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Dissecting neuroblastoma heterogeneity through single-cell multi-omics: insights into development, immunity, and therapeutic resistance

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Neuroblastoma (NB), the most common extracranial solid tumor in children, is characterized by remarkable cellular heterogeneity and clinical variability ranging from spontaneous regression to aggressive progression and relapse. Despite advances in multimodal therapies, including surgery, chemotherapy, radiotherapy, differentiation therapy, and immunotherapy—treatment resistance remains the principal barrier to improving survival in high-risk patients. Recent single-cell and spatial multi-omics studies have revolutionized our understanding of NB by revealing its developmental origins, lineage hierarchy, and adaptive evolution under therapeutic pressure. These technologies have delineated distinct cellular states along an adrenergic–mesenchymal continuum and uncovered the dynamic interplay between tumor cells and their microenvironment. Genetic instability, epigenetic reprogramming, and metabolic plasticity cooperate with immune and stromal remodeling to drive tumor persistence and relapse. At the molecular level, mechanisms such as MYCN-driven chromatin remodeling, super-enhancer reorganization, bypass signaling activation, quiescent persister programs, immune checkpoint engagement, and metabolic rewiring collectively enable therapeutic escape. Importantly, these processes are reversible, highlighting tumor plasticity as both a hallmark and a potential vulnerability of NB. Integrating single-cell transcriptomics, epigenomics, and spatial profiling provides an unprecedented framework to map resistance evolution, identify lineage-specific vulnerabilities, and guide rational combination strategies. Targeting epigenetic regulators, metabolic checkpoints, and immune suppressive networks in a temporally coordinated manner holds promise for converting NB from an adaptive to a controllable disease.

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INTRODUCTION

Neuroblastoma (NB) is one of the most prevalent pediatric solid tumors, arising from neural crest-derived progenitors of the sympathetic nervous system, most commonly in the adrenal medulla, sympathetic chain, or abdominal regions [1, 2]. It represents 8%–10% of all childhood cancers and occurs predominantly in children under five years of age, with earlier onset generally predicting more favorable outcomes [3]. Clinically, NB is characterized by striking heterogeneity, ranging from tumors with spontaneous regression to highly aggressive and lethal forms. At diagnosis, most high-risk cases already present metastatic spread typically to bone marrow, bone, or lymph nodes [4, 5]. Despite steady progress in multimodal therapy, including chemotherapy, radiotherapy, surgical resection, autologous stem cell transplantation, and immunotherapy, long-term outcomes remain poor, with five-year survival in high-risk NB lingering around 40%–50% [6–8].

Targeted therapy for NB is still limited. To date, most molecular approaches have focused on ALK mutations, yet these alterations are restricted to a minority of patients, underscoring the urgent need to identify additional oncogenic drivers and actionable

therapeutic targets [9–12]. Immunotherapy has delivered the most notable breakthrough to date, particularly with anti-GD2 monoclonal antibodies. The pivotal COG ANBL0032 trial demonstrated that dinutuximab, combined with GM-CSF, IL-2, and retinoic acid after autologous transplantation, significantly improved outcomes, raising two-year event-free survival from 46% to 66% and overall survival from 75% to 86% [13]. Subsequent long-term follow-up and meta-analyses confirmed durable benefits, with 2–5-year survival rates increasing by approximately 10–25 percentage points, establishing anti-GD2 therapy as standard post-consolidation treatment [14–16]. Yet, this progress has not eliminated the grim prognosis of high-risk NB: more than half of patients still relapse within five years, and recurrent tumors frequently display heightened chemoresistance [7, 8]. Other immunotherapeutic modalities, such as CAR T-cell therapy, remain experimental, with limited efficacy and significant toxicity [17–20]. These realities underscore that while NB therapy has advanced, curative outcomes remain elusive.

A major impediment is the profound intratumoral heterogeneity of NB, which traditional bulk genomic or transcriptomic

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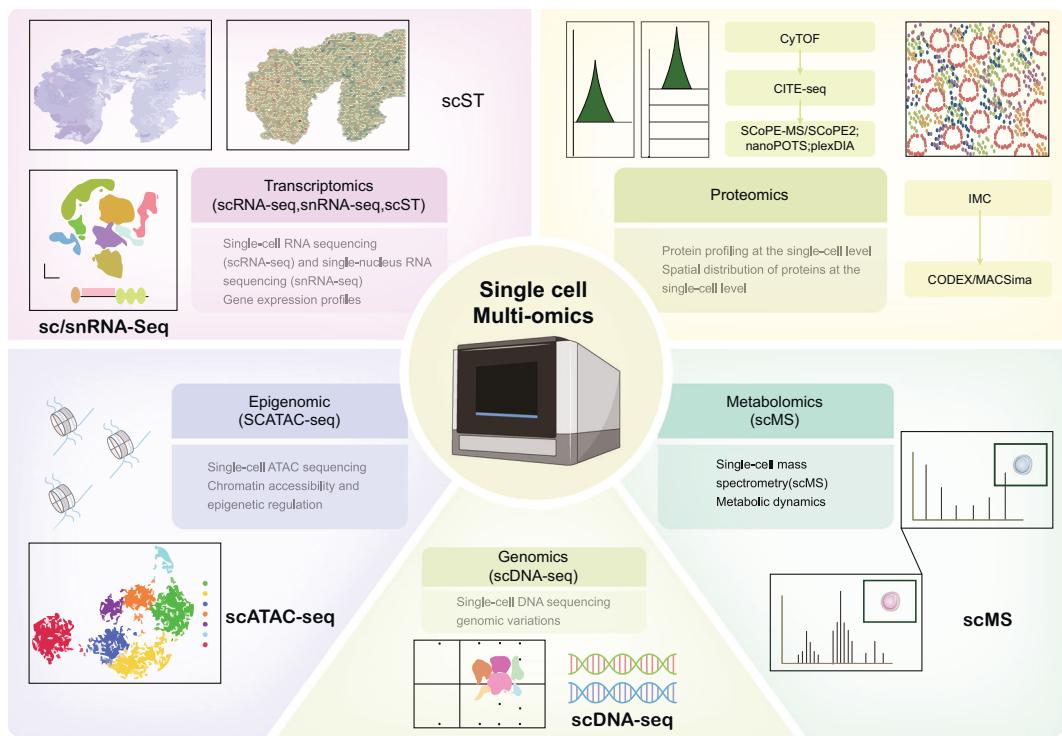


Fig. 1 Overview of single-cell multi-omics technologies. Single-cell multi-omics has expanded beyond transcriptomics (scRNA-seq, snRNA-seq, scST) to include epigenomics (scATAC-seq), genomics (scDNA-seq), proteomics (CyTOF, CITE-seq, SCoPE-MS, IMC, CODEX/MACSimA), and metabolomics (scMS). This integrated framework enables comprehensive characterization of gene expression, chromatin accessibility, genomic alterations, protein distribution, and metabolic dynamics at the single-cell level, thereby providing a powerful platform for dissecting tumor heterogeneity, tumor-immune interactions, and therapeutic responses.

methods cannot capture [21–23]. Recent single-cell RNA sequencing (scRNA-seq) studies have begun to resolve this complexity, revealing diverse cellular lineages, functional plasticity, and intricate interactions within the tumor microenvironment [24, 25]. These insights have uncovered novel mechanisms of tumorigenesis, therapeutic resistance, and relapses, while laying the foundation for precision therapies tailored to tumor architecture at single-cell resolution.

In this review, we discuss recent progress in applying single-cell technologies to NB, highlighting their potential to unravel tumor heterogeneity and inform precision oncology.

COMPARISON OF EXISTING SINGLE-CELL TECHNOLOGIES FOR CANCER RESEARCH

Single-cell technologies have emerged as a disruptive methodological innovation in cancer research. What was once narrowly confined to single-cell transcriptomics has now expanded into a comprehensive framework of “broad-spectrum single-cell omics,” encompassing the transcriptome, epigenome, genome, proteome, and even metabolome (Fig. 1). This multi-omics system provides a technical foundation for systematically dissecting tumor initiation, progression, microenvironmental remodeling, and therapeutic responses at the single-cell level. By uncovering the profound heterogeneity of tumor cell populations and their evolutionary trajectories, it enables detailed mapping of tumor-immune interactions and in-depth exploration of mechanisms such as drug resistance and immune evasion. In doing so, single-cell omics is driving a paradigm shift from population-level observation to individual-level decoding, fundamentally reshaping our understanding of cancer biology.

Single-cell transcriptomic technologies: characterizing cell states and reconstructing lineages

Among single-cell omics approaches, transcriptomic profiling is the most mature and widely used, serving as a key entry point for understanding cellular states and functions [26, 27]. Current platforms include single-cell RNA sequencing (scRNA-seq), single-nucleus RNA sequencing (snRNA-seq), and spatial transcriptomics (scST), which provide complementary perspectives in terms of cellular resolution, tissue adaptability, and spatial organization. As mRNA expressions most directly reflect cellular responses and functional states, scRNA-seq has become the primary tool for dissecting tumor heterogeneity, reconstructing developmental trajectories, and profiling immune cell composition [28–30]. It has been extensively applied across solid and hematological malignancies, supported by a robust analytical toolkit, although it remains constrained by reliance on high-quality tissue dissociation and limits in sequencing depth and resolution [31, 32].

snRNA-seq offers an effective alternative for samples that are difficult to dissociate, structurally dense, or frozen [33]. By capturing nuclear transcripts without enzymatic digestion, it avoids stress-induced artifacts and enhances data stability [26, 34], making it particularly valuable for tumors with complex structures such as NB [35] and glioblastoma (GBM) [36].

Both scRNA-seq and snRNA-seq, however, lack spatial information. scST overcomes this limitation by mapping gene expression to tissue coordinates, integrating cell identity, functional state, and structural context. This provides unprecedented insights into tumor-immune architecture, cell-cell interactions and spatially driven immunosuppressive niches (e.g., studies in medulloblastoma [37]; glioma niche analyses [38]; immune macrophage subpopulation spatial profiling [39]). These technologies have already yielded

important discoveries. Okonechnikov et al. showed that large-scale chromosomal aberrations frequently arise at early stages of medulloblastoma, acting as initiating events of tumor evolution, while single oncogenic alterations more often drive later progression [40]. Van de Velde et al. identified a pathogenic CD4⁺ T-cell subset and ARG1⁺ myeloid cells as critical microenvironmental components sustaining NB development [41].

Taken together, transcriptomic single-cell technologies provide complementary advantages in resolution, tissue compatibility, and spatial context. In NB and other complex tumors, they not only refine cellular lineage mapping and microenvironmental reconstruction but also lay a foundation for integrative multi-omics analyses.

Single-cell epigenomic technologies: chromatin accessibility and fate determination

While transcriptomic profiling provides a precise snapshot of cellular expression states, it does not fully capture the upstream regulatory mechanisms that drive these changes, particularly the early events underlying cell fate specification and state transitions [42]. As a result, research has increasingly expanded toward the epigenomic layer [43] and its derivative, single-nucleus ATAC sequencing (snATAC) and single-cell ATAC sequencing (scATAC-seq) [44], have emerged as powerful tools to interrogate chromatin accessibility, offering key insights into state reprogramming and fate determination [45, 46].

scATAC-seq enables the detection of open chromatin regions, the identification of putative enhancers, promoters, and transcription factor (TF) binding sites, and the inference of regulatory networks and TF activity. It has been broadly applied across cancers to map tumor epigenomes at single-cell resolution. For example, in clear cell renal cell carcinoma (ccRCC), scATAC-seq revealed chromatin remodeling features associated with aggressive phenotypes, pinpointing key regulatory elements and TF networks driving tumor progression [47]. In triple-negative breast cancer, scATAC-seq captured therapy-induced TF reprogramming patterns linked to drug resistance [48]. Beyond tumor cells, scATAC-seq also offers unique advantages in lineage tracing of immune populations. In myeloid cells such as neutrophils, whose phenotypes are tightly coupled with differentiation states, accessibility trajectories provide a powerful framework to resolve developmental pathways and enable fine-grained subpopulation identification and functional characterization [49, 50]. Importantly, integrative analysis of scATAC-seq with scRNA-seq has become a central strategy, allowing prediction of TF activity, reconstruction of regulatory networks, and multi-layered dissection from chromatin accessibility to transcriptional output at the single-cell level [51–53].

Similar to snRNA-seq, snATAC-seq is particularly suited for frozen or dense tissues and is often combined with snRNA-seq. For instance, as noted earlier, Konstantin Okonechnikov employed this approach to distinguish chromosomal events during medulloblastoma initiation and progression [40]. In ccRCC, Wu and colleagues used large-cohort snATAC-seq profiling to demonstrate that BAP1 mutations typically reduce global chromatin accessibility, whereas PBRM1 mutations enhance chromatin openness, displaying a mutually exclusive pattern that may represent distinct mechanisms and transitional stages of disease development [54]. In glioblastoma (GBM), Wang et al. integrated snATAC-seq with spatial transcriptomics and uncovered higher chromatin accessibility and stronger immune evasion signatures at the tumor margin, contrasted with profound immunosuppression in the core [55]. They further identified several region-specific TFs, including RUNX, FOS, and SPI1, as potential drivers of spatially defined tumor programs.

Single-cell genomic technologies: genomic instability and clonal evolution

Beyond chromatin-level alterations, the evolutionary origins of tumor progression also involve genomic abnormalities such as

mutations and copy number variations (CNVs) [56, 57]. Single-cell DNA sequencing (scDNA-seq) has therefore become a key approach for delineating clonal evolutionary trajectories, genetic heterogeneity, and chromosomal instability (CIN). For example, Wang et al. analyzed hepatocellular carcinoma (HCC) samples with scDNA-seq and demonstrated a two-phase model of CNV accumulation characterized by “early catastrophic rearrangements followed by late progressive evolution [58].” Progressive CNV acquisition was strongly associated with recurrence risk, while ploidy reconfiguration events, such as whole-genome duplication, indicated genetic continuity during clonal evolution and further exacerbated tumor heterogeneity [59–61]. Similarly, Zhou et al. developed Alleloscope, an algorithm that integrates scDNA-seq and scATAC-seq data to resolve allele-specific CNVs at single-cell resolution. This method uncovered pervasive allelic imbalance and copy-neutral loss of heterozygosity (LOH) within subclones and enabled tracing of their coordinated changes with chromatin accessibility [62].

It is noteworthy that although NB is characterized by marked genomic instability, such as MYCN amplification, 1p/11q deletions, and 17q gain [63, 64], applications of scDNA-seq in NB remain very limited. On the one hand, most NB clinical specimens are obtained from biopsies or frozen tissues, which often preclude the extraction of high-quality DNA and hinder the technical feasibility of scDNA-seq [65]. On the other hand, compared to adult cancers, NB tends to harbor relatively simple clonal structures and lower mutational burdens, reducing the immediate demand for high-resolution clonal tracing tools [6, 66]. In addition, the availability of extensive bulk WGS and WES datasets in NB provides a partial substitute for single-cell analyses. Nevertheless, given the presence of subclonal expansion, chromosomal aberrations driving tumor progression, and relapse following immunotherapy, future integration of scDNA-seq with transcriptomic and epigenomic modalities to reconstruct clonal evolution in NB remains highly valuable and holds significant potential [67–69].

Single-cell proteomic technologies: functional phenotypes and communication networks

Compared with transcriptomic and epigenomic approaches, proteomics provides a direct readout of cellular functional states and thus represents a critical dimension for understanding tumor heterogeneity and signaling dynamics. Mass cytometry (CyTOF) was among the first technologies applied to single-cell protein profiling and has been widely used to chart immune landscapes across diverse cancers. For instance, Baughn et al. analyzed 34 protein markers in 49 multiple myeloma samples and delineated a protein-level atlas of cellular heterogeneity, identifying subpopulations associated with prognosis and therapeutic response [70]. In HCC, Zhang and colleagues applied CyTOF to peripheral blood immune cells and uncovered immune signatures relevant to tumor detection [71]. Similarly, Garman et al. used CyTOF to characterize tumor-infiltrating immune cells in solid tumors, highlighting its strength in identifying LAG-3⁺ T cells [72]. Moreover, the integration of CyTOF with conventional flow cytometry has been leveraged to build predictive scoring systems for treatment response. In NB, although CyTOF studies remain limited, exploratory work has demonstrated its potential. Nolo et al. reported that P-selectin stimulation induces heterogeneous activation of the PI3K/AKT and MAPK pathways across NB cell lines, providing mechanistic support for P-selectin inhibition as a therapeutic strategy [73]. Nonetheless, CyTOF is restricted by antibody dependence, limited proteome coverage, and throughput constraints, which hinder comprehensive proteomic profiling [74, 75].

Recent advances in single-cell proteomics have sought to overcome these limitations by developing label-free mass spectrometry platforms, joint transcriptome–protein assays and spatially resolved imaging systems. In the label-free field,

Single-Cell Proteomics by Mass Spectrometry (SCoPE-MS) and its upgraded version SCoPE2 have achieved high-throughput quantification of single-cell proteomes with the aid of tandem mass tag (TMT) labeling. Originally developed by Budnik et al. and validated in human cancer cell lines such as U-937 and Jurkat, SCoPE-MS demonstrated the ability to discriminate proteomic differences among cell types and uncover functional heterogeneity during differentiation [76]. Subsequent refinements by Slavov's group led to SCoPE2, which markedly improved throughput, accuracy, and automation, enabling quantification of >1000 proteins across hundreds to thousands of single cells [77]. While the application of SCoPE2 to primary tumor samples remains challenging, it has already been successfully employed in models of immune cell heterogeneity and differentiation. For example, in macrophage differentiation models, the integration of SCoPE2 with scRNA-seq uncovered transcription–protein decoupling patterns, underscoring the importance of post-transcriptional regulation during immune differentiation—an approach readily extendable to tumor-associated macrophages (TAMs) [77]. However, Petelski et al. also noted remaining technical limitations, including relatively large sample processing volumes, multiple LC-MS/MS runs, and ion co-isolation-induced quantification bias. Future advances in microdroplet processing, barcoding strategies, and parallel ion accumulation are expected to further improve performance [78].

In this context, nanoPOTS (Nanodroplet Processing in One Pot for Trace Samples) has emerged as a microfluidics-based platform that reduces processing volumes below 200 nL, minimizing sample loss and enhancing sensitivity for low-abundance proteins. It has been successfully applied to HeLa cells and circulating tumor cells (CTCs), and further refinements have increased recovery and throughput, enabling quantification of >1500 proteins across different cell lines [79]. More recently, plexDIA, which combines data-independent acquisition (DIA) with isotopic labeling, allows parallelized sample analysis and signal sharing across runs, thereby improving data completeness and throughput. plexDIA can now quantify thousands of proteins in a single run and has been applied to high-throughput analysis of human single-cell samples [80]. Collectively, these advances provide strong technological support for mapping proteomic heterogeneity in tumors.

In parallel, CITE-seq has emerged as a powerful alternative by combining antibody-based protein detection with transcriptome profiling, enabling cost-effective multimodal analyses. Unlike conventional flow cytometry, CITE-seq substantially expands the number of detectable proteins (>100) while offering greater quantitative accuracy and reproducibility [81, 82]. It has been widely applied in cancer research to dissect immune heterogeneity, reconstruct TME, and predict therapeutic responses. For example, Leader et al. used CITE-seq in non-small cell lung cancer to delineate T-cell lineages and functional states and to associate immune activation modules with tumor antigen load [83]. In NB, CITE-seq applications remain scarce, but the technology holds strong promise for identifying therapy-related immune subsets and uncovering immune evasion mechanisms, especially when integrated with TCR/BCR sequencing and spatial transcriptomics.

Another critical dimension is spatial proteomics, which complements CyTOF and CITE-seq by preserving spatial context of protein expression within tissues. Imaging Mass Cytometry (IMC), combining CyTOF with laser scanning microscopy, has been used to build spatial immune atlases and prognostic models. For example, Elaldi et al. profiled 35 immune-related markers in breast cancer tissues, demonstrating spatial exclusion of immune cells by tumor density [84]. Xiao et al. applied IMC to melanoma, identifying spatial co-localization patterns among T cells, macrophages, and tumor cells, and linking distinct microenvironmental architectures to clinical outcomes [85]. Similarly, CODEX (CO-Detection by Indexing), a multiplexed antibody-based imaging

system, enables 50+ protein markers to be visualized in three dimensions [86]. Goltsev et al. demonstrated the feasibility of CODEX for high-dimensional proteomic imaging of spleen and tumor tissues [87].

To date, applications of IMC and CODEX in NB remain scarce, likely due to challenges in pediatric tumor tissue acquisition and immature antibody validation frameworks. Nonetheless, given the pronounced immune microenvironment heterogeneity and spatial tissue organization of NB, integrating spatial proteomics platforms into NB research could elucidate immune exclusion zones, tumor–stroma interfaces, and spatial biomarkers of therapeutic response, ultimately informing precision immunotherapy strategies.

Single-cell metabolomic technologies: metabolic reprogramming within the tumor ecosystem

Compared with transcriptomic, epigenomic, and proteomic approaches, metabolomics provides the most direct snapshot of biochemical activity, serving as a crucial window into tumor metabolic reprogramming and microenvironmental adaptation. However, the vast diversity of metabolites, their short half-lives, and rapid fluctuations in abundance pose major challenges for generating high-quality data at single-cell resolution [88, 89]. In recent years, mass spectrometry-based single-cell metabolomics (scMS) has overcome many of these technical bottlenecks in sensitivity and resolution, emerging as a powerful tool to dissect metabolic heterogeneity. Typically employing high-resolution platforms such as Orbitrap or TOF-MS combined with microsampling, scMS enables both targeted and untargeted detection of key pathways, at the single-cell or microscale tissue level [89]. This approach has now been broadly applied to both solid and hematological malignancies [90, 91].

Although large-scale applications of scMS in NB remain limited, the known presence of MYCN-driven metabolic reprogramming and divergent oxidative phosphorylation versus glycolytic states among NB subpopulations suggests that coupling scMS with spatial or proteomic modalities holds strong promise [92–94]. Such integrated approaches may uncover metabolic vulnerabilities and guide the development of precision therapeutic strategies for NB.

Single-cell decoding of neuroblastoma: from developmental arrest to ADR/MES plasticity

To provide a more intuitive overview, the developmental origins and state plasticity of NB can be conceptualized as a continuum from neural crest cell (NCC) arrest to dynamic transitions between adrenergic (ADR) and mesenchymal (MES) phenotypes (Fig. 2). This chapter highlights the lineage complexity and state interconversion that underpin tumor heterogeneity, while directly linking ADR/MES plasticity to therapy sensitivity and resistance.

Developmental origins and trajectory heterogeneity in NB

Accumulating developmental evidence indicates that NB arises from arrested differentiation of NCCs within the sympatho-adrenal lineage [95, 96]. This framework accounts for several biological features of NB, including phenotypic diversity, mutational profiles, spontaneous regression, and sensitivity to differentiation-inducing agents. NB was first recognized as a distinct tumor in the mid-19th century, and by 1910 was classified as neuronal in origin based on fiber-like structures resembling sympathetic ganglion neurons [97]. Its capacity to differentiate into mature neurons was noted early on, and the prevailing consensus now holds that NB complexity reflects the diverse and dynamic trajectories of neural crest-derived lineages [6]. Jansky et al. demonstrated that NB closely resembles developing NCC progenitors, with clinical phenotypes mapping to distinct stages of normal neural crest differentiation [98].

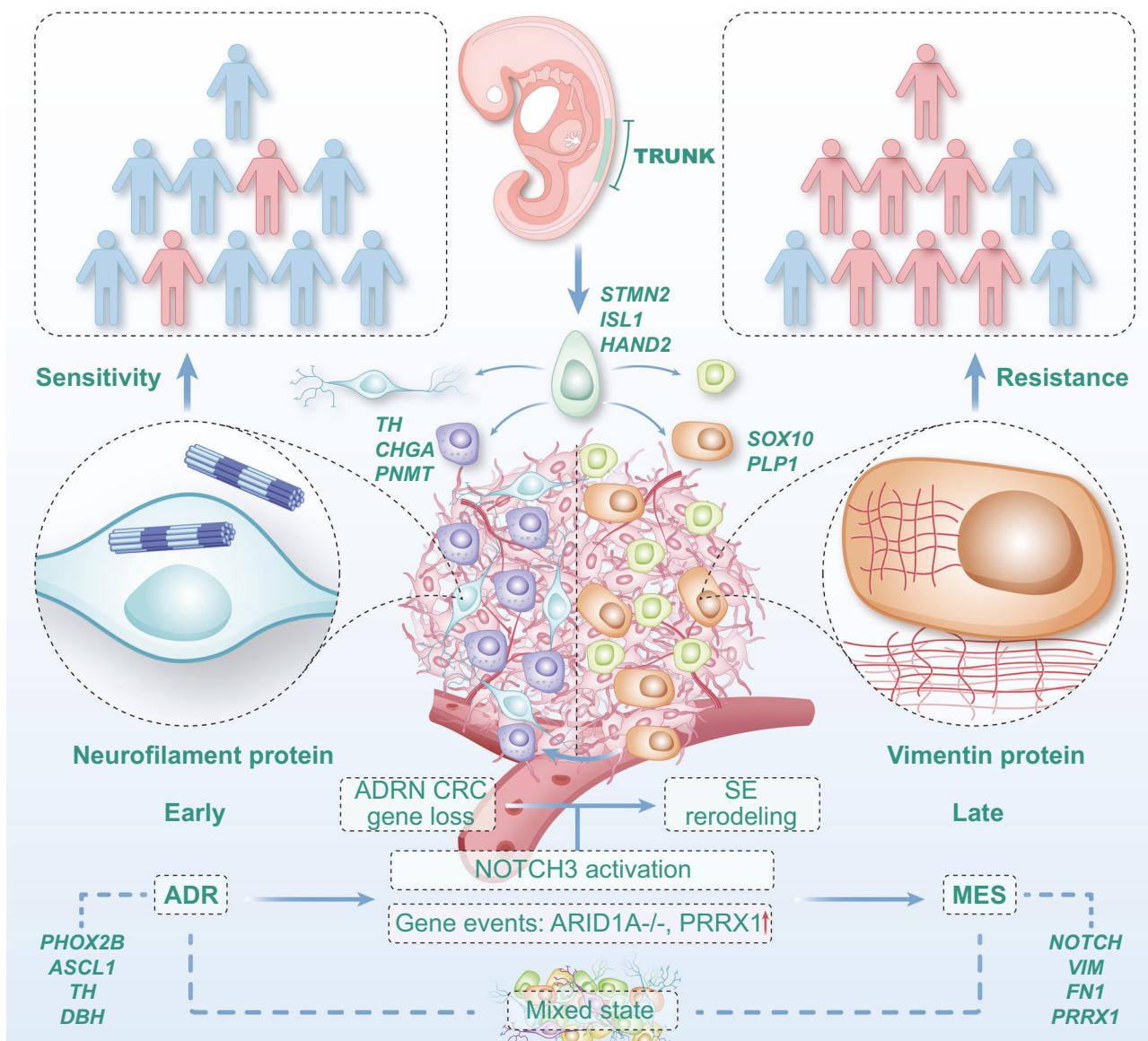


Fig. 2 Developmental origins and state plasticity of NB. NB arises from developmental arrest of NCCs within the sympatoadrenal lineage, leading to tumor cells trapped at intermediate differentiation states. NCC-derived progenitors such as sympathoblasts and Schwann cell precursors (SCPs) exhibit dual potential, giving rise to adrenergic (ADR) and mesenchymal (MES) lineages. ADR cells express neurofilament proteins and catecholamine biosynthesis markers (TH, CHGA, PNMT) and are associated with early developmental trajectories and chemotherapy sensitivity. In contrast, MES cells express vimentin and stromal markers (SOX10, PLP1), correspond to late developmental states, and confer therapy resistance. Single-cell multi-omic analyses have revealed that ADR and MES states are stabilized by distinct core regulatory circuits (CRCs) and super-enhancers (SEs) yet remain interconvertible. Key genetic and epigenetic events, including ARID1A loss, PRRX1 upregulation, and NOTCH3 activation, promote ADR → MES transitions via enhancer remodeling, resulting in therapy-induced MES enrichment. Conversely, a fraction of MES cells can revert to ADR upon microenvironmental changes, rebuilding tumor heterogeneity. Transitional and hybrid states coexist within tumors, highlighting dynamic fate plasticity and the continuum from developmental arrest to ADR/MES interconversion as determinants of NB progression, therapy sensitivity, and relapse.

NCCs are proliferative, multipotent progenitors whose fates are determined by both axial position and non-cell-autonomous cues [99, 100]. For example, cranial NCCs generate bone and cartilage, while sacral NCCs form enteric neurons. Cross-regional transplantation experiments further show their plasticity: trunk NCCs transplanted to the cranial region can produce bone/cartilage, whereas cranial NCCs placed in the trunk region generate the full sympatoadrenal lineage, including sympathetic neurons, chromaffin cells, Schwann cells, and melanocytes [96]. Thus, migratory pathways and microenvironmental cues are tightly coupled to lineage choice. Trunk-derived sympathoblasts possess dual potential, giving rise to both neurons and mesenchymal-like cells.

A critical question therefore arises although sympathetic neurons and chromaffin cells normally differentiate along mutually exclusive lineages, this segregation may not hold in NB. Indeed, NB cells can simultaneously exhibit sympathetic neuronal and chromaffin/mesenchymal features, suggesting “rewiring” of their developmental trajectories [101]. Two complementary explanations have been proposed: (i) intrinsic dysregulation of developmental programs that reactivates NCC multipotency and drives aberrant (trans)differentiation; and (ii) extrinsic signals from the tumor microenvironment, such as Schwann cell- or stroma-derived cues, which promote fate switching linked to aggressiveness and therapy resistance [102]. Together, these mechanisms

underscore the pivotal role of the microenvironment in fate plasticity within NB.

Single-cell studies have provided direct lineage evidence. Schwann cell precursors (SCPs) have been identified as the predominant progenitors of both adrenal chromaffin cells and sympathoblasts [98, 103–105], and also contribute to parasympathetic ganglia formation [106]. NB tumor transcriptomes resemble noradrenergic chromaffin cells, with malignant states typically arrested at intermediate stages of sympatho-adrenal differentiation [107]. Although both SCP-derived and NCC-derived pathways exist, the resulting sympathoblast phenotypes are highly similar, contributing to intertumoral complexity. NCC-derived cells traverse multiple transcriptionally distinct states, progressing from undifferentiated progenitors to fully differentiated, fate-committed cell types [101, 104]. Projecting NB cells onto this continuum reveals that the degree of progression from SCP to differentiated noradrenergic cells strongly correlates with clinical outcome: high-risk NB often retains immature sympathoblast features, whereas low-risk NB is enriched for more differentiated states [98, 107].

Further intratumoral evidence indicates that developmental heterogeneity is recapitulated within tumors. Kameneva et al. described a “fork-like” transitional state in which single cells co-express key markers of both SCPs and chromaffin/sympathoblast lineages [105]. Multi-omic single-cell profiling of human NB samples further identified aneuploid SCP-like subclones with unique transcriptional signatures and clonal expansion, integrating precursor-like and transitional states into NB progression [108]. These findings align with large-scale genomic studies showing parallel clonal evolution between primary and metastatic lesions, with therapy-resistant clones driving relapse [68, 109, 110]. However, genomic data alone cannot resolve the phenotypes of post-therapy persister cells. Integrated sc/snRNA-seq approaches have begun to characterize their transcriptional states and roles in clonal selection and relapse [67]. Collectively, these observations highlight the temporal and spatial continuity between developmental arrest, lineage plasticity, and tumor reconstruction, reinforcing the tight coupling of NB clinical progression to its developmental and lineage context, a theme further elaborated in subsequent sections.

Tumor heterogeneity and cellular plasticity in NB

Building on its developmental origins, NB manifests at the tumor level as heterogeneity and plasticity of differentiation outcomes. Since the 1980s, when primary cultures and cell lines were first established, NB heterogeneity has been consistently observed. Early in vitro studies broadly classified tumor cells into neuron-like (N-type) and substrate-adherent (S-type) categories [111–114]. N-type cells express neurofilament proteins and catecholamine biosynthesis markers (e.g., TH, CHGA, PNMT) and exhibit neuronal differentiation potential, whereas S-type cells are enriched for vimentin and stromal markers (e.g., SOX10, PLP1), resembling non-neuronal neural crest derivatives [100, 115]. However, this binary framework cannot explain three critical aspects: the long-term coexistence and therapy-induced reshaping of subpopulations, the reversible interconversion and existence of intermediate states, and the regulatory drivers and causal mechanisms underlying these dynamics.

With the advent of single-cell transcriptomic, chromatin accessibility, and epigenomic profiling, NB “types” are now better defined as plastic states maintained by core regulatory circuits (CRCs) and super-enhancers (SEs) [116, 117]. Multi-omic integration has revealed two antagonistic but interconvertible networks: ADR (also termed ADRN) and MES [101, 118]. Mirroring the early N/S paradigm, ADR and MES states are also reversible: van Groningen et al. demonstrated bidirectional spontaneous switching in cell lines [119], while Durbin et al. used longitudinal cultures with sn/snRNA-seq to quantify transition rates, showing that ADR

→ MES transitions are generally faster than MES → ADR [101]. Clinical and *in vivo* data support functional associations: ADR correlates with chemotherapy sensitivity, whereas MES is linked to tolerance and resistance [116]. Under cytotoxic pressure, ADR cells are preferentially eliminated, while low-proliferative/quiescent MES cells are retained, leading to post-treatment MES enrichment and phenotypic shift [120]. Upon drug withdrawal or microenvironmental change, a fraction of MES cells can revert to ADR, rebuilding heterogeneity and driving relapse [102, 118].

Mechanistically, ADR maintenance requires intact CRC/SE architecture [117]. Its disruption increases the probability of MES conversion, whereas stabilization or reinforcement of ADR-SE peaks helps sustain the ADR state [101]. Two representative studies provide causal nodes with sequencing evidence: van Groningen et al. mapped the SE landscape of NB using H3K27ac ChIP-seq with RNA-seq, showing that PRRX1 upregulation reprograms enhancers and transcriptional profiles to drive ADR → MES, with MES preferentially enriched in post-treatment/relapsed tumors [116]. Shi et al. reported that ARID1A (SWI/SNF) loss promotes ADR → MES conversion and enhances platinum resistance in ADR cells, supported by RNA-seq and multi-target ChIP-seq evidence pointing to enhancer-mediated transcriptional reprogramming [121]. Furthermore, van Groningen’s group proposed a NOTCH3 feed-forward module as a key switch for CRC interconversion, where NOTCH3 activation triggers genome-wide SE remodeling, downregulates ADR-CRC members, and upregulates MES networks; inhibition of this axis partially blocks the transition [119]. Notably, there is currently no single transcription factor proven to induce stable MES → ADR conversion, suggesting that reverse stabilization likely requires multilayered regulation rather than a single TF.

Importantly, owing to the complexity of intrinsic genetic regulation and extrinsic environmental cues, recent studies have captured intermediate states, ADRN/MES subtypes, or novel hybrid programs, linking them to NB plasticity. For instance, Thirant et al. identified a subset of adrenergic cells with mesenchymal features using 10x scRNA-seq, validated in vitro [122]. Chapple et al. applied an acNMF algorithm to scRNA-seq maps, showing that “weak mesenchymal-like” programs frequently coexist within ADR-dominant cells and can be induced *in vivo* within 24 h of chemotherapy, indicating that therapy-related hybrid populations constitute early escape mechanisms [123]. Large-scale atlas efforts, such as the NBAtlas and integrative spatial/multi-omic maps, have further confirmed co-expression and neighborhood-specific distribution of states in patient samples, providing tissue-level evidence for hybrid subpopulations [124].

In summary, single-cell omics have reframed NB from a binary classification into a developmental continuum of plastic states. They enable dissection of ADR, MES, and transitional subpopulations at single-cell resolution, anchoring them to CRC/SE regulatory levers such as NOTCH3, PRRX1, and ARID1A. This shift transforms our understanding from observing heterogeneity to uncovering actionable mechanisms, offering conceptual footholds for therapeutic intervention in clinical NB.

CELLULAR MECHANISMS OF NEUROBLASTOMA: FROM GENETIC PREDISPOSITION TO THE METASTATIC MICROENVIRONMENT

Developmental arrest and ADR/MES plasticity not only shape the molecular and cellular landscape of NB but also provide a conceptual framework for understanding its biological complexity. However, how these molecular and developmental alterations translate into the clinical heterogeneity and symptoms observed in patients requires further elucidation at the level of cellular regulatory mechanisms. Such insights are essential to clarify the core dynamics of tumorigenesis and progression, while also identifying

potential entry points for targeted therapies, immunotherapies, and interventions in TME. In this regard, single-cell omics technologies have recently demonstrated unique advantages.

From the perspective of genetic predisposition, familial NB (FNB) represents a rare subtype whose defining mechanisms have been elucidated by single-cell studies. Using scRNA-seq, Zhang et al. mapped the cellular atlas of FNB and revealed marked differences from sporadic NB, FNB was characterized by a reduced proportion of neuroendocrine cells and an increased proportion of immune cells, with the identification of a cancer-associated fibroblast (CAF) subtype, Fib-4, with prognostic relevance [125]. In the context of risk stratification, Bedoya-Reina et al. employed snRNA-seq to construct a reference atlas of postnatal human adrenal cells and compared it to NB. Their analysis showed that low-risk NB resembled sympathetic neurons and chromaffin cells, whereas high-risk NB was enriched for TRKB⁺ cholinergic progenitors. This highlighted the link between aberrant developmental lineages and NB risk and underscored the potential of single-cell omics for risk prediction [126]. Mechanistically, George et al. summarized the molecular hallmarks of high-risk NB, including MYCN amplification, chromosomal instability, and activation of telomere maintenance mechanisms, and proposed novel therapeutic strategies targeting telomere biology [127]. Integrating these with single-cell multi-omics approaches may provide a more refined understanding of oncogenic drivers and actionable vulnerabilities. Moreover, Dong et al. analyzed relapsed NB by scRNA-seq and demonstrated that residual tumor cells acquire drug-resistant transcriptional states that interact with an immunosuppressive microenvironment, emphasizing the utility of single-cell profiling in capturing resistant populations during tumor evolution [107].

Metastasis represents the ultimate stage of cancer evolution, accounting for the majority of NB-related mortality and frequently accompanied by treatment resistance. Importantly, the altered bone marrow (BM) microenvironment in which disseminated NB cells reside can reshape their transcriptional programs and thereby promote resistance. Recent advances in scRNA-seq have enabled high-resolution profiling of the NB BM niche, which is particularly relevant given the strong predilection of NB for BM metastasis. Mei et al. revealed enrichment of tumor-associated neutrophils, macrophages, and exhausted T cells in metastatic BM, alongside an increase in regulatory T cells and a decrease in B cells and identified malignant-cell markers associated with poor prognosis [128]. Similarly, Fetahu et al. combined single-cell transcriptomics with epigenomic profiling to uncover the central role of monocytes in BM metastasis, highlighting their dual functions in promoting inflammation and mediating immunosuppression [129]. Complementarily, Kildisiute et al. [130] constructed a large-scale single-cell atlas and demonstrated that noradrenergic tumor cells maintain a stable phenotype in BM metastases, yet their interaction networks with immune cells undergo profound rewiring, implicating the metastatic microenvironment as a critical determinant of resistance and progression.

Collectively, these findings establish that NB metastasis is not merely a passive process of cellular dissemination but is intimately coupled with extensive remodeling of the immune microenvironment, predominantly orchestrated by myeloid lineages. This paradigm provides fresh perspectives on the molecular and cellular dynamics underlying metastasis, reinforces the importance of dissecting the NB TME, and lays the conceptual groundwork for developing therapies that target tumor to microenvironment interactions.

THE TUMOR MICROENVIRONMENT OF NEUROBLASTOMA: CELLULAR COMPOSITION AND FUNCTIONAL HETEROGENEITY

Single-cell atlas of the neuroblastoma microenvironment

Over the past decade, the rapid advancement of single-cell technologies has profoundly enhanced our understanding of the

complex ecosystems of solid tumors. A series of landmark studies have demonstrated that constructing high-resolution single-cell atlases not only reveals intratumoral heterogeneity but also elucidates the key mechanisms governing tumor evolution and microenvironmental interactions. For instance, in glioblastoma, Neftel et al. [131] proposed four dynamically interchangeable cellular states, highlighting the coupling between tumor cell plasticity and microenvironmental cues. In melanoma, Tirosh et al. [132] delineated multidimensional interaction networks among malignant, immune, and stromal cells, thereby uncovering the ecological basis of immune evasion. In breast cancer, Azizi et al. [133] and Wagner et al. [134] systematically mapped the functional continuum of tumor-infiltrating immune cells, linking immune gradients to therapeutic responsiveness. In pancreatic ductal adenocarcinoma, Elyada et al. [135] identified distinct subsets of CAFs with immunomodulatory functions, emphasizing the central role of stromal cells in immune exclusion. Similarly, Ma et al. [136] integrated single-cell transcriptomic data from hepatocellular carcinoma to show that lineage diversity within tumor cells drives microenvironmental remodeling and differential responses to immunotherapy. Collectively, these studies have defined the current paradigm of single-cell atlas research in solid tumors, from dissecting cellular composition and state plasticity to stratifying immune and stromal function and linking atlas-derived features to clinical outcomes and offering a multidimensional framework for decoding tumor biology.

TME consists of both cellular and non-cellular components that collectively orchestrate tumor initiation, progression, invasion, metastasis, and therapeutic resistance [137–139]. Neuroblastoma is composed of diverse cell types, including malignant neuroblasts, stromal cells, endothelial cells, CAFs, and various immune cell populations residing within the tumor microenvironment [112, 140]. The NBAtlas, which integrates single-cell transcriptomic profiles from 61 patients across seven independent studies, provides a unified reference for defining the cellular composition of NB. It highlights the extensive intratumoral heterogeneity and immune complexity of the disease and establishes correlations between transcriptional programs and clinical outcomes, serving as an invaluable benchmark for annotating new single-cell datasets [124]. Beyond compositional mapping, single-cell studies targeting distinct biological contexts have expanded the functional dimension of the NB atlas. Bedoya-Reina et al. [126] compared single-nucleus transcriptomes of human postnatal adrenal glands with NB samples, revealing that low-risk tumors share transcriptional similarities with sympathetic neurons and chromaffin cells, whereas high-risk tumors are enriched for TRKB⁺ cholinergic progenitor-like populations, linking aberrant developmental trajectories to clinical risk phenotypes. Moreover, in studies focusing on the metastatic bone marrow niche, Mei et al. [128] and Fetahu et al. [129] identified a coordinated immunosuppressive network involving tumor-associated neutrophils (TANs) and the monocyte–macrophage axis as a key driver of NB metastasis. Mei et al. showed that metastatic lesions are enriched in TANs and monocyte–macrophage populations. TANs highly express CXCL8 and S100A8/A9, activating the CXCR2–MAPK/NF-κB pathway to induce immune exhaustion and upregulate ARG1 and PD-L1, reinforcing suppression. Concurrently, monocyte–macrophage cells secrete SPP1, MIF, and CCL2, which signal through MIF–CD74/CD44 and SPP1–ITGAV axes to promote M2 polarization, T-cell inhibition, and ECM remodeling, forming an immune-excluded “myeloid barrier.” Complementarily, Fetahu et al. identified SPP1⁺ macrophages and CD163⁺/IL-10⁺ monocytes that drive immune evasion and angiogenesis via TGF-β, VEGF, and IL-10 pathways, and regulate ECM organization through the SPP1–ITGB1–STAT3 axis. Together, these findings position the TAN–macrophage interaction network as a central mediator linking immune suppression and stromal remodeling in metastatic NB.

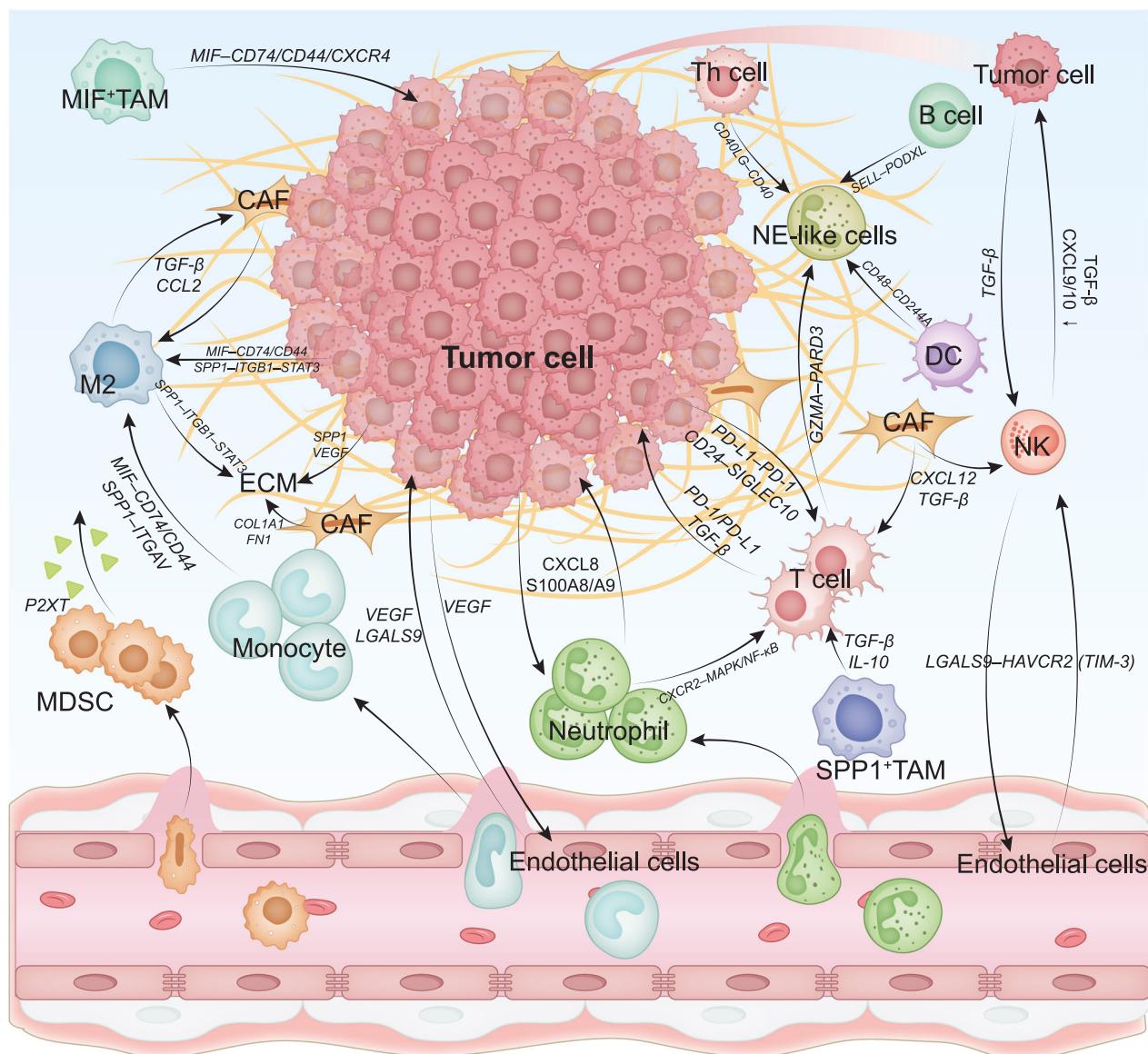


Fig. 3 The immunosuppressive tumor microenvironment of neuroblastoma. Single-cell and spatial multi-omics analyses have revealed a complex immunosuppressive network in the NB TME. Tumor-associated macrophages (TAMs) and neutrophils act as central mediators of immune evasion and stromal remodeling. MIF⁺ TAMs interact with tumor and stromal cells through MIF-CD74/CD44/CXCR4 signaling, while SPP1⁺ TAMs promote angiogenesis and extracellular matrix (ECM) reorganization via SPP1-ITGB1-STAT3 and VEGF pathways. Neutrophils secrete CXCL8 and S100A8/A9, activating the CXCR2-MAPK/NF- κ B axis to induce immune exhaustion and PD-L1 upregulation. Cancer-associated fibroblasts (CAFs) further reinforce immune exclusion by releasing TGF- β , CXCL12, COL1A1, and FN1, forming a collagen-rich physical barrier that limits effector T-cell infiltration. Endothelial-immune crosstalk, exemplified by LGALS9-HAVCR2 (TIM-3) interactions, contributes to vascular immune suppression. In parallel, monocytes and MDSCs support macrophage recruitment and differentiation through MIF-CD74/CD44 and SPP1-ITGAV signaling. Collectively, these coordinated cellular and molecular interactions establish a highly suppressive microenvironment that promotes immune escape, angiogenesis, and tumor progression in high-risk and relapsed NB.

The immunosuppressive microenvironment promotes tumor immune evasion

As illustrated in Fig. 3, recent studies based on NB patient samples and murine models have progressively revealed the complex immune architecture of the TME, emphasizing the remarkable functional heterogeneity among immune cell populations [140]. In addition to myeloid-derived suppressor cells (MDSCs), the NB TME is enriched with multiple immunosuppressive components, including TANs, regulatory T cells (Tregs), dysfunctional natural killer (NK) cells, and TAMs [128, 140]. Among them, macrophages display high plasticity and can dynamically switch between pro-inflammatory M1 (CD68⁺, IL-1 β ⁺, TNF- α ⁺) and anti-inflammatory M2 (CD163⁺, CD206⁺, IL-10⁺) phenotypes. While M1 macrophages

primarily mediate inflammation and immune activation, M2 macrophages are involved in tissue remodeling and immunosuppression. Although high TAM infiltration generally predicts poor prognosis, certain pro-inflammatory myeloid subsets have been correlated with favorable outcomes, suggesting that macrophages may exert context-dependent dual functions [141, 142].

Recent single-cell and multi-omics studies have further refined this immunosuppressive landscape. Yu et al. [143], through longitudinal single-cell multi-omics profiling of high-risk NB, identified TAMs as the dominant immune population within the TME. Their abundance markedly increased after therapy and exhibited pronounced subtype heterogeneity, with subsets enriched in immunosuppressive axes such as MIF-CD74/CD44/

CXCR4 and NECTIN2–TIGIT. Wienke et al. [142] analyzed 24 NB tumors (pre- and post-chemotherapy) using scRNA-seq and revealed therapy-induced immune remodeling characterized by the upregulation of suppressive pathways in both myeloid and T-cell compartments. Giudice et al. [144], using a GPC2 CAR-T therapy model, demonstrated that treatment-driven TME reprogramming reshaped immune cell composition and effector states, highlighting the plasticity of immunosuppressive circuits. Strijker et al. [145] further showed that pharmacological inhibition of M2-derived MIF enhanced CAR-T efficacy, providing experimental evidence for targeting TAM-associated druggable pathways. In spatial multi-omics analyses, Yu et al. [145] and Wienke et al. [142] consistently reported upregulation of SPP1⁺ TAMs and the MIF–CD74/CD44 axis in high-risk and relapsed NB, which were closely associated with T-cell exhaustion phenotypes. Spatial transcriptomics also revealed enriched LGALS9–HAVCR2 (TIM-3) interactions between endothelial and NK/T cells within immune-excluded regions, underscoring the importance of vascular–immune crosstalk in maintaining suppression [146]. Additionally, Liu et al. [147] demonstrated in gastric cancer that activation of the MIF–CD74 pathway drives macrophage reprogramming toward an immunosuppressive phenotype—a mechanism also observed in NB TAMs—indicating a conserved axis across solid tumors. Batchu et al. [148] confirmed this in a pan-cancer integrated analysis, showing broad upregulation of MIF–CD74/CD44 signaling associated with macrophage recruitment and immune evasion, providing a comparative framework for NB myeloid regulation.

Beyond the myeloid compartment, other immune cell types—such as eosinophils, mast cells, and monocytes—can be recruited to tumor sites through cytokines including IL-1, IFN- γ , and TNF- α , thereby sustaining chronic inflammation and facilitating metastasis [149–151]. Meanwhile, a subset of cancer-associated fibroblasts (CAFs) within the NB microenvironment exhibits immunosuppressive features [140]. Integrative single-cell and bulk transcriptomic analyses [152] established a CAF-related prognostic model, linking TGFB1 and CXCL12 secretion to distinct immune infiltration patterns. Two CAF subtypes were defined: one expressing TGF- β , CXCL12, COL1A1, and FN1, forming an immune-excluded “collagen barrier,” and another ACTA2⁺ myofibroblastic subtype associated with ECM cross-linking and tissue stiffening.

Single-cell and spatial studies in other solid tumors have also revealed conserved mechanisms of CAF-mediated immune exclusion. Elyada et al. [135] identified MHC II⁺ antigen-presenting CAFs (apCAF) in pancreatic ductal adenocarcinoma that engage TAMs through TGF- β and CCL2 signaling feedback loops. Peng et al. [153] found in colorectal cancer that inflammatory CAFs (iCAF) negatively correlate with NK and monocyte infiltration, suggesting cytokine-driven immune exclusion zones. Zhang et al. [154] reported spatial co-localization of FAP⁺ CAFs and SPP1⁺ macrophages, mediated by ECM-related ligand–receptor networks that regulate immune tone. Jain et al. [155] further confirmed, through single-cell and spatial transcriptomics of glioblastoma, the presence of CAF-like fibroblasts—an unexpected finding in a neural malignancy traditionally considered fibroblast-poor. These CAF-like cells localized near endothelial and M2 macrophage clusters, jointly promoting local immunosuppression and stromal stiffening.

MECHANISMS OF THERAPEUTIC RESISTANCE IN NEUROBLASTOMA: CELLULAR LINEAGE EVOLUTION AND ADAPTIVE PLASTICITY

Despite the substantial improvement in overall outcomes achieved through multimodal therapies, therapeutic resistance remains the major obstacle to long-term survival in patients with high-risk and relapsed NB. Recent single-cell and spatial multi-

omics studies have delineated the dynamic trajectory of resistance evolution. At diagnosis, NB tumors are predominantly composed of highly proliferative ADR cells, with limited immune infiltration. Following induction chemotherapy, the cellular composition undergoes profound remodeling, characterized by an increased proportion of mesenchymal-like and persister-like tumor subpopulations, accompanied by the gradual enrichment of myeloid cells, particularly SPP1⁺ macrophages and neutrophils, within the immune microenvironment. In relapse and metastatic stages, tumor cells display markedly elevated heterogeneity and plasticity, coinciding with the expansion of tumor-associated neutrophils, immunosuppressive macrophages, and fibroblasts in the bone marrow niche, while effector T and NK cells are progressively depleted [128, 129, 143]. This temporal shift in cellular composition, from a “tumor-dominant” to an “immunosuppressive and stromal-reprogrammed” ecosystem, highlights the ecological remodeling of NB under therapeutic pressure and provides a spatiotemporal framework for understanding treatment failure and relapse (as illustrated in Fig. 4).

Genetic and structural remodeling

Single-cell DNA and RNA sequencing have revealed marked structural and clonal dynamics accompanying the development of NB resistance. Avitabile et al. performed single-cell RNA analysis of etoposide- and cisplatin-resistant NB cell lines and identified transcriptional clusters clearly segregated from parental cells, enriched in pathways related to DNA repair, nucleosome organization, and chromatin maintenance, suggesting that genomic remodeling contributes to the establishment of drug resistance [156]. Another integrative DNA/RNA sequencing study of NB cell lines and patient samples demonstrated that heterogeneity in extrachromosomal DNA (ecDNA) copy number drives MYCN expression variability and cell-state transitions, indicating that ecDNA-mediated genomic plasticity underlies adaptive phenotypic switching [157].

Epigenetic and Core Regulatory Circuit Remodeling

Epigenetic plasticity is one of the core mechanisms underlying the formation and maintenance of drug resistance. Integrative analysis shows that neuroblastoma undergoes extensive remodeling of super-enhancers and CRCs during treatment, characterized by the activation of stress-related transcriptional axes (PRRX1/NOTCH) and the suppression of neural-lineage factors (ASCL1/PHOX2B) [158, 159]. This state transition, formerly referred to as the adrenergic–mesenchymal transition (AMT), actually lies within a broader epigenetic continuum involving multiple reversible developmental states. Pharmacological studies have shown that targeting epigenetic regulators (such as BET, HDAC, or EP300) can partially restore epigenetic balance, thereby reinducing the sensitivity of resistant cells to therapy. Meanwhile, resistance stratification analysis has revealed a specific pattern of rapid attenuation in transcription factor regulatory activity (such as BAZ1A, HCFC1, MAZ, and ZNF146), and their silencing significantly inhibits the proliferation of NB cells *in vitro*, suggesting that this pathway participates in the stabilization of epigenetic maintenance and drug insensitivity [160]. At the single-cell level, the imbalance of demethylation and chromatin assembly–related axes accompanies the occurrence of AMT, further supporting the critical role of the reversible “super-enhancer–CRC–cell fate” loop in drug resistance [156, 160].

Target loss and bypass activation

Integrated single-cell multi-omics analyses indicate that therapeutic resistance in NB often arises from signal network rewiring rather than the inactivation of a single pathway. Studies have revealed that, under ALK inhibition or chemotherapy pressure, a subset of NB cells can restore downstream cascades through bypass receptor–ligand axes, thereby maintaining MAPK and

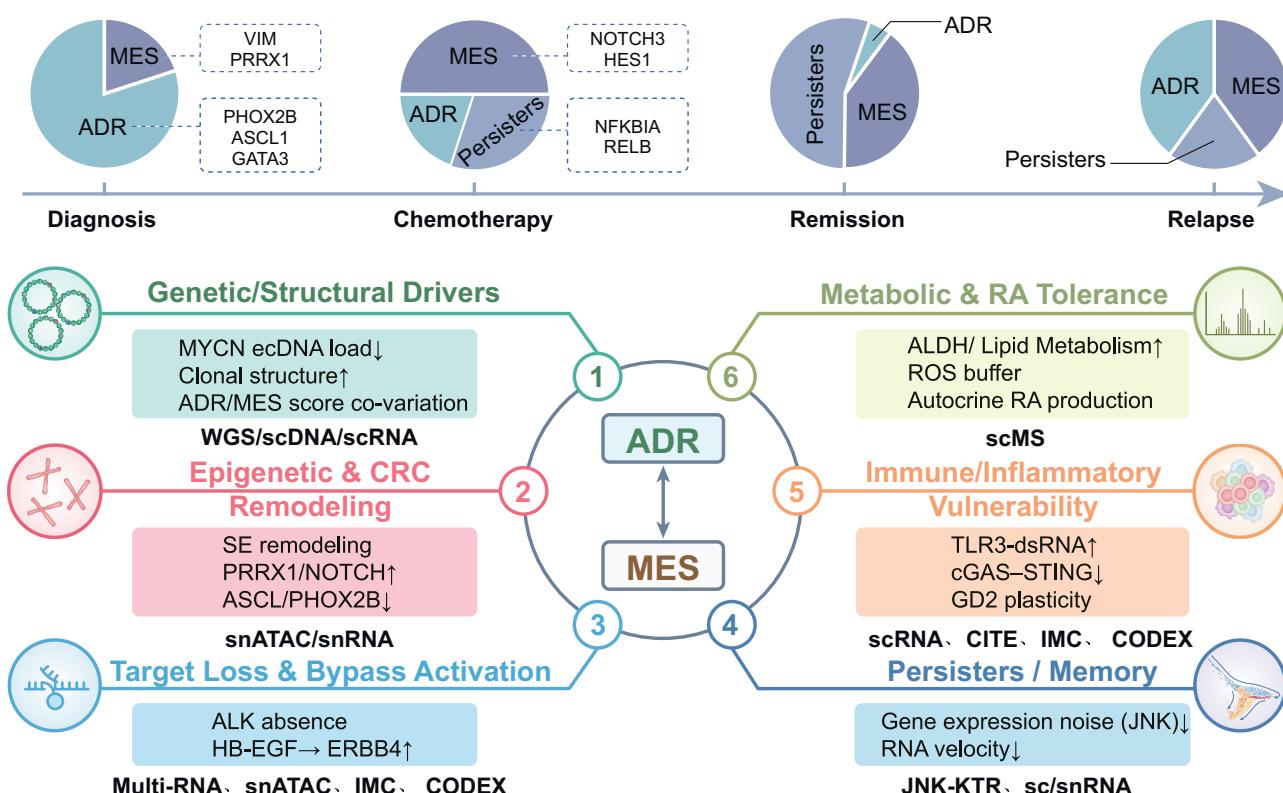


Fig. 4 Cellular lineage evolution and multi-omic mechanisms of therapeutic resistance in neuroblastoma. Schematic representation of NB cell-state transitions and resistance mechanisms under therapeutic pressure. During disease progression from diagnosis to relapse, ADR tumor cells progressively convert to MES and persister-like states, accompanied by epigenetic reprogramming and ecological remodeling. Six major resistance mechanisms are highlighted: (1) Genetic and structural drivers, MYCN ecDNA variation and clonal evolution; (2) Epigenetic and CRC remodeling, super-enhancer (SE) reorganization and PRRX1/NOTCH activation with ASCL1/PHOX2B suppression; (3) Target loss and bypass activation, receptor-ligand rewiring (e.g., HB-EGF–ERBB4) sustaining MAPK/PI3K signaling; (4) Persister and memory programs, low RNA velocity and JNK-dependent transcriptional noise reduction; (5) Immune and inflammatory vulnerability, TLR3-dsRNA activation, cGAS-STING deficiency, and GD2 plasticity; and (6) Metabolic and retinoic acid tolerance, enhanced lipid metabolism, ROS buffering, and autocrine RA synthesis. Together, these multi-layered processes drive adaptive resistance and therapeutic escape in NB.

PI3K–AKT signaling and sustaining cell survival. A representative example is that TAMs secrete HB-EGF, which in turn activates ERBB4 signaling in tumor cells through paracrine interaction, triggering ERK pathway activation and proliferation. Functional validation demonstrated that both an HB-EGF neutralizing agent (CRM197) and pan-ERBB inhibitors (e.g., afatinib) markedly suppressed NB cell growth, indicating that the HB-EGF–ERBB4 axis acts as a key bypass route mobilized under therapeutic stress [143].

In etoposide/cisplatin-resistant models, scRNA-seq profiling further revealed that resistant clusters form transcriptionally distinct subpopulations from their parental cells, with significant enrichment of pathways related to DNA repair (such as the BARD1/BRCA1/PARP1 axis) and drug target modification. These alterations are accompanied by cell identity reprogramming and concurrent signaling rewiring, supporting a model of plastic resistance driven by functional redundancy [156].

In the context of ALK-targeted therapy, secondary ALK mutations often coexist with downstream signal rerouting. System-level genomic studies have revealed that ALK pathway mutations not only confer inhibitor resistance but also expose collateral vulnerabilities, providing rationale for multi-target combination strategies or function-centric pathway blockade approaches [161].

At the spatial level, integrated analyses combining imaging mass cytometry (IMC) with single-cell and spatial transcriptomics have shown that bypass activation frequently coincides with stromal remodeling and immune exclusion. In NB bone marrow

metastatic niches, myeloid cells, particularly SPP1⁺ macrophages and neutrophils—are enriched alongside upregulation of immunosuppressive signaling axes and chromatin accessibility reprogramming, establishing a ligand-rich and receptor-rich microenvironmental scaffold that facilitates bypass signaling [129].

Persister and memory-like cell programs

Drug resistance in NB is not merely the result of clonal selection but also arises from a dynamic equilibrium of cell states. Single-cell lineage tracing and RNA velocity analyses have shown that a subset of cells can reduce RNA dynamics and transcriptional noise, thereby entering a low-metabolic and low-proliferative persister state [162]. Computational modeling and single-cell imaging further suggest that, even after chemotherapy, gene expression noise persists, which can drive the emergence of resistant phenotypes within a clone without relying on rare gene expression states. Among these, defective JNK signaling is closely associated with persistence and regrowth, and JNK-deficient subpopulations can be detected even in untreated cells, exhibiting a form of “resistance memory” [162]. Restoring JNK activity or lowering the apoptotic threshold has been shown to significantly increase drug sensitivity, offering a promising therapeutic approach to eliminate latent residual cell populations.

Across resistant models and longitudinal clinical samples, a set of recurrent “survival-type epigenetic programs” has emerged, characterized by decreased cell cycle scores, markedly reduced or stagnant RNA velocity, and globally diminished chromatin accessibility. A subset of surviving cells, after exposure to

chemotherapy, shows a pronounced decline in cell-cycle activity and enters a state of slow proliferation or quiescence. RNA velocity analyses reveal minimal transcriptional flux and a lack of directional transitions toward new cellular states [156]. Concurrently, multiple single-cell ATAC-seq, snATAC-seq, or joint multi-omic studies have demonstrated that these residual or drug-tolerant cells often exhibit a more closed chromatin landscape, with fewer accessible regions and attenuated enhancer activity [108, 129, 143]. Collectively, these findings indicate that NB resistance involves not only transcriptional and metabolic adaptation but also an epigenetically stabilized quiescent state, representing a reversible yet resilient survival program that bridges transient persistence and long-term resistance.

Immune and inflammatory vulnerabilities

The TME plays a critical role in the development of therapeutic resistance. Single-cell and spatial multi-omics studies have revealed a deep coupling between inflammatory and immune signaling and the evolution of resistance. The epigenetic state determines inflammatory sensing, with ADR and MES lineages exhibiting distinct capacities in innate immune pathways. Specifically, most NB cells lack cGAS-STING-mediated DNA sensing, whereas MES-state cells display a stronger TLR3-dependent recognition of double-stranded RNA (dsRNA), accompanied by higher basal inflammatory levels and a more active pro-inflammatory transcriptomic profile. Reprogramming ADR cells into the MES state restores dsRNA responsiveness, induces cytokine secretion, and enhances T cell-mediated cytotoxicity, indicating an immune vulnerability window linked to lineage plasticity [163].

In resistant lesions, immunosuppressive signaling axes are frequently “switched on.” Integrated single-cell transcriptomic interaction analyses have identified NECTIN2-TIGIT as a key immune checkpoint pathway in NB, and functional assays have shown that combined blockade can partially reverse immune escape and restore effector T cell activity. This finding is consistent with the bone marrow metastatic niche, where SPP1⁺ macrophages and neutrophils are enriched, immunosuppressive signaling is upregulated, and chromatin accessibility is reprogrammed, together forming a microenvironmental foundation that facilitates bypass activation and immune exclusion [142].

Moreover, the epigenetic plasticity of tumor-associated differentiation antigens provides a manipulable opportunity for combinatorial immunotherapy. Studies have demonstrated that EZH2 inhibition can reprogram lineage states and upregulate GD2 expression, thereby restoring or enhancing anti-GD2 therapeutic responses. This discovery links epigenetic modulators with antibody-based therapy, offering a strategic anchor to counteract AMT/MES-associated resistance [164].

Metabolic and retinoic acid tolerance

Metabolic adaptation represents a crucial layer in the development of drug resistance in NB, particularly evident in MES and persister-like subpopulations. These cells undergo extensive metabolic rewiring, forming a state characterized by enhanced oxidative phosphorylation (OXPHOS) and fatty acid oxidation (FAO) dependence, increased antioxidant capacity, and activation of endogenous all-trans retinoic acid (RA) synthesis, enabling long-term survival under chemotherapeutic and oxidative stress.

Studies have shown that MYCN-amplified NB cells exhibit significant metabolic reprogramming at the energetic level, characterized by enhanced mitochondrial respiration, increased dependence on FAO, and elevated glycolytic flux. This type of metabolic remodeling supports sustained energy production and stress resistance, while simultaneously exposing new metabolic vulnerabilities. For instance, the CPT1A-mediated FAO pathway has been identified as a potential therapeutic target [93, 165]. Moreover, the upregulation of lipid metabolism promotes

membrane biosynthesis, lipid signaling molecule generation, and energy compensation, providing long-term metabolic support for resistant cells.

In terms of oxidative stress defense, resistant NB cells often display persistent activation of the glutathione (GSH)-Nrf2-HO-1 antioxidant axis. As a master regulator of oxidative stress, Nrf2 upregulates multiple antioxidant and detoxification-related genes, thereby eliminating reactive oxygen species (ROS) and maintaining intracellular redox homeostasis. This feature is recognized as a hallmark of cell persistence and chemoresistance across multiple cancer types [166].

More importantly, MES-type NB cells possess an intrinsic ability to synthesize endogenous RA. Recent studies have shown that this subpopulation forms an autocrine RA loop through ALDH1A1/ALDH1A3-mediated retinal oxidation, leading to pronounced differentiation resistance to exogenous RA *in vitro*. Endogenous RA, in turn, maintains the MES transcriptional state and promotes cell migration and survival, constituting a major mechanism of resistance to differentiation therapies such as ATRA [167].

EMERGING BIOMARKERS AND THERAPEUTIC TARGETS

Identification of emerging biomarkers driven by single-cell omics

NB exhibits remarkable molecular and clinical heterogeneity, which has long limited the ability of conventional bulk gene expression analyses to precisely delineate its key drivers and therapeutic response patterns [168]. In recent years, advances in single-cell multi-omics integration—encompassing transcriptomic, epigenomic, and proteomic data—have enabled researchers to simultaneously dissect the dynamic states and molecular interactions of tumor, immune, and stromal cells. This shift from population-level to cell-specific resolution has redefined biomarker discovery, allowing reconstruction of NB’s evolutionary trajectory and treatment response at a molecular scale. Such an approach not only reveals core regulatory nodes of tumor initiation and progression but also provides a high-resolution framework for risk stratification and precision therapy.

Among these studies, several transcriptional regulators, including STMN2, TUBA1A, PAGE5, and ETV1, have been identified as key drivers of NB tumorigenesis and intratumoral heterogeneity, suggesting their potential as therapeutic targets. Moreover, ITGB1, which is highly expressed in M2-like macrophages, has been associated with favorable prognosis, underscoring its dual potential as a diagnostic biomarker and immunotherapeutic target [169].

Recent single-cell analyses have further illuminated critical signaling pathways mediating immune evasion and therapeutic resistance within the NB tumor microenvironment. The NECTIN2-TIGIT axis has been recognized as a major immune checkpoint pathway that promotes T cell exhaustion and attenuates antitumor immunity [142]. *In vivo* experiments demonstrated that co-targeting TIGIT and PD-L1 markedly suppressed tumor growth and induced complete remission, even in chemotherapy-resistant NB models, highlighting the translational potential of this pathway for immunotherapy.

At the molecular subtype level, pathway analysis has revealed that specific transcriptional signatures can delineate highly aggressive NB subtypes associated with poor prognosis, providing new insights for precision-targeted interventions [170]. Notably, the PCLAF⁺ neuroendocrine cell subpopulation has been identified as a major driver of NB progression. Further studies demonstrated that this subpopulation is highly susceptible to cuproptosis, a newly characterized form of copper-dependent cell death, suggesting a promising metabolic vulnerability that may be exploited for therapeutic intervention in refractory NB.

Table 1. Overcoming Challenges and Shaping Future Directions in Neuroblastoma.

Research aspect	Key challenges	Potential solutions
Technical limitations	Loss of cell viability, sample contamination, and high sequencing cost	Optimize tissue handling and cell preservation techniques; employ microdissection-based enrichment and low-input sequencing strategies
Data reliability	Insufficient sequencing depth and high intercellular variability	Increase sequencing depth; integrate temporal and spatial multi-omics data to improve normalization and consistency
Tumor heterogeneity	Complex microenvironmental and intercellular diversity	Develop cross-layer multi-omics frameworks to capture tumor–microenvironment interactions
Multi-omics integration	Limited application beyond scRNA-seq	Expand multi-omics coverage and develop hierarchical data integration algorithms
Computational resources	High computational demand and limited scalability of algorithms	Incorporate AI/ML-assisted high-throughput computing platforms and construct interpretable analytical frameworks
Translational validation	Lack of robust clinical cohorts and functional validation experiments	Establish longitudinal clinical cohorts integrating spatial omics and experimental models for mechanistic validation

Computationally powered prognostic signature identification

With the rapid advancement of computational science and artificial intelligence (AI), machine learning algorithms have become widely applied across various aspects of cancer research, including diagnosis, classification, prognosis prediction, and treatment response assessment. In NB, this trend is particularly pronounced. By integrating multi-omics datasets, such as bulk RNA sequencing, methylome, proteome, radiomics, and histopathology, researchers have developed accurate risk-stratification models that enable data-driven decision-making in clinical diagnosis and outcome evaluation [171–174].

At the single-cell resolution, however, computational approaches remain in a phase of rapid expansion, primarily serving to elucidate therapy resistance mechanisms and identify cell-specific biomarkers. For example, using the acNMF algorithm, researchers constructed a predictive model of drug response and discovered that adrenergic gene expression patterns were conserved between clinical and preclinical NB models, while drug-resistant mesenchymal transcriptional programs were largely confined to tumor-associated non-malignant cells [123]. In high-risk NB, chemotherapy induced a “weak-mesenchymal” transcriptional state within adrenergic cells, suggesting that treatment may drive a reversible mesenchymal transition. This finding not only clarified the cellular origin of post-treatment relapse and resistance but also revealed a potential intervention window to reprogram reversible transcriptional states.

At the same time, traditional biomarker discovery methods are often expensive and time-consuming, which limits their translational efficiency. Consequently, high-throughput computational models have emerged as effective alternatives for identifying key prognostic genes. By combining multi-omics integration with machine learning, multiple molecular signatures with strong translational potential have been developed in NB. For instance, Li et al. [175] established a machine learning model based on differential enhancer methylation, which accurately distinguished INSS stage 4 from stage 4S NB and identified methylation markers with dual diagnostic and prognostic value. Xia et al. [176] developed a four-gene prognostic model incorporating the mRNA-based stemness index (mRNAsi), revealing a close coupling between tumor stemness and immune infiltration. Similarly, Cheng et al. [177] integrated ferroptosis-related gene expression and found that high levels of MYCN and RRM2 constituted a core molecular basis of chemoresistance in NB. In addition, Wang et al. [178] proposed a mitochondria-related gene (MRG) signature, which outperformed the traditional MYCN-based classification in risk prediction and identified FEN1 as a crucial regulator linking metabolic reprogramming and immune suppression in NB. Furthermore, Cai et al. [179] integrated E2F family transcription factor-related genes to construct a prognostic model capable of

stratifying distinct risk subtypes and pinpointing potential druggable targets for individualized therapy. In a related approach, Aierken et al. [180] developed an 11-gene neutrophil extracellular trap (NETs)-related model, which maintained stable performance across cohorts and revealed the association between NETs signaling and immunosuppressive TME. Finally, Chen et al. [181] proposed a T-cell exhaustion (TEX)-related risk signature, accurately predicting both immunotherapy response and patient prognosis.

Collectively, these computationally powered strategies have accelerated the discovery of prognostic biomarkers and therapeutic targets in NB. By integrating high-dimensional omics data with machine learning frameworks, researchers can now delineate complex molecular networks, improve precision in risk prediction, and identify actionable pathways—offering new opportunities to enhance the survival of high-risk and relapsed NB patients who remain refractory to multimodal therapy.

CHALLENGES AND FUTURE PERSPECTIVES

Despite the remarkable progress achieved through single-cell studies in NB, substantial technical and translational challenges remain (Table 1). At the technical level, maintaining cell viability, preventing sample contamination, and the high cost and long turnaround time of single-cell sequencing continue to limit study scale and reproducibility [182–187]. Moreover, intrinsic variability in single-cell data—arising from differences in cell-cycle stage, sequencing depth, and sample heterogeneity—complicates interpretation and necessitates advanced algorithms and standardized workflows to minimize bias [187]. The pronounced heterogeneity of NB further increases analytical complexity, requiring simultaneous characterization of developmental hierarchies among malignant lineages and dynamic interactions among immune, stromal, and vascular components within TME [6, 25, 102, 188, 189]. Achieving robust multidimensional data integration from limited pediatric samples remains a major bottleneck for clinical translation.

Future multi-omics research should prioritize cross-layer integration and dynamic tracing. Integrating single-cell transcriptomics with epigenomics will help delineate regulatory networks involving chromatin accessibility and epigenetic modifications, thereby revealing key transcriptional nodes driving tumor evolution and therapeutic resistance. The inclusion of single-cell proteomics and metabolomics will further bridge the gap between gene expression and signaling execution, offering a functional perspective on cellular states. In parallel, the convergence of spatial transcriptomics and imaging mass cytometry allows *in-situ* reconstruction of the cellular ecosystem of NB, enabling real-time mapping of immune infiltration, metastatic

progression, and therapeutic response, thereby offering novel avenues for precision intervention [29, 190–192]. However, multi-omics approaches are not universally applicable. When research goals focus on validating specific pathways, conducting low-throughput mechanistic studies, or achieving rapid clinical testing, comprehensive multi-omics profiling may introduce unnecessary noise, cost, and analytical burden. In such contexts, hypothesis-driven targeted multi-omics or layered bulk–single-cell validation strategies are more appropriate, balancing data dimensionality with biological interpretability [193, 194]. Particularly in pediatric NB, where tissue access and sample size are often constrained, excessive sequencing depth should be avoided in favor of cell enrichment, snRNA-seq, or organoid-based models to enhance signal-to-noise ratio and representativeness [195, 196].

To overcome current analytical and technical limitations, the integration of artificial intelligence (AI) and machine learning (ML) has emerged as a pivotal strategy. AI-driven frameworks enable nonlinear feature extraction and multimodal pattern recognition, substantially improving the identification of rare cell subsets and transient phenotypic transitions [197, 198]. Through deep neural networks (DNNs) and self-supervised learning paradigms, these models can automatically uncover latent correlations across transcriptomic, methylomic, and proteomic layers, reconstructing lineage trajectories and disease evolution. Meanwhile, spatiotemporal AI models, such as graph neural networks (GNNs) and multimodal Transformers, have shown promise in systematically reconstructing tumor–immune–stromal interaction networks and predicting key signaling pathways and actionable therapeutic targets [199]. Furthermore, AI facilitates quality control, anomaly detection, and visualization, correcting sequencing bias, mitigating batch effects, and enhancing denoising accuracy [200]. Collectively, these advances markedly improve the reproducibility and standardization of single-cell analyses, establishing a scalable computational foundation for NB multi-omics research.

Looking ahead, progress in NB research will depend on the synergistic integration of bioinformatics, clinical oncology, and basic science. From the bioinformatics perspective, developing scalable algorithmic frameworks and open-access data-sharing platforms will enable large-scale, multi-center integration of NB datasets. Clinically, establishing single-cell-driven patient cohorts will facilitate biomarker validation and personalized therapeutic guidance. At the mechanistic level, exploring the dynamic interplay among tumor, immune, and metabolic networks will provide essential functional validation for multi-omics discoveries. As deep learning and spatial multi-omics continue to converge, NB research is poised to evolve from descriptive molecular profiling toward predictive and mechanistic modeling, paving the way for precision oncology and improved long-term survival in high-risk patients.

CONCLUSION

Recent advances in single-cell technologies have fundamentally reshaped our understanding of neuroblastoma by revealing its remarkable cellular diversity and molecular complexity. Beyond traditional bulk transcriptomic approaches, single-cell and spatial multi-omics have enabled precise mapping of malignant, immune, stromal, and endothelial cell populations, uncovering their lineage relationships and functional plasticity during tumor initiation, progression, and relapse. These insights have not only clarified the developmental origins of neuroblastoma but also illuminated how transcriptional reprogramming, metabolic adaptation, and immune modulation collectively drive disease heterogeneity.

At the molecular level, integrative single-cell analyses have identified multiple coexisting malignant programs, ranging from proliferative, neuronal, and mesenchymal-like to stress-adaptive and persister phenotypes, reflecting the dynamic equilibrium of tumor ecosystems under therapeutic pressure. Alterations in

chromatin accessibility, metabolic flux, and inflammatory signaling converge to promote cell survival, immune evasion, and therapeutic resistance. These findings highlight that treatment failure in neuroblastoma arises not from a single resistant clone but from a coordinated network of adaptive states across the tumor microenvironment.

Looking forward, the continued integration of transcriptomic, epigenomic, proteomic, and metabolomic data, combined with artificial intelligence–driven modeling, will be essential to capture the spatial and temporal evolution of neuroblastoma with greater precision. Such approaches will accelerate the discovery of predictive biomarkers, identify actionable therapeutic vulnerabilities, and facilitate personalized treatment strategies. Ultimately, the synergy between single-cell technologies, computational biology, and translational validation is expected to transform neuroblastoma management from static classification to dynamic, precision-guided intervention.

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AUTHOR CONTRIBUTIONS

GQH conceived of the presented idea and manuscript preparation. SJH, XYJ and YLD conducted the primary review of the literature and gave intellectual input. XG, JG and WZ conceived and organized the manuscript. All authors reviewed the results, provided critical feedback and comments, and approved the final version of the manuscript.

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COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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