles at the
218 single cell level. The signature enrichment of these 280 programs was calculated in
219 every individual tumor cell of the whole dataset. Next, based on the enrichment
220 scores, we hierarchically clustered the programs and identified 10 metaprograms
221 (**Fig. 4A**). Genes were ranked according to their frequency of being present within
222 one metaprogram. The metaprograms spanned a narrow range of functions (**Table**
223 **S5**) including neuronal development (M1: BMPR1B, ROBO1; M2: NRG1, NTNG1;
224 M3: FGF14, ROBO1; M8: SYT1, CTNNA2; M10: HDAC9, RORA), ion channel
225 activity (M4: RYR2, PDE4B; M5: CACNA2D3, CHRM3), hormone synthesis (M9: TH,
226 GCH1) and proliferation (M6: BRIP1, HELLS). Metaprogram seven (M7) could not be
227 associated with a significant ontology term.

228 The hierarchical clustering of the metaprogram-scored cells unveiled two major
229 clusters separating RET from SDHB tumor cells (**Fig. 4B**). The subclusters within the
230 RET-branch segregated along the patient samples. In the SDHB-branch, however,
231 solely sample P313 formed a discrete subcluster, while the tumor cells of other
232 SDHB patients formed mixed subclusters. Surprisingly, the SDHB tumor cells
233 (originating from various anatomical locations) are less heterogenous than their RET
234 counterparts (originating from the adrenal gland).

235 The most pronounced differences in the average enrichment scores between the
236 RET (cluster 1) and the SDHB (cluster 2) cluster were evident at metaprograms M2,
237 M3, M4 and M5 (**Fig. 4C**). The 'ion channel activity' of the M4-M5 metaprograms is
238 highly enriched among the RET tumor cells indicating a high secretory activity of the
239 adrenal RET-pheochromocytoma tumor cells. The M9 'hormone synthesis' program
240 was more enriched among the SDHB tumor cells, but mainly due to patient P313. A
241 very small fraction of cells scored high for the 'proliferation' (M6) metaprogram,
242 revealing a low but appreciable proliferative capacity of the PCPG tumor cells.
243 Several metaprograms were associated with 'neuronal development' ontology terms
244 and were shared in both branches of the tumor group separations.

245 In sum, the NMF analysis revealed two main transcriptional programs in PCPG that
246 separated RET from SDHB tumor cells. Genes associated with ion channel activities
247 (secretion) were more enriched in RET tumors. We also observed that 'neuronal
248 development' was a highly represented transcriptional program in both PCPG tumor
249 cells.

250

251 **PCPG Tumor Cells Display Early Adrenal Developmental Signatures**

252 The NMF analysis revealed several metaprograms that were associated with
253 neuronal development but showed different enrichments scores among the mutation
254 groups. This implies the developmental signature as an important element of the
255 tumor cells' transcriptome, but the differences between the mutation groups were not
256 reflected in the ontology terms. To shed light on the developmental aspects of the
257 SDHB and RET-PCPG tumors, we compared the transcriptome of the cell types
258 identified in the developing human adrenal gland (8-21 weeks [17] with the PCPG
259 tumors. We applied logistic regression and calculated probability scores for cell type
260 matches (**Fig. 5A**). The analysis revealed that tumor cells were most similar to the
261 cells at the junction to sympathoblast and chromaffin cells, called the 'bridge cells'
262 [18]. The cell types of the PCPG microenvironment showed high similarity with their
263 normal cell counterparts in the developmental adrenal gland dataset.

264 The difference between the SDHB and RET-PCPG became even more evident when
265 the tumor cells of each patient were compared to the chromaffin developmental cell
266 types (**Fig. 5B**). The logistic regression confirmed that the RET-PCPGs were more
267 similar to the reference chromaffin cells, while the SDHB-PCPGs scored highest with
268 both the chromaffin and the bridge cell types, suggesting an earlier developmental
269 state.

270 **DISCUSSION**

271 We performed snRNA-seq and mapped the transcriptional landscape of PCPG to
272 investigate the tumor heterogeneity and to identify the transcriptomic programs that
273 are associated with the mutation group of the tumor. We explored the transcriptional
274 heterogeneity by the analysis of the transcriptomic profiles of 50,868 single nuclei
275 from 11 patients (counting all cell types from 5 RET- and 6 SDHB PCPG tissue
276 samples). This is the first study unveiling the PCPG heterogeneity and the
277 consequences of germline mutations at the single-cell level.

278 Neuroendocrine cells, the largest population in the dataset, were identified as tumor
279 cells on the basis of marker genes and in particular by inferring copy number
280 variations from gene expression levels [19]. The iCNV profiles revealed two important
281 features: firstly, the lack of tumor cell sub-clusters within patients suggests a single
282 initial catastrophic event that led to the birth of the tumor cells. Secondly, apart from
283 very few recurring aberrations, we identified rather patient-specific iCNV patterns,
284 marking the level of inter- and intratumoral heterogeneity in PCPG cellulome that
285 provided a challenge for tumor classification.

286 To identify the patterns of the single-nuclei transcriptomic profiles based on the tumor
287 cells, we applied NMF, an unsupervised learning approach that is employed to
288 approximate high-dimensional datasets in a reduced number of meaningful
289 components [7, 20, 21]. The analysis of single nuclei transcriptomes of >30,000
290 tumor cells resulted in 10 metaprograms across the entire tumor set. The
291 transcription programs related to ion channel activity (transmembrane transport)
292 separated SDHB and RET tumor cells. Based on biochemical analysis of plasma,
293 urinary and tissue samples, we previously [22] found that RET tumors produce (and
294 contain) higher concentrations of catecholamines but secrete them at a lower rate
295 than SDHB tumors. Our cohort was not split by the hormone synthesis metaprogram,
296 moreover (due to a single patient) it showed a higher mean enrichment in the SDHB
297 subset. However, it was split along the ion channel (transmembrane transport)
298 programs that we associated with secretion [23]. Metaprograms linked to neuronal
299 development were found to be active throughout the tumor cells irrespective of their
300 mutational groups.

301 To explore the developmental status of the tumor, we utilized published datasets of
302 the developing adrenal gland as reference. Applying logistic regression revealed that
303 the RET-PCPG tumor cells are transcriptionally more similar to developed adrenal
304 chromaffins, while SDHB-PCPG tumor cells appear to be in an earlier phase of
305 adrenal development. Our results suggest that PCPG tumor cells had a primarily
306 chromaffin-like phenotype suggesting that the chromaffin cell development state
307 maybe related to the mutation-associated prognosis.

308 In summary, we revealed extensive levels of heterogeneity among PCPG tumor cells
309 and identified transcriptional programs related to neuronal development as key
310 processes active in these tumor cells. We speculate that in RET-PCPG, the mutation
311 caused a development block during late chromaffin development as compared to the
312 'more immature' SDHB-PCPG tumors. To differentiate this developmental block from
313 alternative transformative events that could also lead to the modified transcriptomes
314 of the tumor cells, investigation of larger cohorts is needed. Understanding the origin

315 of the tumor and the sources of its heterogeneity may help the development of
316 targeted therapies.

317 **DATA AND MATERIAL'S AVAILABILITY**

318 **Lead Contact**

319 Further information and requests for resources and reagents should be directed to
320 and will be fulfilled by the Corresponding author.

321

322 **Materials Availability**

323 This study did not generate new unique reagents.

324

325 **Data and Code Availability**

326 The high-throughput datasets have been deposited in the European Genome-
327 phenome Archive. The accession number for the single cell expression data of
328 PCPG tumor samples reported in this study is EGAS00001006230. This study did
329 not generate any unique code. All software tools used in this study are freely or
330 commercially available.

331

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346

347 **ETHICS DECLARATIONS**

348 **Ethical Statement**

349 The study was approved by the Institutional Review Board of the Radboud University
350 Medical Center and informed consent was obtained from each patient (protocol no
351 9803-0060)

352

353 **Human Tumor Samples**

354 Tumor tissue samples of patients with a biochemically / histologically proven
355 diagnosis of pheochromocytoma/paraganglioma were investigated. Fresh samples
356 were snap frozen, usually within 1 hour after resection.

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- 443

444 **FIGURES**

445 **Figure1.**

- 446 A. UMAP visualization of all the 50,868 cells grouped according to their cluster
- 447 annotation and colored by their clusters, location of origin, mutation group or
- 448 patient ID
- 449 B. Violin plots displaying the expression levels of canonical markers of
- 450 representative cell types
- 451 C. Distribution of cell types across the merged dataset and per sample
- 452 D. UMAP visualization of all the 50,868 cells highlighting the cells annotated to
- 453 the main cell types. The UMAP clusters of NEs are also marked by their most
- 454 representative patient IDs

455
456 **FigureS1.**

- 457 A. UMAP visualization of the merged dataset separately annotated by patient
- 458 (blue: 'SDHB group', red: 'RET-group')
- 459 B. Fraction of cells per sample populating the UMAP-clusters
- 460 C. Fraction of cells per UMAP-clusters found per sample

461
462 **FigureS2.**

- 463 A. UMAP visualization of the PCPG immune cells subcluster after re-clustering
- 464 (no batch-correction). The arrows point at the cells annotated as
- 465 macrophages, found in tumors from similar anatomical locations. (blue: from
- 466 an SDHB-tumor, red: from a RET-tumor)
- 467 B. UMAP visualization and the relative expression levels of canonical cell type
- 468 markers across the PCPG immune cells subcluster
- 469 C. UMAP visualization of the PCPG stromal cells subcluster after re-clustering
- 470 (no batch-correction)
- 471 D. UMAP visualization and the relative expression levels of canonical cell type
- 472 markers across the PCPG stromal cells subcluster

473
474 **Figure2.**

- 475 A. Heatmap of inferred CNVs of NE cells (immune clusters and stromal clusters
- 476 were applied as reference). The patient IDs are colored by the mutation
- 477 groups.

478
479 **Figure3.**

- 480 A. UMAP visualization of the PCPG tumor cells subcluster after re-clustering (no
- 481 batch-correction), annotated by patient ID, tumor location, mutation group and
- 482 cluster
- 483 B. Hierarchical clustering of the differentially expressed genes for UMAP-clusters
- 484 across the PCPG tumor cell subclusters
- 485 C. Hierarchical clustering of the differentially expressed genes for RET and
- 486 SDHB mutation groups (sn-markers) across the PCPG tumor cells

487
488 **Figure4.**

- 489 A. Heatmap showing the correlation and hierarchical clustering of the 280 factors
- 490 calculated in our NMF-analysis of the tumor cells individual samples, across all

- 491 mutation groups. Metaprograms are numbered M1-M10 and annotated by
492 their representative ontology terms.
493 B. Heatmap showing scores of PCPG tumor cells for the 10 metaprograms
494 identified from the NMF analysis of individual samples.
495 C. Violin-plots showing scores of PCPG tumor cells for the 10 metaprograms
496 identified from the NMF analysis grouped per mutation group (black dots mark
497 mean, *Wilcoxon* $p < 2.2e-16$ within each Metaprogram).
498

499 **Figure5.**

- 500 A. Heatmap showing similarity scores (logistic regression and logit scale) of the
501 signatures of developing cell types from [17] (fetal adrenal dataset) (x axis) to
502 PCPG cells (y axis)
503 B. Heatmap showing similarity scores (logistic regression and logit scale) of the
504 signatures of developing adrenal cell types from [17] (fetal adrenal dataset) (x
505 axis) to PCPG tumor cells, by patient (y axis)
506

507 **Additional Data**

508 **Table S1.** Clinical information and snRNAseq quality parameters of
509 processed/analyzed samples

510 **Table S2.** Top50 differentially expressed markers of the 20 clusters in the complete
511 merged dataset

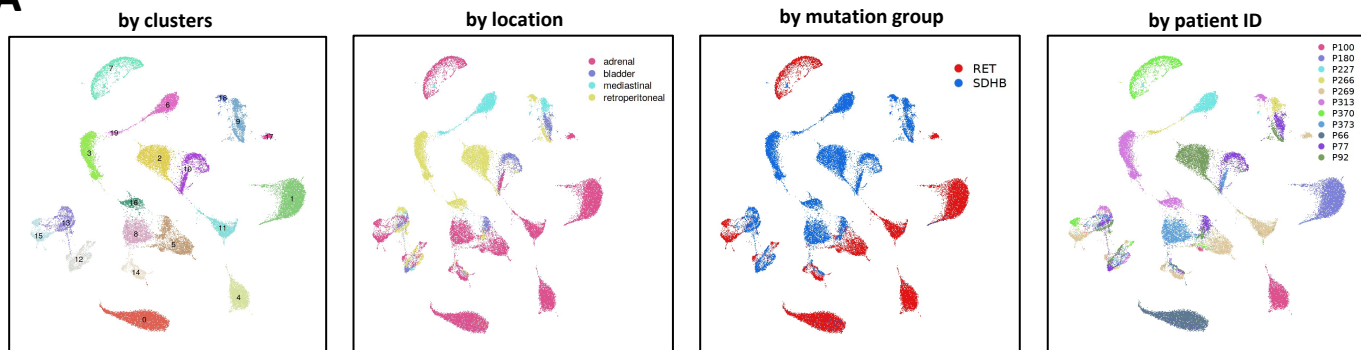
512 **Table S3.** Top50 differentially expressed markers of the tumor cell sub-clusters

513 **Table S4.** Differentially expressed markers of the of mutation groups based on the
514 tumor cells

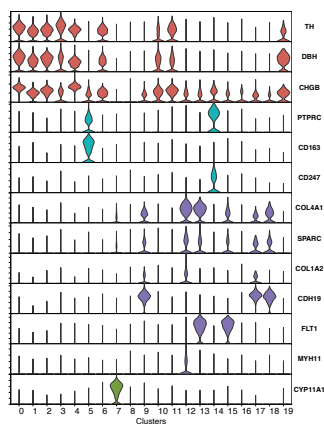
515 **Table S5.** Gene lists of the 10 metaprograms identified in the tumor cells. The cells
516 with unique markers (across the metaprograms) are colored blue.

Figure 1

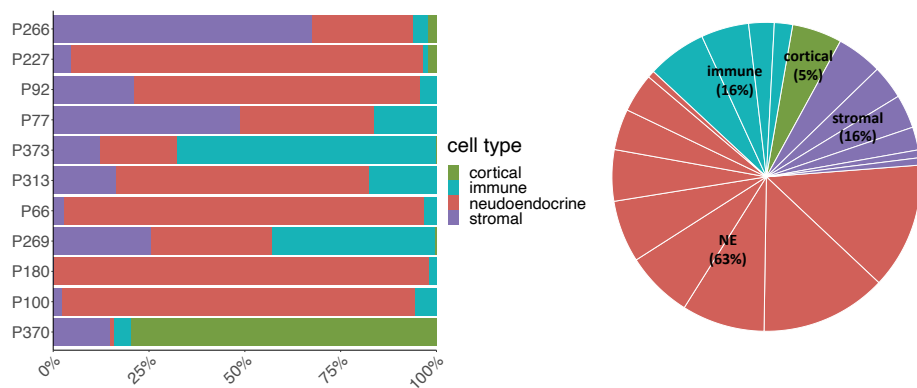
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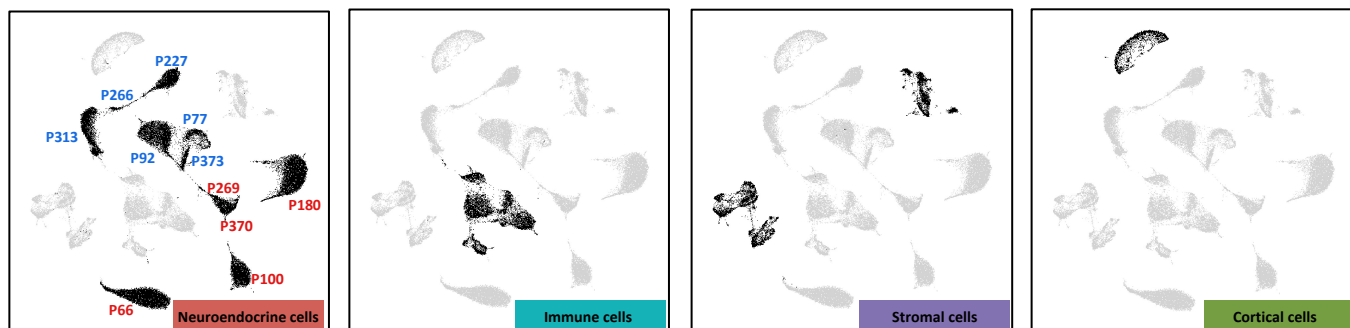
B



C



D



Supplemental Figure 2

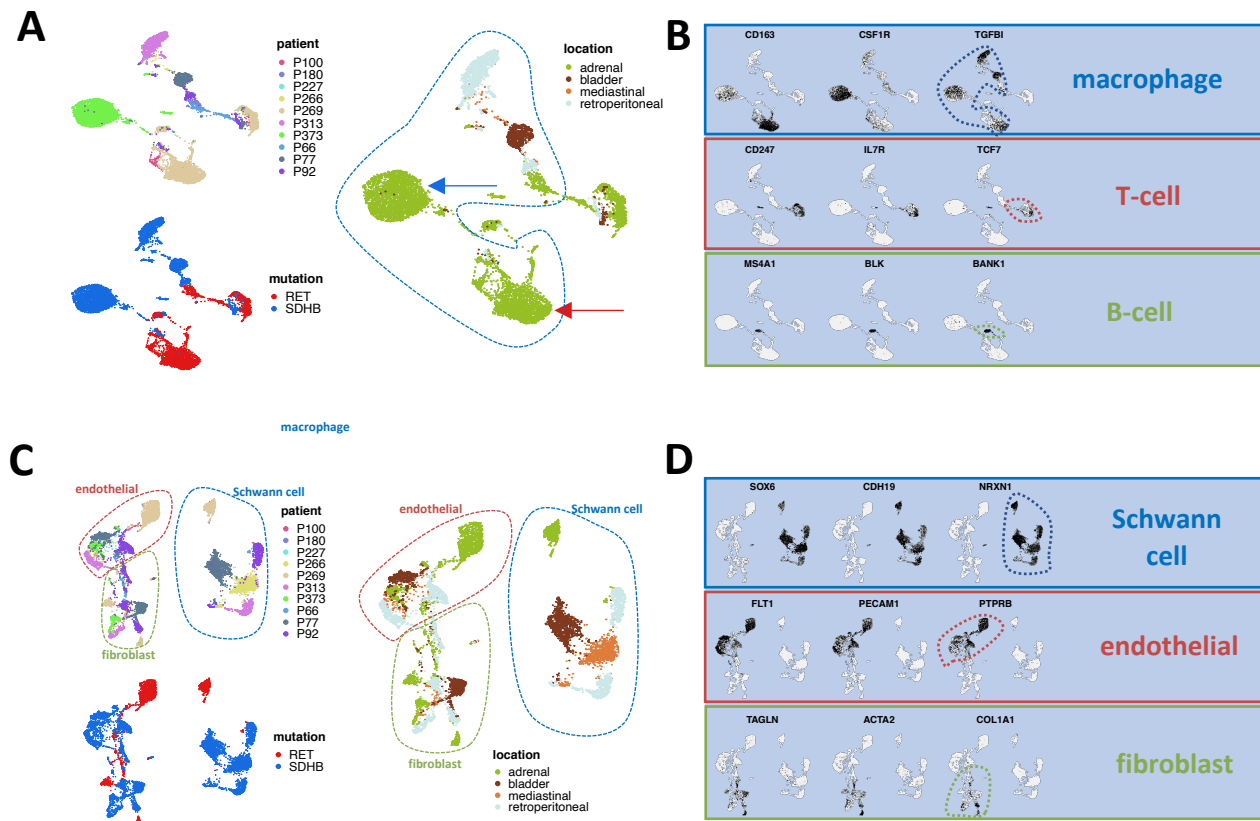


Figure 2

A

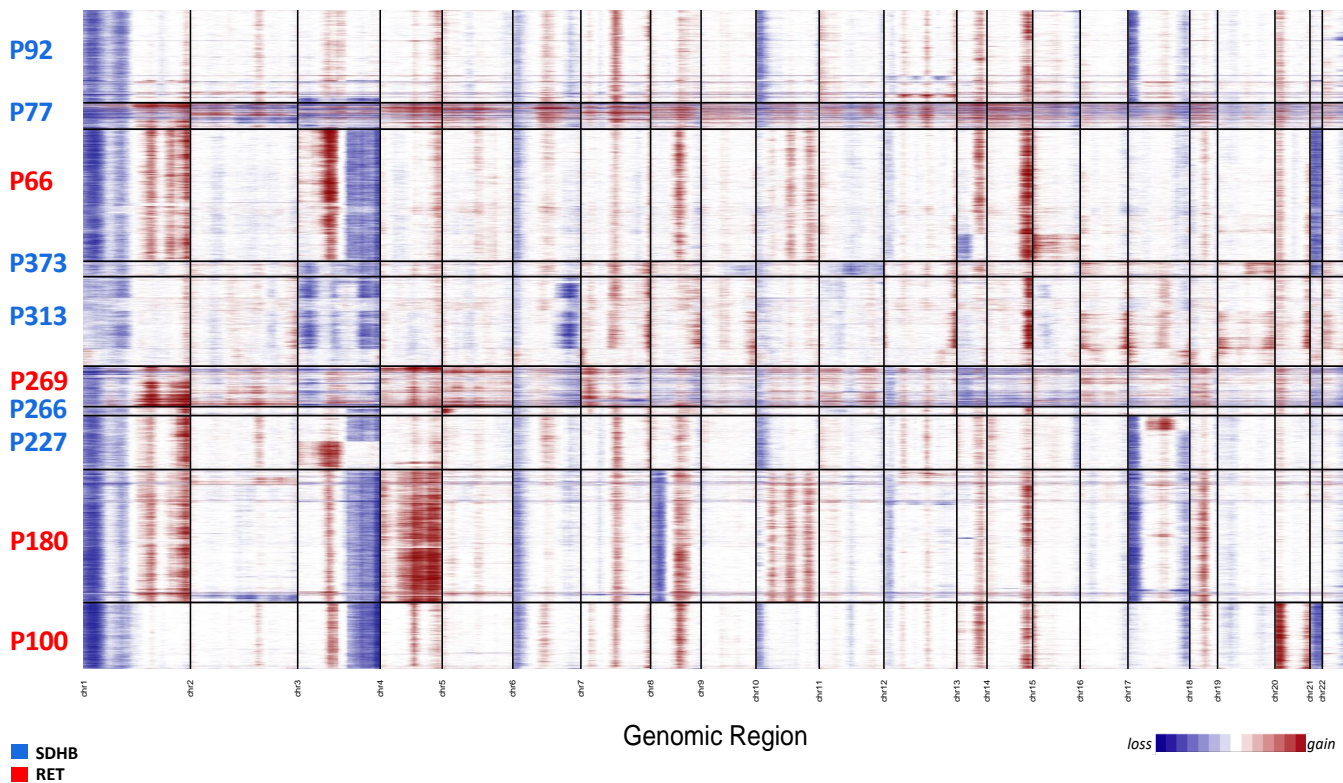
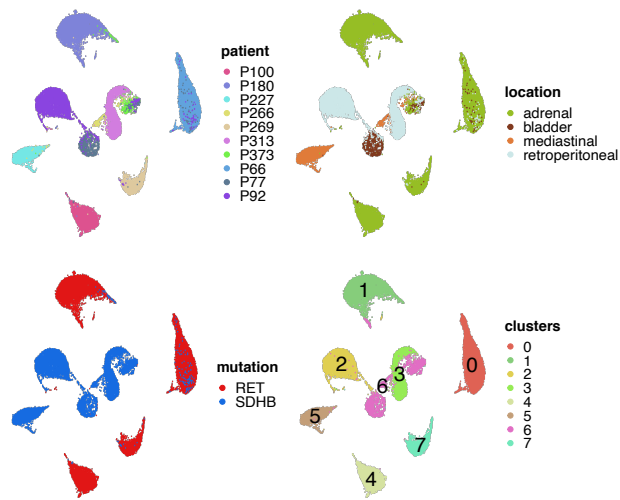
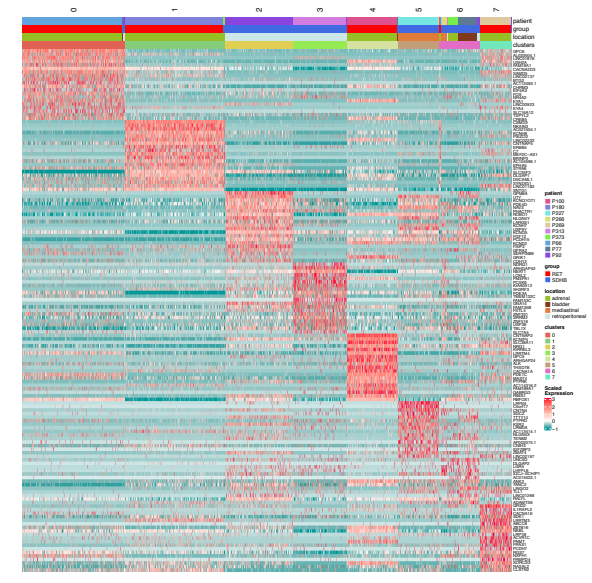


Figure 3

A



B



C

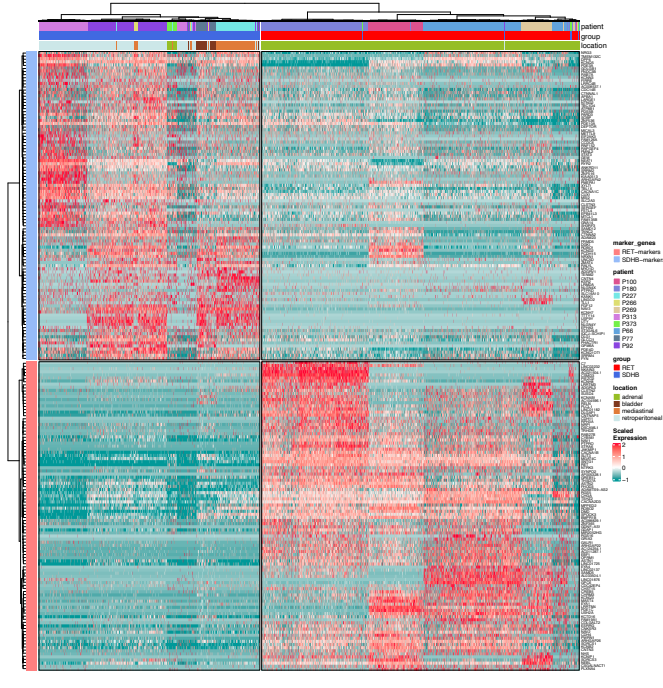


Figure 4

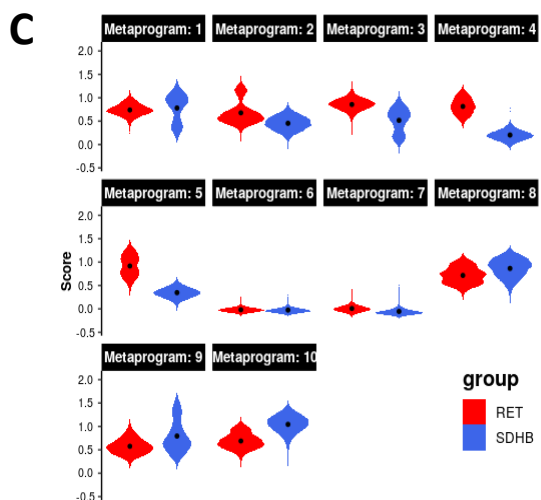
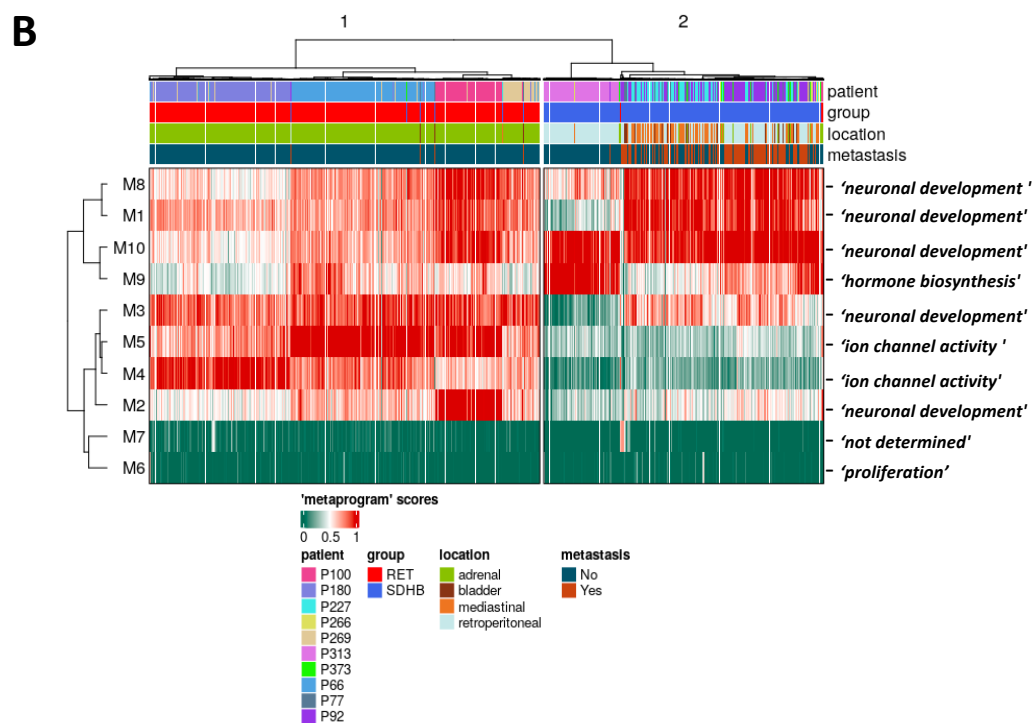
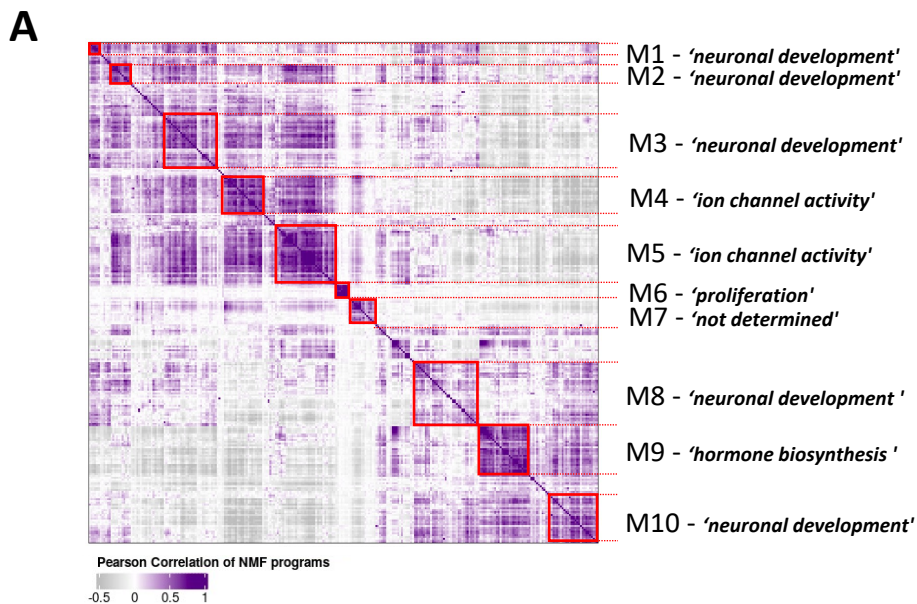


Figure 5

