Settima edizione di

G.d.L. Biologia Cellulare Molecolare

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ASSOCIAZIONE ITALIANA EMATOLOGIA ONCOLOGIA PEDIATRICA

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Deciphering chemotherapy resistance in neuroblastoma by single-cell transcriptomics

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Disclosures of Ferdinando Bonfiglio

Company name	Research support	Employee	Consultant	Stockholder	Speakers bureau	Advisory board	Other

Neuroblastoma (NB) is one of the most prevalent pediatric tumors, arising from neural crest cells of the sympathetic nervous system

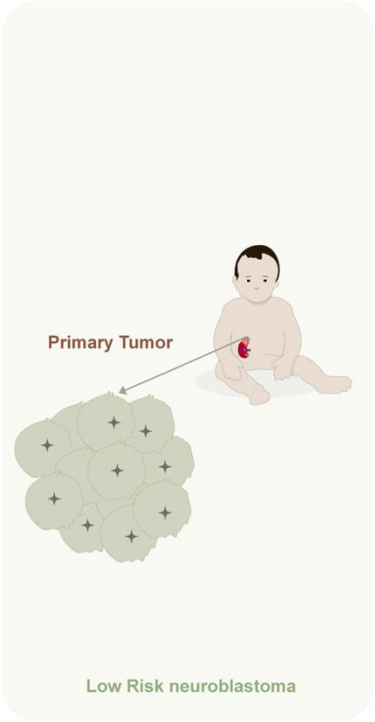
- Mostly **sporadic**, 2% familial cases (mutations in *ALK* and *PHOX2B*).

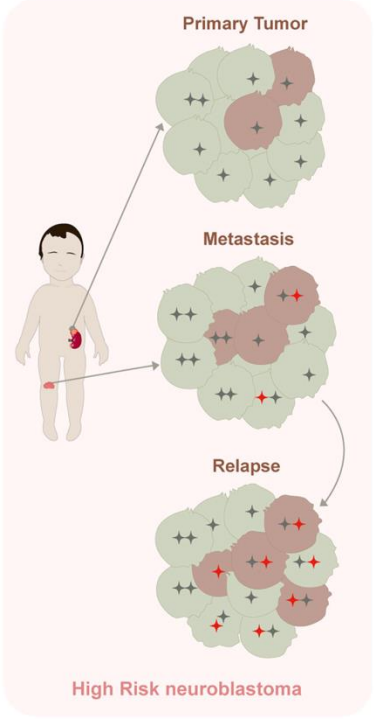
- **50% of NB are high-risk** with <50% survival at 5-years, despite recent advances in standard care

-recurrent genetic alterations (MYCN amp, -1p, -11q, +17q)

- cellular plasticity (adrenergic ⟺ mesenchymal transition)

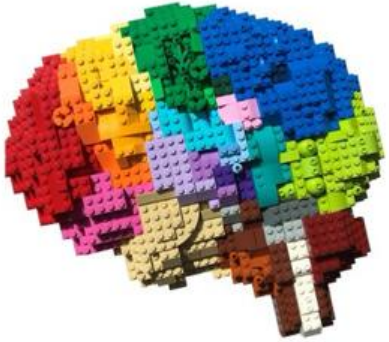
- A major challenge is the **intratumoral heterogeneity**, which cannot be adequately resolved using conventional bulk genomic or transcriptomic approaches.





Gomez et al, 2022

Recent single-cell RNA sequencing (scRNA-seq) studies have begun to resolve this complexity, revealing diverse cellular lineages and interactions within the NB



functional tissue

bulk RNA-seq

- Get a general idea about gene expression but lacks cellular resolution
- cost effective

single-cell RNA-seq

- Characterize the cell types in a tissue, discover new cell types, define differences across development and disease
- expensive
- high technical requirements
- complex data analysis

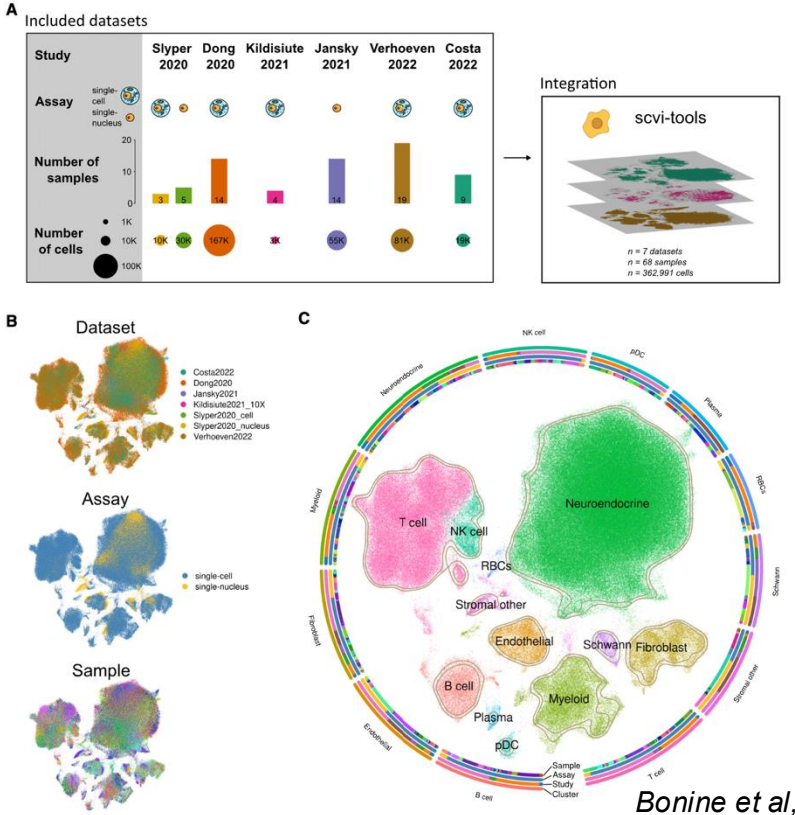
scRNAseq advanced our understanding of the NB intratumoral heterogeneity

- **NBAtlas**, a large harmonized single-cell/single-nucleus RNA-seq atlas of NB

- integrates datasets from 61 patients and 363k cells

- distinct tumor cell transcriptional states linked to clinical behavior and prognosis, providing a **detailed view of neuroblastoma heterogeneity** at single-cell resolution.

- a **reference resource** used for automated cell-type annotation and comparative analyses in future neuroblastoma and pediatric cancer studies.



Bonine et al, 2024

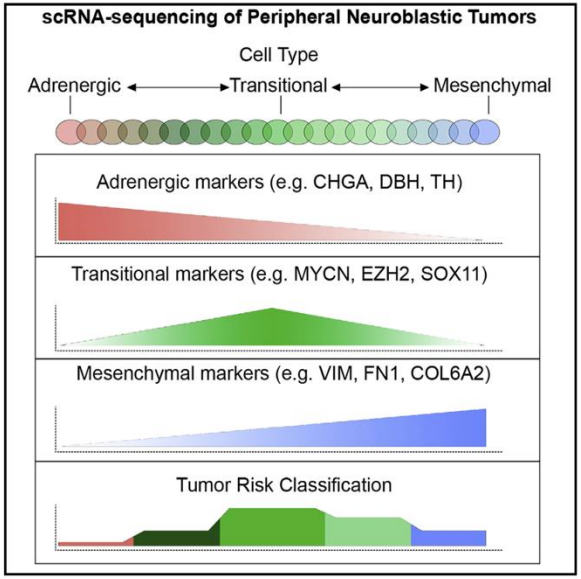
scRNAseq helped to characterize two core neoplastic adrenergic-like (ADR) and mesenchymal-like (MES) states in NB

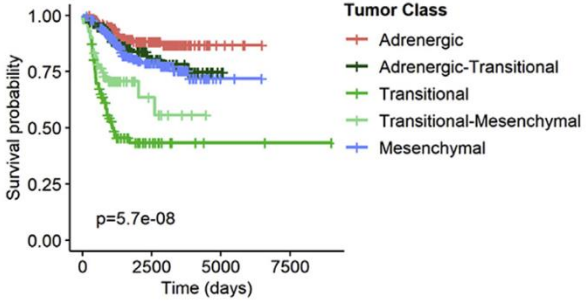
- N=10, malignant and non-malignant **cell heterogeneity**

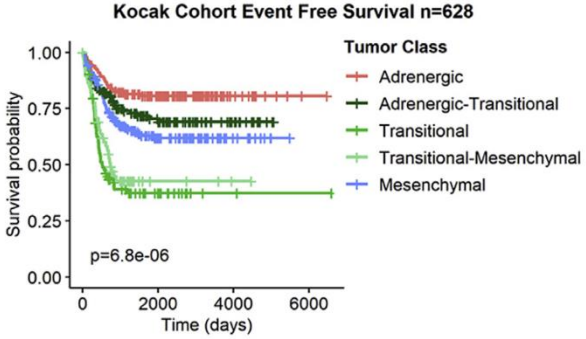
- "Classic" ADR state associated with better prognosis, MES state associated with higher risk

- A **malignant transitional cell state** bridges adrenergic and mesenchymal neuroblasts and associate with **aggressive disease**

- the concept: **NB cells dynamically shift along an ADR-to-MES trajectory**, epigenetic state transitions are central to tumor evolution and may represent therapeutic vulnerabilities.







Yuan et al, 2022

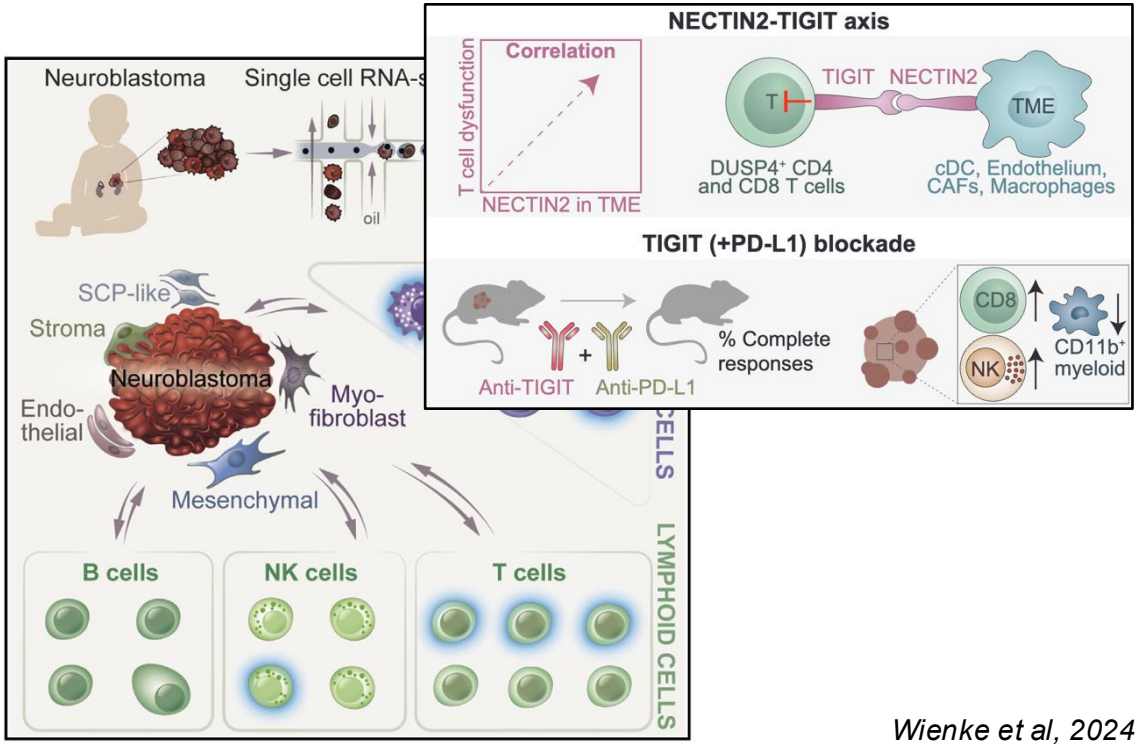
single-cell profiling of NB microenvironment has uncovered distinct immune subset and identified potential targets for immunotherapy

- 27 different immune subtypes → **complex microenvironment**

- Inflammatory monocytes correlate with disease progression, Active NK cells correlate with improved survival

- Interaction analysis identifies the **NECTIN2-TIGIT** axis as crucial immune checkpoint

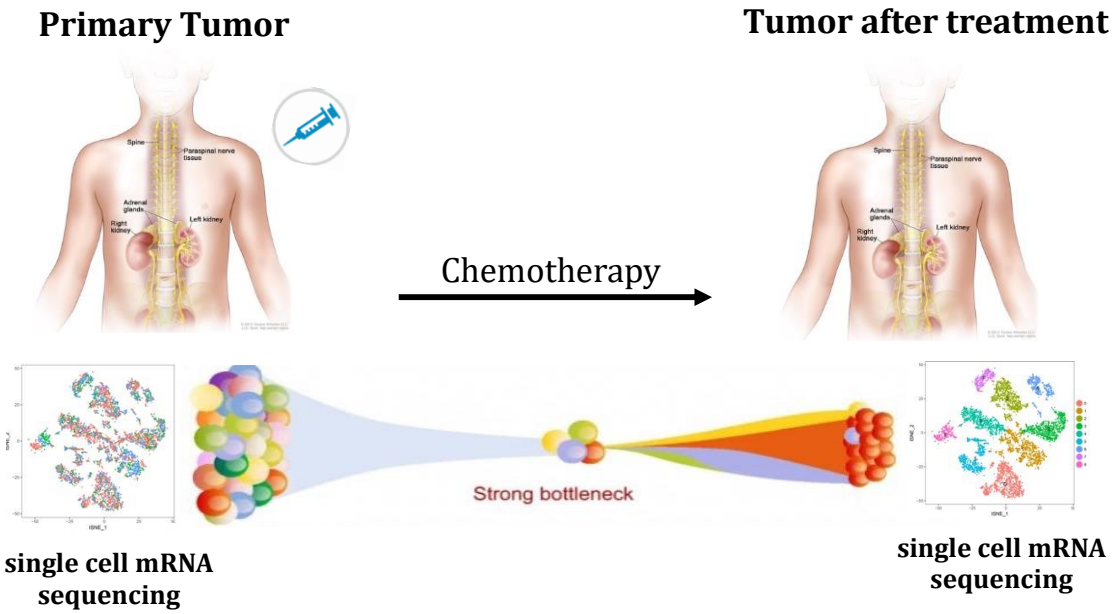
- TIGIT (+PD-L1) blockade **enhances immune responses against NB** in vivo



Wienke et al, 2024
Verhoeven et al, 2022

The interplay between NB cells and the role of **chemotherapy** to promote treatment resistance remain **unclear** due to the limited availability of studies using **matched pre- and post-therapy patient** samples.

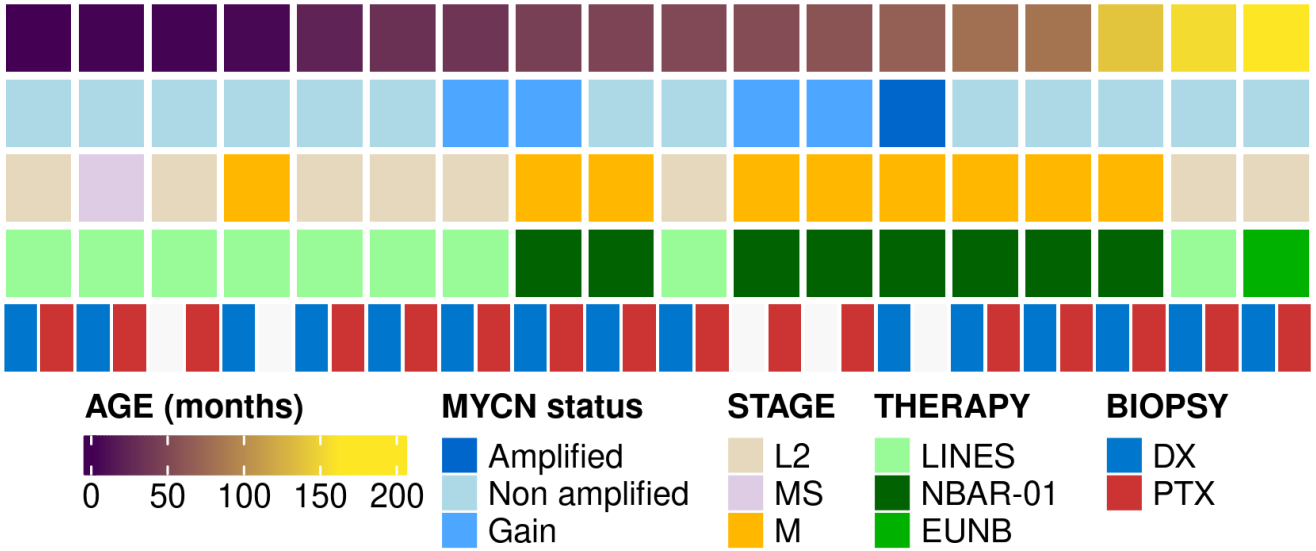
Comparative Single-Cell Transcriptomics of NB Tumors at Diagnosis and after Chemotherapy



Chemotherapeutic treatment: a combination of vincristine, carboplatin, etoposide, cyclophosphamide, and cisplatin

STUDY DESIGN

- 33 NB samples from 18 patients (15 pre- and 18 post-treatment), 13 matched
- mean age of 60 months
- 5 MYCN amplified tumors
- 50% stage M



Outline of the study

- GEXscope technology (microfluidic based)

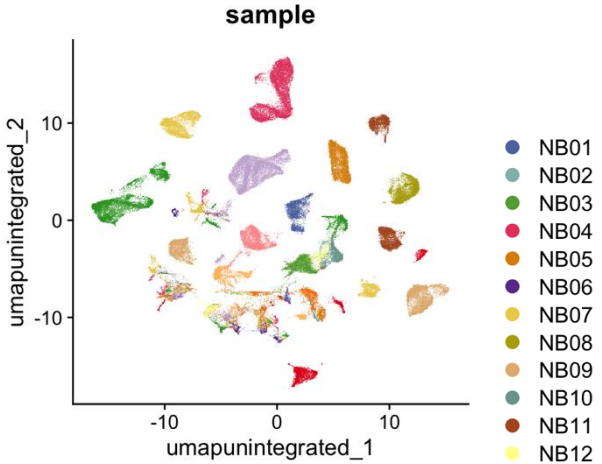
- **single nuclei RNA seq** (does not require the preservation of cellular integrity during sample preparation)

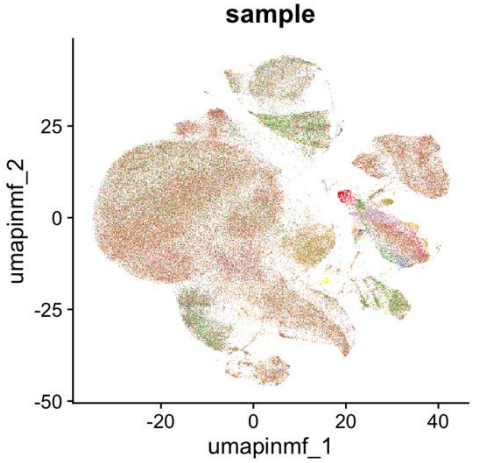
- 214,181 cells sequenced from 33 samples

- stringent quality control (QC) to remove low-quality cells, sequencing artifact, etc…

- total number of high-quality cells after QC: **111,604**

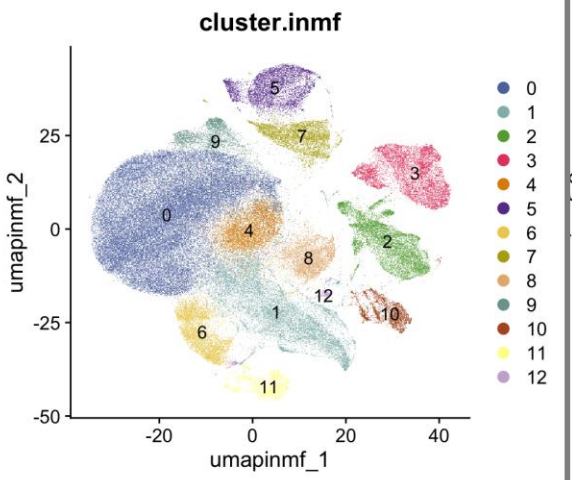
Unintegrated data



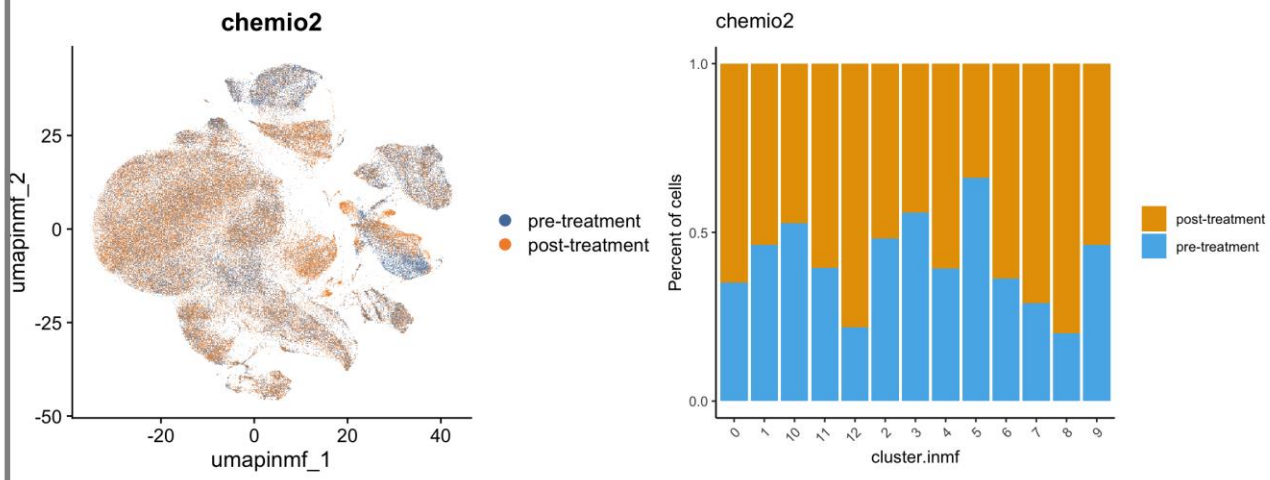


*Data from multiple samples successfully integrated with **our custom pipeline***

Clustering and abundance analysis



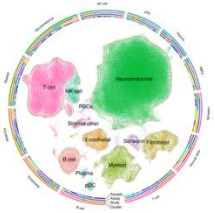
13 cell clusters identified



Clusters show differential abundance of cells pre- and post-chemo

unpublished data please do not tweet or shar

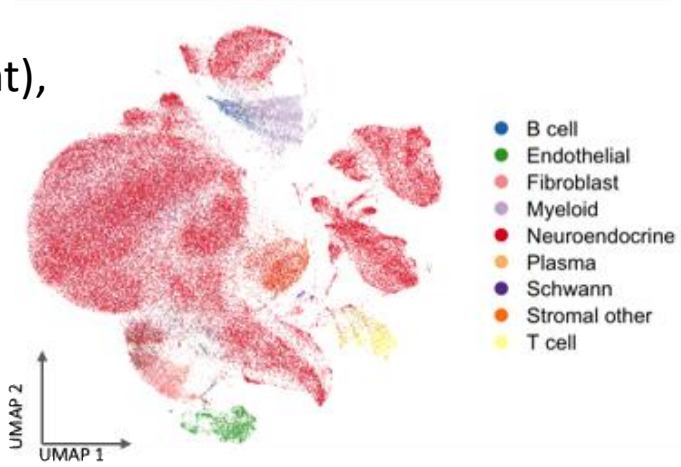
What kind of cells do we find in these samples?

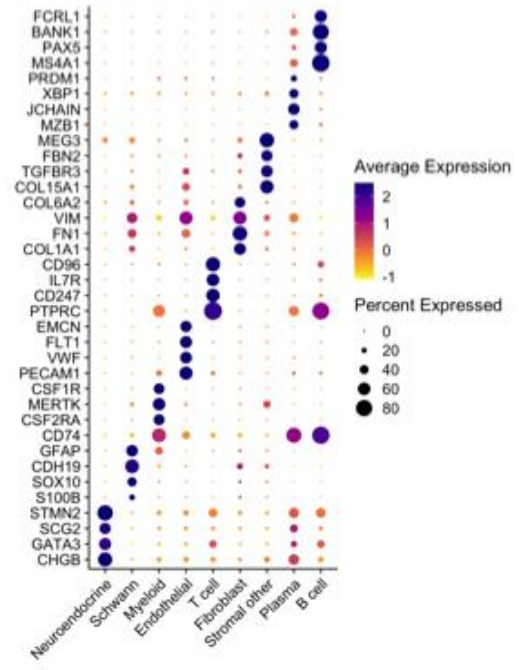


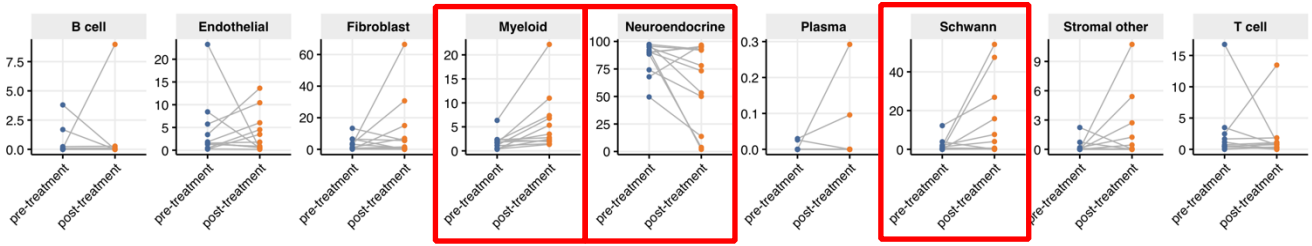
NBAtlas (reference)

CELL TYPE ANNOTATION

- 70% Neuroendocrine (Malignant), 20% Stroma, 10% Immune cells.
- cell marker analysis
- Differential abundance pre- vs post-chemo of neuroendocrine, myeloid and shwann cells

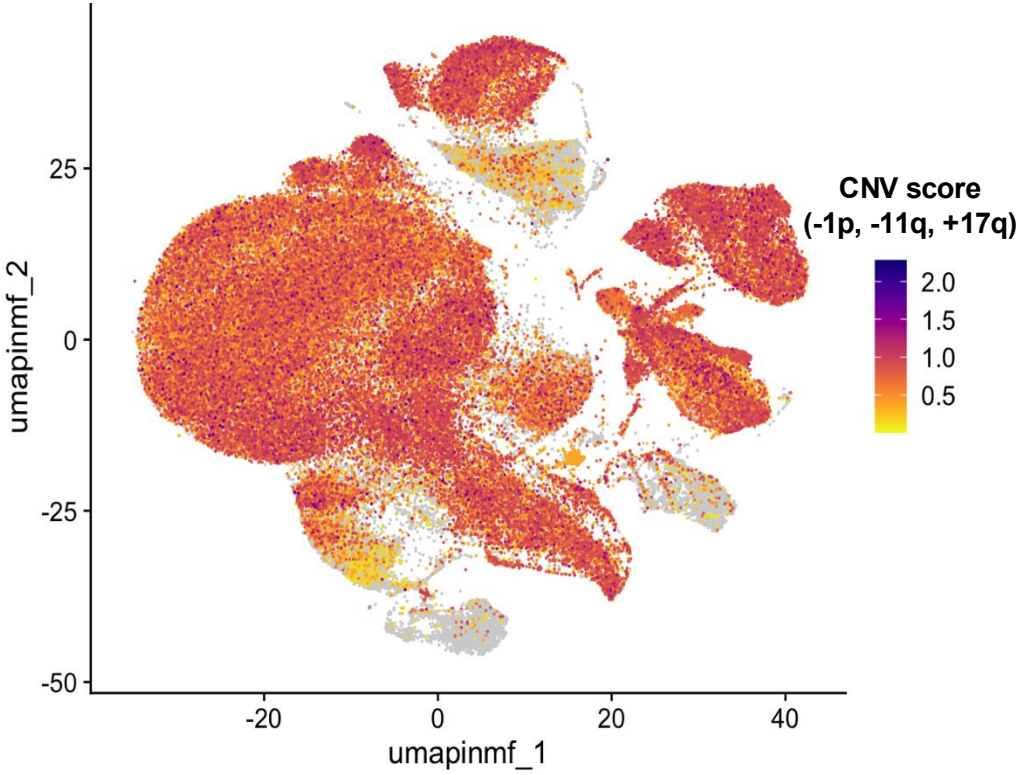






scRNAseq allows Copy Number Variation (CNV) analysis

CNV ANALYSIS
Neuroendocrine cells show hallmark NB-associated CNV (1p loss, 11q loss, 17q gain)

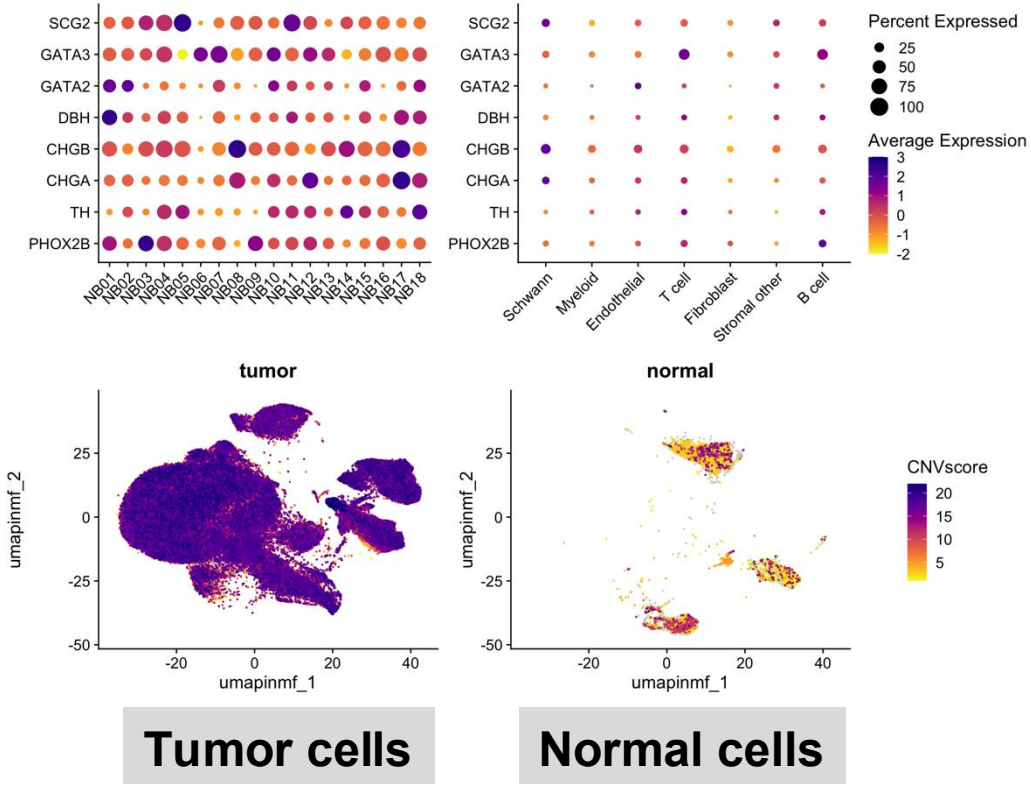


Tumor cells were selected according to annotation, CNV and clustering

Tumor cells selected according to:
- CNV score > 0
- "malignant" annotation in reference datasets

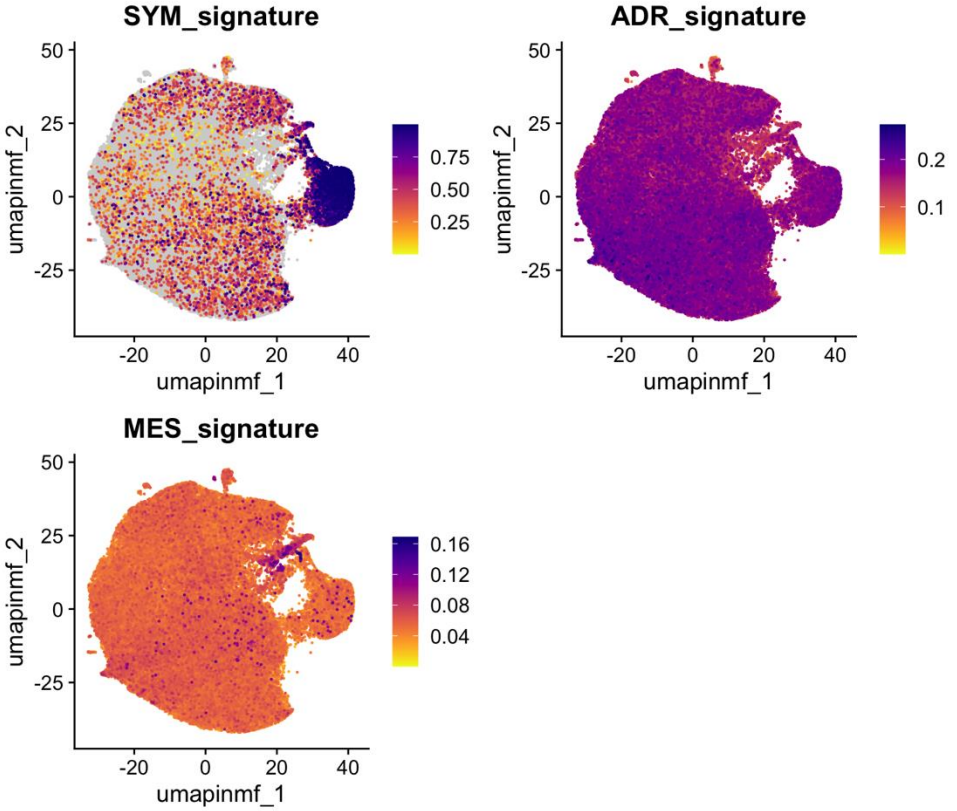
A total of **78,955** tumor cells (70.7%)

tumor cells express a typical NB gene **expression signature** (PHOX2B, TH, CHGB, GATA, etc…) and **high CNV score**



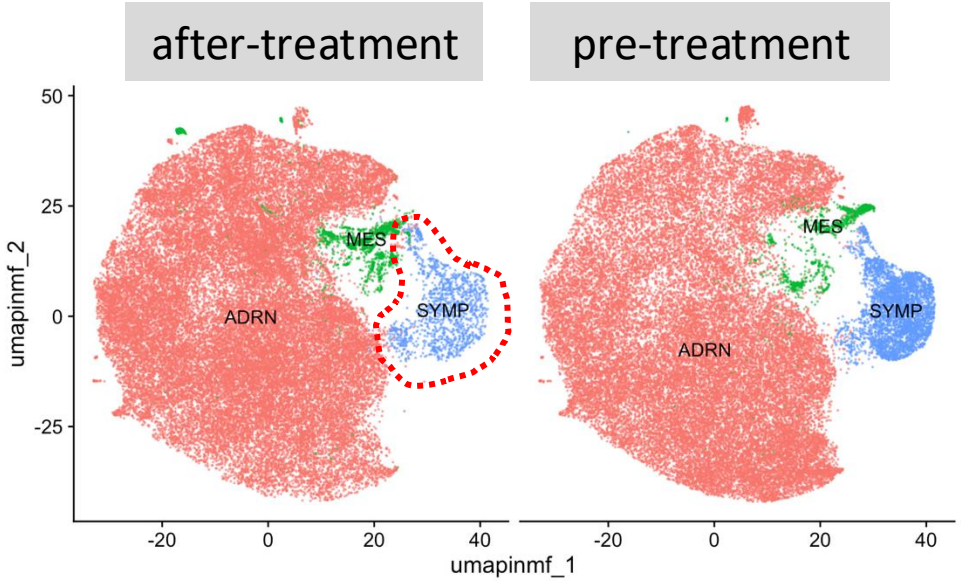
Tumor only analysis - NB lineage signatures

- tumor cells reclustered
- most cells with ADR lineage signature (90%)
- 4% MES lineage signature
- 6% SYM signature (proliferating sympathoblasts, sub-population characterized by highly proliferative status)



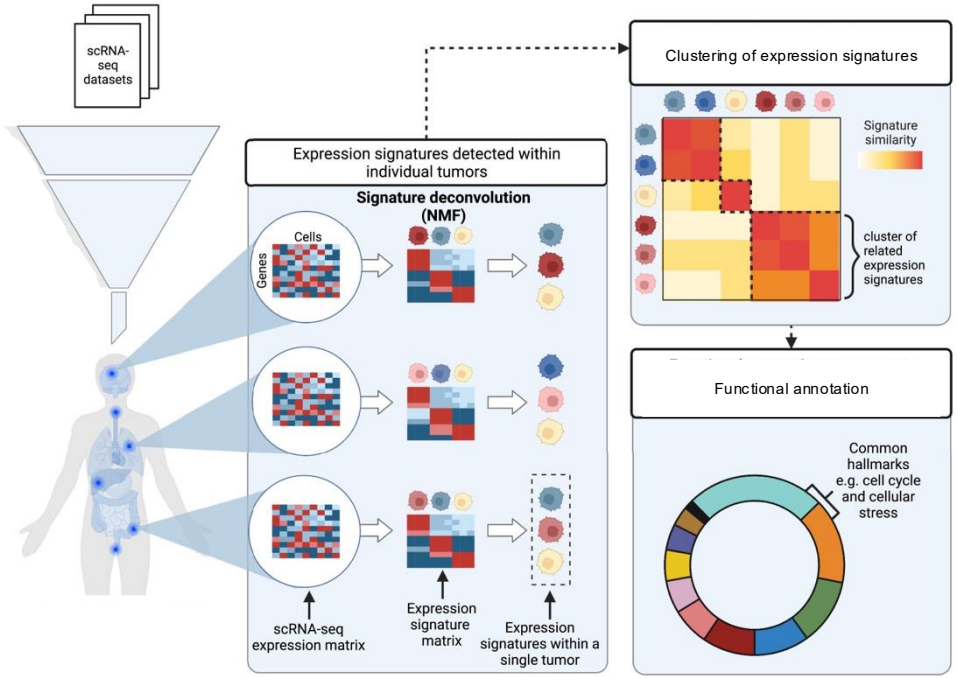
Tumor only analysis - NB lineage signatures (ADR, MES and SYM)

- tumor cells reclustered
- most cells with ADR lineage signature (90%)
- 4% MES lineage signature
- 6% SYM signature (proliferating sympathoblasts, sub-population characterized by highly proliferative status)
- SYM population significantly decreased after chemotherapy (P=0.008)



Defining meta-programs in high-risk NB tumor cells

- *"Recurrent patterns of gene expression that explain a significant proportion of transcriptional heterogeneity observed across multiple single-cell datasets."*



Adapted from Jones & Mcgranahan 2023

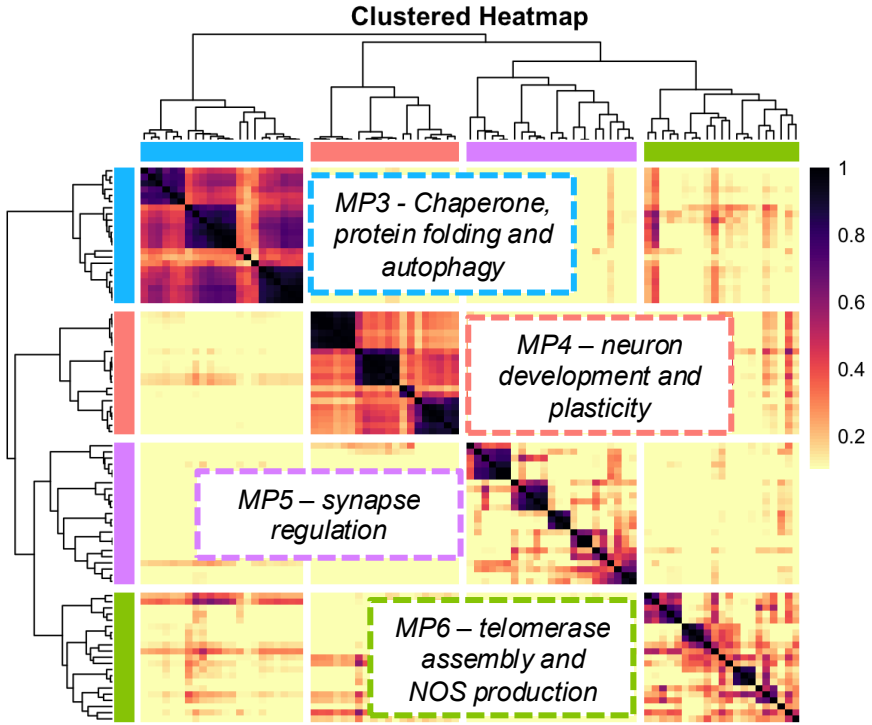
4 high-quality meta-programs found in high risk NB post-chemo

>MP3
MTATP6P1 GAPDH CLU NEFL HSPA8 ACTG1 HSP90AB1
SCG2 PRPH PSAP PEBP1 TUBB TUBA1A TPI1 FTH1 ATP1B1
HSP90AA1 B2M YWHAH TUBA1B B4GAT1 EID1 SYT4
HSPA5 NFE2L1 ATP6AP2 PCSK1N NORAD CDKN1C TIMP2
CYCS UBC INSIG1 TUBB2B TUBB2A LGALS1 TMSB10
S100A6 NDUFA4 HSPB1 TMSB4X

>MP4
CHRM2 IQCJ-SCHIP1 EPHA6 SEMA3C PLD5 PTPRG PDE4B
KIRREL3 ST6GALNAC3 ARPP21 PLXNA2 RYR3

>MP5
GLCCI1 CD44 NXPH1 ASTN2 ICA1 CNTN1 FAM163A NRG1
LRGUK SOX6 PLXNC1 ADGRB3 LRRTM4

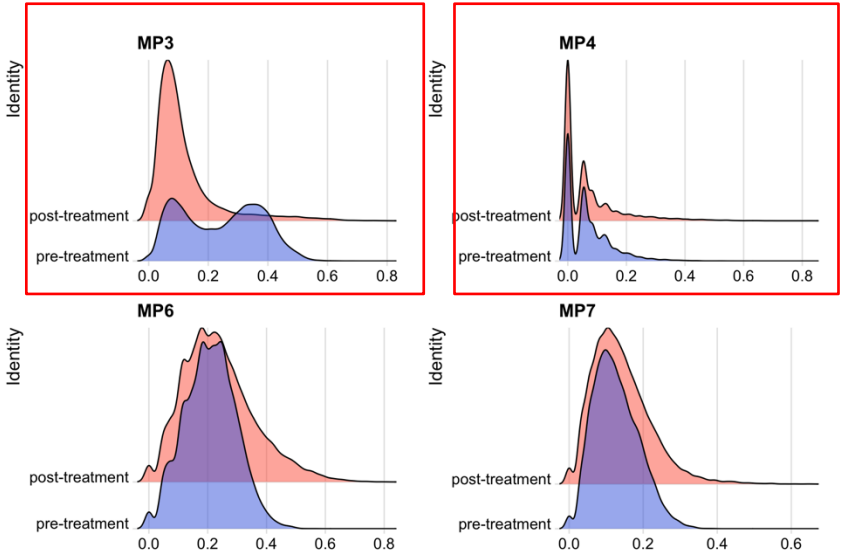
>MP6
CPNE8 CTNNAL1 LRRC4C HSP90AB1 KCNB2 OSBPL3
HSP90AA1 FTH1 SLC18A2 FAM163A ZDBF2 MAP1B LIN7A
PRKCA



MP4 and MP5 both related to neurodevelopment

High quality MPs: numberGenes >=4 & silhouette>=0.1 & sampleCoverage>=0.3

MP3 and MP4 are differentially expressed in NB cells before and after chemotherapy



Association results: random intercept and slope

term	estimate	std.error	statistic	p.value	fdr
MP3	-8.241343	3.5815739	-2.3010395	0.021389399502	0.03422303920
MP4	3.941879	0.8912934	4.4226500	0.000009749764	0.00007799811
MP5	-5.904847	5.4596580	-1.0815415	0.279456324669	0.31937865676
MP6	-7.183296	3.9736445	-1.8077348	0.070647780003	0.09419704000

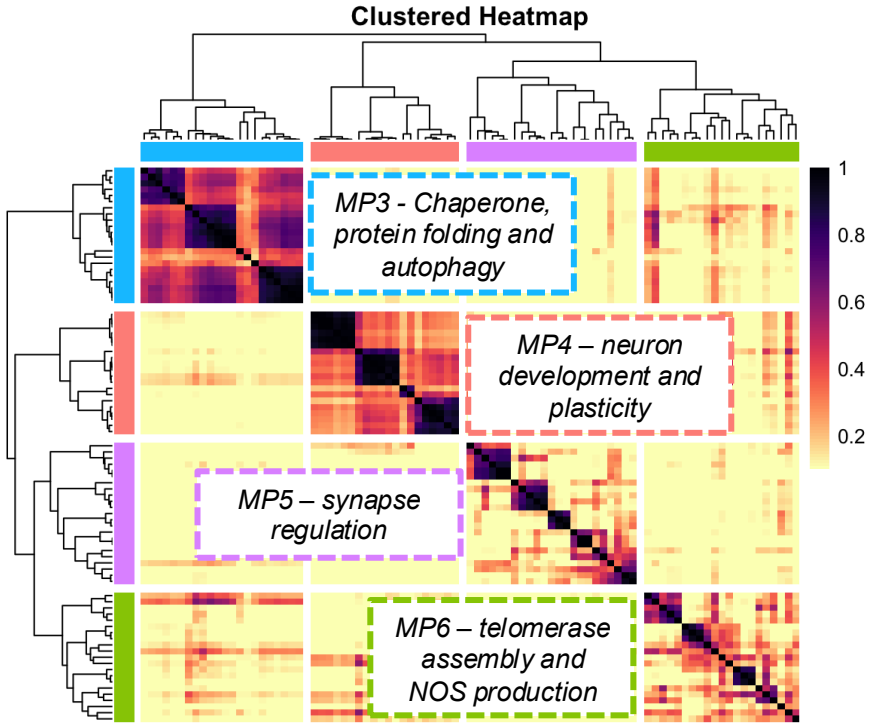
4 high-quality meta-programs found in >2 samples (high risk NB post-chemo)

>MP3
MTATP6P1 GAPDH **CLU** NEFL **HSPA8** ACTG1 **HSP90AB1**
SCG2 PRPH PSAP PEBP1 TUBB TUBA1A TPI1 FTH1 ATP1B1
HSP90AA1 B2M YWHAH TUBA1B B4GAT1 EID1 SYT4
HSPA5 NFE2L1 ATP6AP2 PCSK1N NORAD CDKN1C TIMP2
CYCS UBC INSIG1 TUBB2B TUBB2A LGALS1 TMSB10
S100A6 NDUFA4 **HSPB1** TMSB4X

>MP4
CHRM2 IQCJ-SCHIP1 EPHA6 SEMA3C PLD5 PTPRG PDE4B
KIRREL3 ST6GALNAC3 ARPP21 PLXNA2 RYR3

>MP5
GLCCI1 CD44 NXPH1 ASTN2 ICA1 CNTN1 FAM163A NRG1
LRGUK SOX6 PLXNC1 ADGRB3 LRRTM4

>MP6
CPNE8 CTNNAL1 LRRC4C HSP90AB1 KCNB2 OSBPL3
HSP90AA1 FTH1 SLC18A2 FAM163A ZDBF2 MAP1B LIN7A
PRKCA

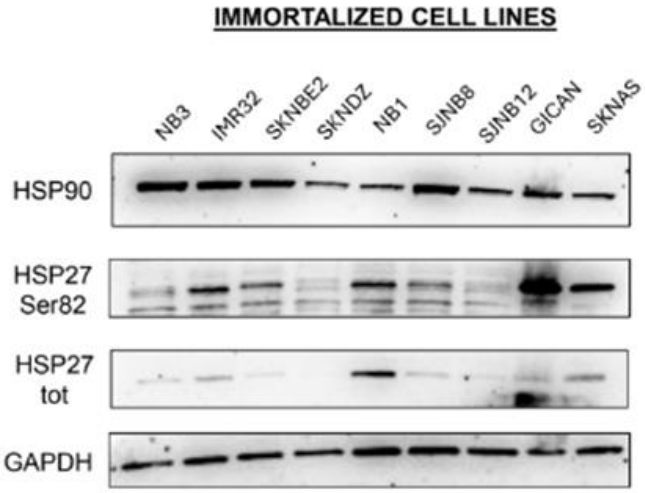


MP4 and MP5 both related to neurodevelopment

High quality MPs: numberGenes >=4 & silhouette>=0.1 & sampleCoverage>=0.3

Chaperon-related genes (*CLU, HSPA8, HSP90AB1, HSP90AA1, HSPA5, HSPB1*) selected for functional studies

- preliminary data show high expression of HSP90 in NB cells and a more specific expression pattern for HSP27
- pharmacological studies with IVERMECTIN are ongoing
- analysis of the other proteins (H*SPA5, HSPA8, CLU*) is also ongoing



CONCLUSIONS

- snRNAseq of matched pre- and post-chemotherapy NB samples revealed significant transcriptional remodeling associated with treatment exposure (↑ tumor cells, ↓ myeloid, etc.).
- NB tumors remain predominantly ADR-like, while proliferative SYM populations significantly decrease after chemotherapy, suggesting selective treatment pressure on highly proliferative cells.
- Integrated analysis identified meta-programs linked to protein folding/autophagy and neuronal plasticity, highlighting potential mechanisms of adaptation and resistance.
- Chaperone-related genes as candidate therapeutic targets and are currently under functional validation.
- Analysis of the tumor microenvironment is also ongoing (see selected abstract)

ACKNOWLEDGEMENTS

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