

1 **Cell lineage as a predictor of immune response in neuroblastoma**

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3 Satyaki Sengupta^{1,2,*}, Sanjukta Das^{1,2,*}, Angela Crespo^{2,3}, Brian Miller^{4,5,6,7}, Bandana Sharma^{1,2},
4 Shupe Zhang⁸, Ruben Dries^{1,2}, Hao Huang^{1,2}, Małgorzata Krajewska^{1,2}, David N. Debruyne^{1,2},
5 Luigi Soriano¹, Malkiel A. Cohen⁸, Rogier Versteeg⁹, Rudolf Jaenisch^{8,10}, Stefani Spranger^{10,11},
6 Judy Lieberman^{2,3}, Rani E. George^{1,2,12}

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8 ¹Department of Pediatric Oncology, Dana-Farber Cancer Institute, Boston, MA 02215, USA

9 ²Department of Pediatrics, Harvard Medical School, Boston, MA 02215, USA

10 ³Program in Cellular and Molecular Medicine, Boston Children's Hospital, Boston, MA, USA.

11 ⁴Broad Institute of MIT and Harvard, Cambridge, Massachusetts 02142, USA.

12 ⁵Department of Medical Oncology, Dana-Farber Cancer Institute and Harvard Medical School,
13 Boston, Massachusetts 02215 USA.

14 ⁶Department of Immunology, Blavatnik Institute, Harvard Medical School, Boston, MA, USA

15 ⁷Evergrande Center for Immunological Diseases, Harvard Medical School and Brigham and
16 Women's Hospital, Boston, MA, USA.

17 ⁸Whitehead Institute for Biomedical Research, 455 Main Street, Cambridge, MA 02142, USA

18 ⁹Department of Oncogenomics, Amsterdam UMC University of Amsterdam, Meibergdreef 9,
19 1105 AZ Amsterdam, The Netherlands

20 ¹⁰Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02142,
21 USA

22 ¹¹Koch Institute for Integrative Cancer Research, Cambridge, MA 02142, USA

23 *These authors contributed equally

24 ¹²Corresponding author: rani_george@dfci.harvard.edu

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27 **SUMMARY**

28 Immunotherapy for patients with neuroblastoma has met with limited success, partly due to an
29 incomplete understanding of the mechanisms underlying immune responsiveness in this clinically
30 and genetically heterogenic tumor. Here, we undertook an unbiased analysis using dimension
31 reduction and UMAP visualization of transcriptional signatures derived from 498 primary
32 neuroblastoma tumors. Four distinct clusters based on differentially expressed genes emerged,
33 of which one, representing about 30% and comprising mainly of *MYCN*-nonamplified tumors, was
34 notable for the high expression of genes associated with both immune response activation and
35 suppression. This capacity to elicit a productive immune response resided exclusively in tumors
36 with dominant populations of undifferentiated, neural crest-like or mesenchymal cells; by contrast,
37 tumors comprising primarily of committed, adrenergic neuron-like cells were less immunogenic.
38 Mesenchymal neuroblastoma cells were enriched for innate and adaptive immune gene
39 signatures, demonstrated engagement with cytotoxic T and natural killer cells, and induced
40 immune cell infiltration in an immunocompetent mouse model. Transcriptional or targeted therapy-
41 induced reprogramming of adrenergic cells to the mesenchymal state led to reactivation of tumor
42 cell-intrinsic immune genes. Key immune response genes in adrenergic tumor cells were found
43 to be epigenetically silenced by the PRC2 complex, and such repression could be relieved by
44 either mesenchymal cell state reprogramming or EZH2 inhibition, leading to increased activation
45 of natural killer cells by the tumor cells. These data identify cell lineage as a major determinant of
46 the immunogenic potential in neuroblastoma that could be used to stratify patients who are most
47 likely to benefit from immunotherapy.

48 **INTRODUCTION**

49 The anti-disialoganglioside GD2 monoclonal antibody dinutuximab has significantly improved
50 event free survival rates in neuroblastoma¹. Derived from the developing neural crest, this
51 common solid tumor of childhood manifests as an extracranial mass arising in the adrenal medulla
52 or sympathetic ganglia. Approximately half of all patients have high-risk features associated with
53 a poor outcome - age >18 months, distant metastases and unfavorable histologic and genetic
54 factors including amplification of the *MYCN* oncogene². The success of anti-GD2 therapy that
55 relies on immune cell-mediated cytotoxicity suggests that patients with neuroblastoma would
56 benefit from other forms of immunotherapy; however, treatment results with use of cytotoxic CD8+
57 T lymphocytes directed against neuroblastoma antigens³, adoptive transfer of chimeric antigen
58 receptor (CAR)-modified T cells⁴⁻⁶ or checkpoint inhibition^{7,8} have been suboptimal.

59

60 Major impediments to the effectiveness of immunotherapy in neuroblastoma are the immune
61 evasion tactics deployed by the tumor cells as well as the tumor microenvironment (TME)⁹. These
62 include downregulation of major histocompatibility complex (MHC) class I molecules and defects
63 in antigen-processing machinery (APM) that render neuroblastoma cells resistant to T-cell-
64 mediated cytotoxicity¹⁰⁻¹², downregulation of cell-surface ligands required for natural killer (NK)
65 cell receptor activation¹³, upregulation of checkpoint proteins that exert a protective role from NK
66 cell-mediated lysis¹⁴, inefficient homing of cytotoxic T-cells to the tumor site¹⁵ or tumor cell
67 overexpression of the leukocyte surface antigen CD47, which enables avoidance of macrophage-
68 mediated phagocytosis¹⁶. Moreover, infiltration of suppressive immune cells such as T regulatory
69 cells¹⁷, tumor-associated macrophages (TAMs)^{18,19}, myeloid-derived suppressor cells²⁰ and
70 secreted immunosuppressive factors such as TGF-β, contribute to the generation of a TME that
71 hinders an effective immune response and further dampens the effects of adoptive cell
72 therapies²¹.

73

74 Amplification of the MYCN oncogene poses another distinct challenge to immunotherapy in
75 neuroblastoma. This transcription factor is amplified in approximately 50% of high-risk cases and
76 is associated with aggressive disease and a poor clinical outcome^{22,23}. *MYCN*-amplified tumors
77 consistently evade immune destruction by downregulating MHC class I molecules¹⁰ and are
78 associated with poor infiltration of cytotoxic CD8⁺ T cells^{24,25} and reduced expression of NK cell
79 ligands²⁶. Interestingly, approximately half of high-risk neuroblastomas do not express amplified
80 *MYCN*, and their capacity to induce a productive immune response remains unclear. In a recent
81 study that analyzed the immune gene expression programs associated with *MYCN*-nonamplified
82 tumors from high-risk patients, tumors with low as well as high functional tumor *MYCN* signatures
83 were observed to have significantly higher levels of NK and CD8+ T-cell infiltrates compared to
84 *MYCN*-amplified tumors; although, somewhat counterintuitively, these findings translated into a
85 better outcome only in patients with high *MYCN* tumor signatures^{25,27}.

86

87 Thus, although many of the mechanisms of immune evasion in neuroblastoma are known,
88 further understanding of the tumor-host interaction will be crucial to enhancing the ability of
89 immunotherapy to target and eliminate tumor-initiating and propagating cell populations.
90 Especially challenging is the genetic and biologic heterogeneity of this tumor which makes it
91 difficult to identify factors that consistently indicate the likelihood of an effective immune response
92 and hence identify patients who are most likely to benefit from this form of therapy. Thus, we
93 undertook an unbiased analysis of gene expression signatures across diverse clinical subtypes
94 of primary tumors and identify tumor cell state as an important predictor of immune
95 responsiveness in neuroblastoma.

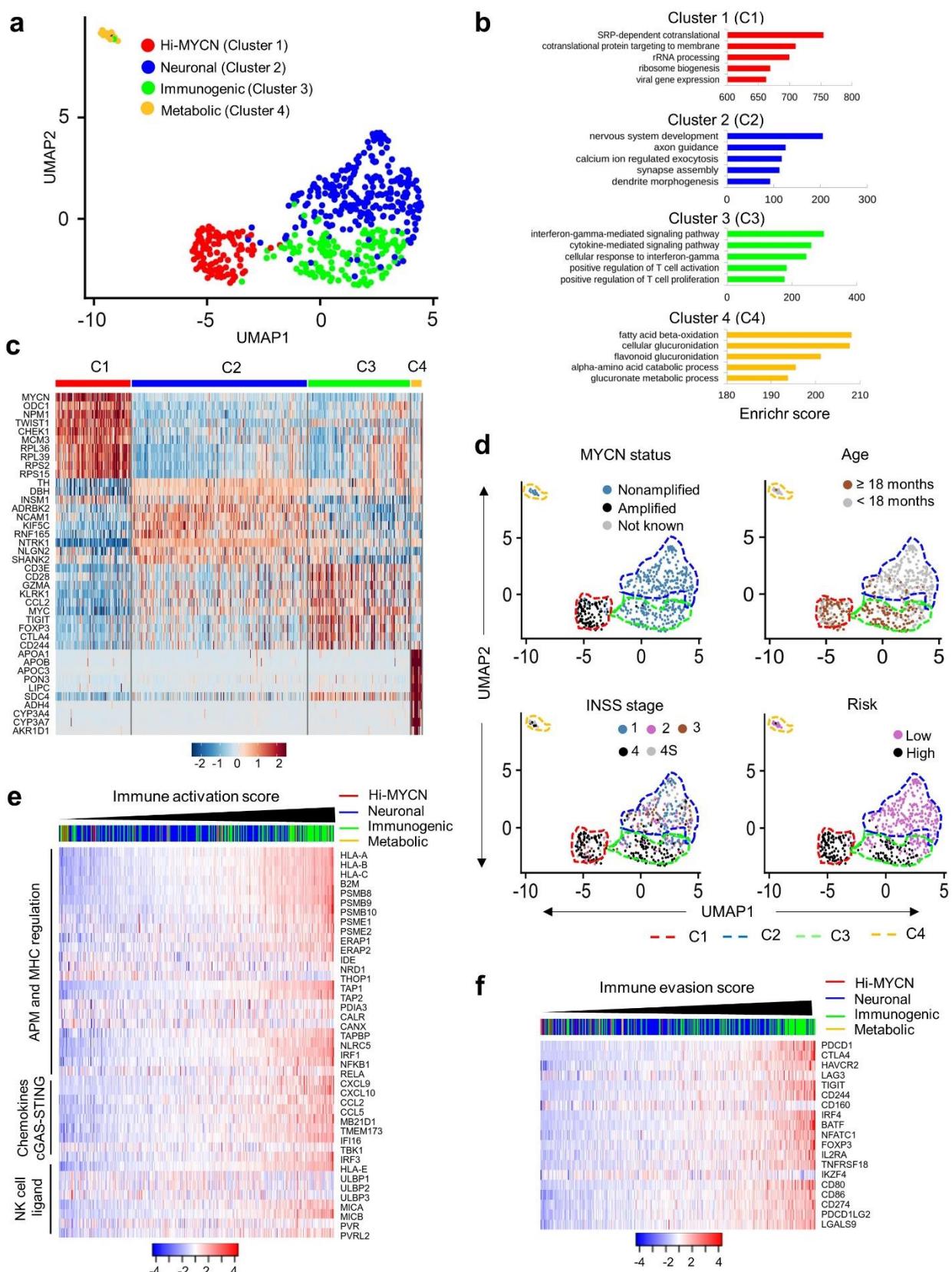
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97 **RESULTS**

98 **A subset of primary neuroblastomas express markers of a productive immune response**

99 To determine whether neuroblastomas are capable of eliciting a productive immune response,
100 we first examined bulk RNA-sequencing data from 498 well-annotated primary human tumors
101 representing diverse clinical and genetic subtypes (SEQC-498; GSE49711; **Supplementary Fig.**
102 **1a**) to quantify tumor-to-tumor gene expression variability and cluster tumor types based on gene
103 expression profiles (see also Methods). In this unbiased analysis, all tumors within one cluster
104 would share similar gene expression profiles, while being dissimilar to those of tumors within other
105 clusters. Specifically, we first identified the top 5000 highly variably expressed genes within this
106 dataset based on the premise that these would be most likely to contribute to distinct molecular
107 subtypes^{28,29} (**Supplementary Fig. 1b; Supplementary Table 1**). The data were dimensionally
108 reduced using principal component analysis (PCA) and the top 20 leading principal components
109 selected for clustering analysis (**Supplementary Fig. 1c**). Four distinct clusters were identified
110 and visualized using 2D-Uniform Manifold Approximation and Projection (UMAP), a non-linear
111 dimension-reduction tool^{30,31} (**Fig. 1a**). To explore the transcriptional differences between the
112 clusters, we identified the differentially expressed genes (DEGs) in each cluster and noted that
113 tumors in cluster 1 (C1; n = 103), termed *Hi-MYCN*, were enriched for MYCN target genes
114 involved in cell proliferation and biosynthesis, and comprised 20% of the tumor set (**Fig. 1a-c**;
115 **Supplementary Fig. 1d; Supplementary Table 2**). Not surprisingly, this cluster segregated with
116 MYCN-amplified tumors in patients aged ≥ 18 months with stage 4 disease [according to the
117 international neuroblastoma staging system (INSS)]³² and annotated “high risk” status (based on
118 the Children’s Oncology Group risk classification)³³ (**Fig. 1d; Supplementary Fig. 1e**). The
119 remaining clusters consisted of MYCN-nonamplified tumors (**Fig. 1a**) of which, cluster 2 (C2, n =
120 241), or *neuronal*, made up the largest proportion of tumors, 48%, and comprised tumors that
121 were enriched for DEGs with roles in nervous system

Fig. 1.



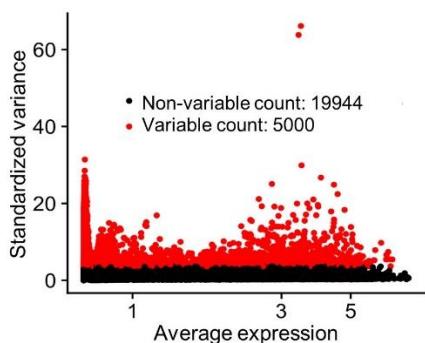
122 **Fig. 1. A subset of neuroblastomas exhibits a productive immune response. (a)** Two-
123 dimensional UMAP representations of the gene expression profiles in 498 neuroblastoma (NB)
124 tumors. Each dot represents a tumor. The top 5000 highly variable genes were selected based
125 on the variance-stabilizing method³⁴ and the 20 significant principal components (PCs) selected
126 and processed in UMAP to generate four clusters representing four NB subtypes. The DEGs were
127 identified for each cluster using the receiver operating characteristics (ROC) curve to compare
128 one cluster with other three ($\log_2 FC > 0.25$). **(b)** Gene ontology (GO) analysis of top DEGs in the
129 four clusters. **(c)** Heat map of expression values of 10 representative DEGs within each cluster.
130 Rows are z-score scaled average expression levels for each gene in all four clusters. **(d)** UMAP
131 visualization of the distribution of the indicated prognostic features in NB among the four different
132 clusters. **(e, f)** Heat map of z-score transformed \log_2 normalized expression values of immune
133 activation **(e)** and evasion **(f)** genes in MYCN-nonamplified NBs (n=401). Tumors were ranked
134 based on increasing immune activation or evasion scores. Cluster annotations of the tumors are
135 indicated on the top horizontal bar.

Supplementary Fig. 1.

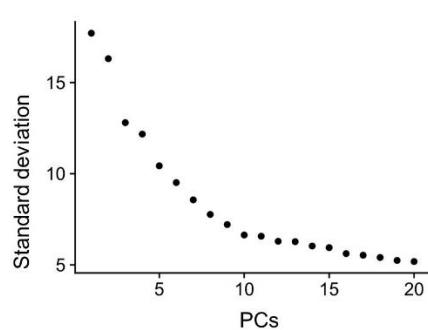
a

Tumor (498)	MYCN (%)		Age (%)		Risk (%)		INSS stage (%)					
	Nonamp	Amp	< 18 mo.	≥ 18 mo.	Low	High	1	2	3	4	4S	
	401 (80.5)	92 (18.4)	300 (60.3)	198 (39.7)	322 (64.6)	176 (35.4)	121 (24.3)	78 (15.7)	63 (12.6)	183 (36.7)	53 (10.6)	

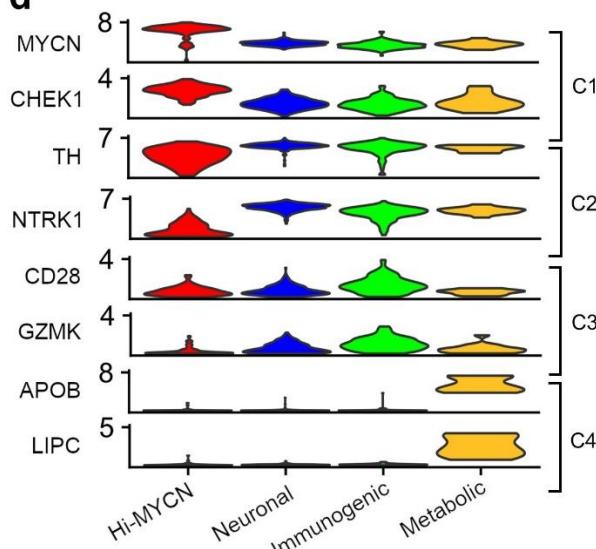
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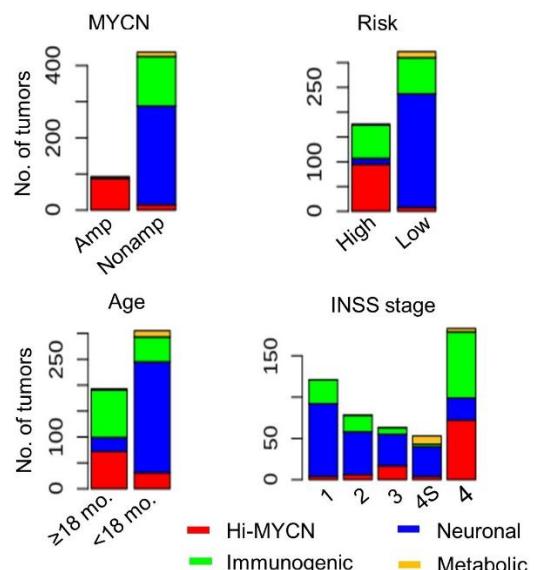
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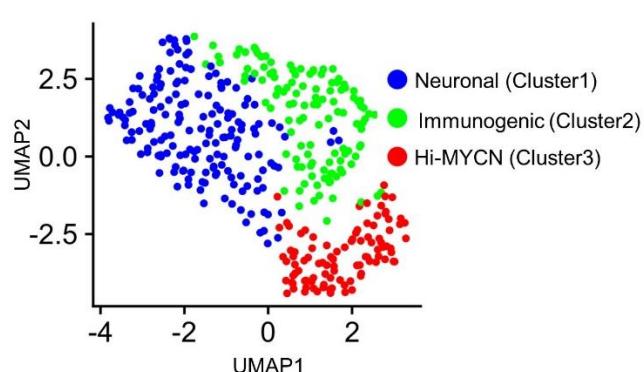
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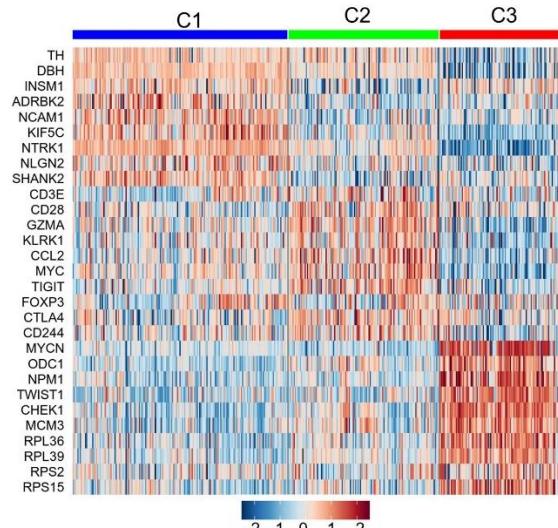
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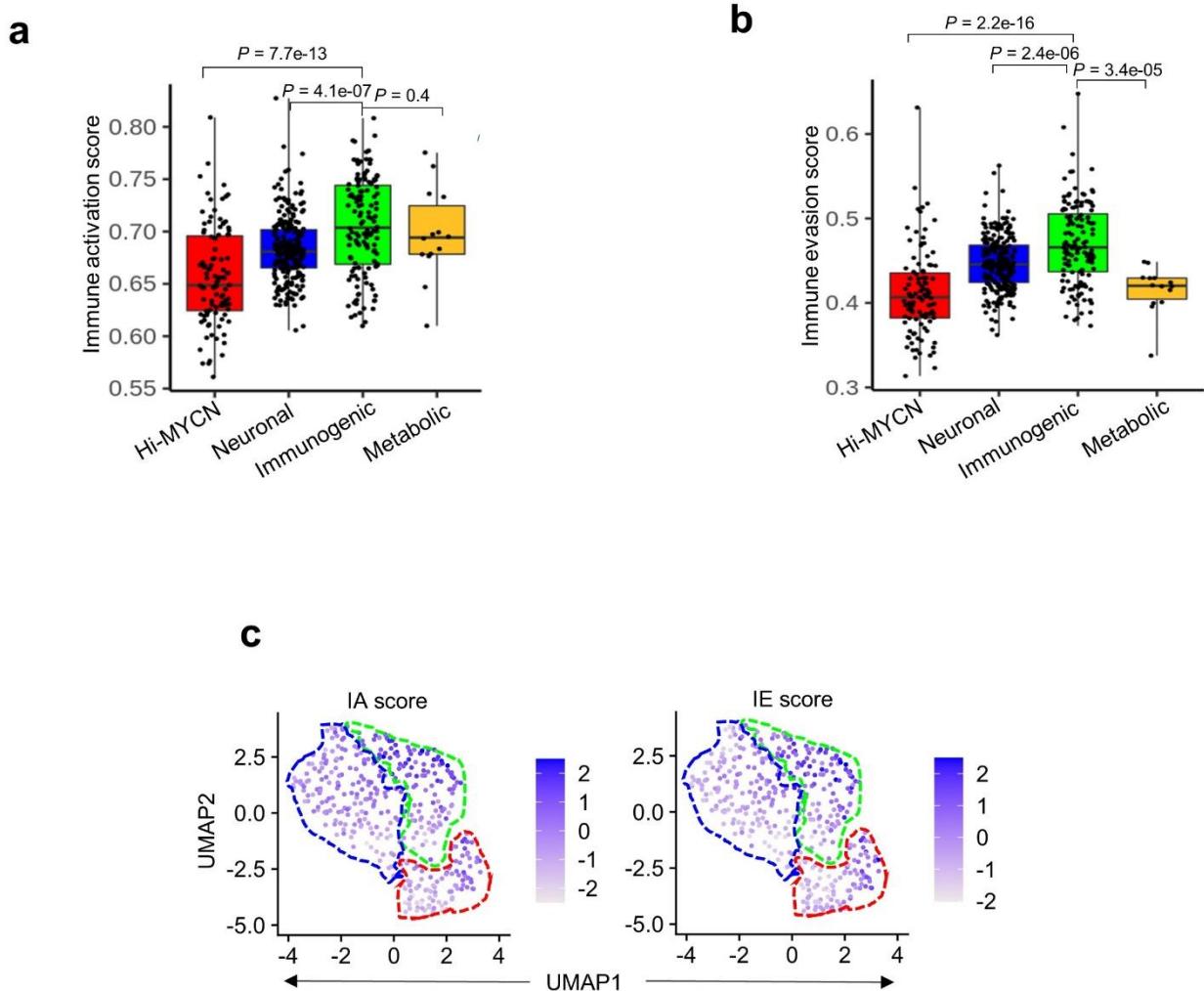


g



136 **Supplementary Fig. 1. Tumors within the immunogenic cluster express both immune
137 activation and evasion markers.** **(a)** Distribution of the 498 primary NB tumors in the data set
138 (SEQC-498; GSE49711) within the indicated prognostic categories. **(b)** Scatter plot of the
139 standardized variance in expression of all protein coding genes within 498 tumors. Red dots
140 indicate the top 5000 variably expressed genes. **(c)** Elbow plot representing the percentage
141 variance for the top 20 principal components, PCs. **(d)** Violin plots showing the expression of
142 representative marker genes across the four clusters. **(e)** Stacked bar plots showing the
143 distribution of tumors within the defined prognostic features within each cluster. Amp, amplified;
144 Nonamp, nonamplified. **(f)** Two-dimensional UMAP representations of the gene expression
145 profiles in 394 NB tumors (GSE120572). Each dot represents a tumor. The top 3000 highly
146 variable genes were selected based on the variance-stabilizing method³⁴ and the 20 significant
147 principal components (PCs) selected and processed in UMAP to generate three clusters
148 representing three NB subtypes. The DEGs were identified for each cluster using the receiver
149 operating characteristics (ROC) curve to compare one cluster with other two ($\log_2 FC > 0.25$). **(g)**
150 Heat map of expression values of 10 representative DEGs within each cluster. Rows are z-score
151 scaled average expression levels for each gene in all three clusters.
152

Supplementary Fig. 2.



153 **Supplementary Fig. 2. The immunogenic tumors are associated with markers of both**
154 **immune activation and evasion. (a, b)** Box plots comparing immune activation **(a)** and evasion
155 **(b)** scores within the four clusters from the SEQC-498 tumor data set. All box plots are defined
156 by center lines (medians), box limits (25th and 75th percentiles), whiskers (minima and maxima;
157 the smallest and largest data range). Significance was determined by the Wilcoxon rank-sum test.
158 **(c)** UMAP visualization of the distribution of IA and IE scores among the three tumor clusters
159 derived from the 394 NBs in the GSE120572 dataset. Color bar represents normalized z-scores.
160 Values <2.5 and >2.5 were set to -2.5 and +2.5 respectively, to reduce the effects of extreme
161 outliers.

162

163 development **Fig. 1a-c; Supplementary Fig. 1d**). Cluster 3 (C3, n = 140), accounting for 28%
164 of the tumors, was enriched for tumors whose DEGs were involved in immune function, such as
165 interferon-gamma (IFN-γ) response and T cell inflammation and activation, and hence were
166 designated *immunogenic* (**Fig. 1a-c; Supplementary Fig. 1d**) . Cluster 4 (C4; n=14; 3%) was
167 clearly distinct from the other three clusters, and largely consisted of the spontaneously
168 regressing stage 4S tumors that were predominantly enriched for genes involved in fatty acid and
169 cholesterol homeostasis and hence were termed *metabolic* (**Fig. 1a-c; Supplementary Fig. 1d**).
170 The neuronal and metabolic tumors arose predominantly in children <18 months of age, were of
171 stages 1-3 and 4S, while the tumors within the immunogenic cluster were associated with patient
172 age ≥18 months and metastatic disease (n= 66; 47%) (**Fig. 1d; Supplementary Fig. 1e**). Thus,
173 our DEG-based analysis of almost 500 tumors categorized neuroblastoma into four largely distinct
174 groups that included a distinct subset, accounting for approximately one-third of the entire cohort,
175 whose gene expression profiles were closely linked to immune responsiveness. To ensure that
176 these results were not confined to one data set, we analyzed an independent data set of 394
177 tumors (GSE120572) using similar clustering methods. This cohort also segregated into *Hi-*
178 *MYCN*, *neuronal* and *immunogenic* clusters, again denoting the presence of immune response
179 gene expression in a subset of primary neuroblastomas, the majority of which lack MYCN
180 amplification (**Supplementary Fig. 1f, g**).
181

182 To pursue the immune genes that were differentially enriched in the immunogenic cluster, we
183 generated an immune activation (IA) score based on the relative expression of a curated set of
184 41 genes known to have major roles in tumor cell-intrinsic immune functions, such as regulation
185 of MHC expression, antigen processing and presentation, NK cell recognition and T and NK cell
186 infiltration (**Supplementary Table 3**). After assigning an IA score to each of the *MYCN*-
187 nonamplified tumors (n = 401) in the SEQC-498 data set and arranging them in ascending order
188 (**Supplementary Table 4**), we observed that a significant number with the highest IA scores

189 predominantly fell within the immunogenic and metabolic clusters (**Fig. 1e; Supplementary Fig.**
190 **2a**), while those with intermediate or lower scores were associated with the neuronal and Hi-
191 MYCN clusters, respectively (**Fig. 1e; Supplementary Fig. 2a**). Because a cytotoxic immune
192 response is generally accompanied by immune suppression or evasion^{35,36}, we determined
193 whether immune suppression was also represented in the MYCN-nonamplified tumors by ranking
194 them in ascending order of an immune evasion (IE) score based on the relative expression of 19
195 genes, most of which were markers of T-cell dysfunction (**Supplementary Table 3**). Again, the
196 immunogenic tumor cluster had significantly higher IE scores compared with the neuronal and
197 metabolic clusters (**Fig. 1f; Supplementary Fig. 2b**). Moreover, we observed enrichment for IA
198 and IE scores in the immunogenic cluster in the additional data set (GSE120572)
199 (**Supplementary Fig. 2c**), thus strengthening our premise that these tumors maybe capable of
200 eliciting an immune response.

201
202 Consistent with the known poor immunogenicity of *MYCN*-amplified tumors^{24,25}, we also
203 observed that tumors within the Hi-MYCN cluster had, on the whole, the lowest IA and IE scores
204 (**Supplementary Fig. 2a, b**). Surprisingly, however, a small subset within this cluster had scores
205 that were comparable to the highly immunogenic tumors within the immunogenic cluster [13 of
206 103 (12.6%) above the median for immunogenic tumors] (**Supplementary Fig. 2a, b**). Thus, while
207 the majority of neuroblastomas do not possess an immune response gene signature, a subset
208 has significantly increased expression of both immune activation and evasion markers, pointing
209 to their ability to induce an anti-tumor immune response.

210
211 **The mesenchymal lineage is preferentially associated with immune response signatures**
212 **in neuroblastoma**

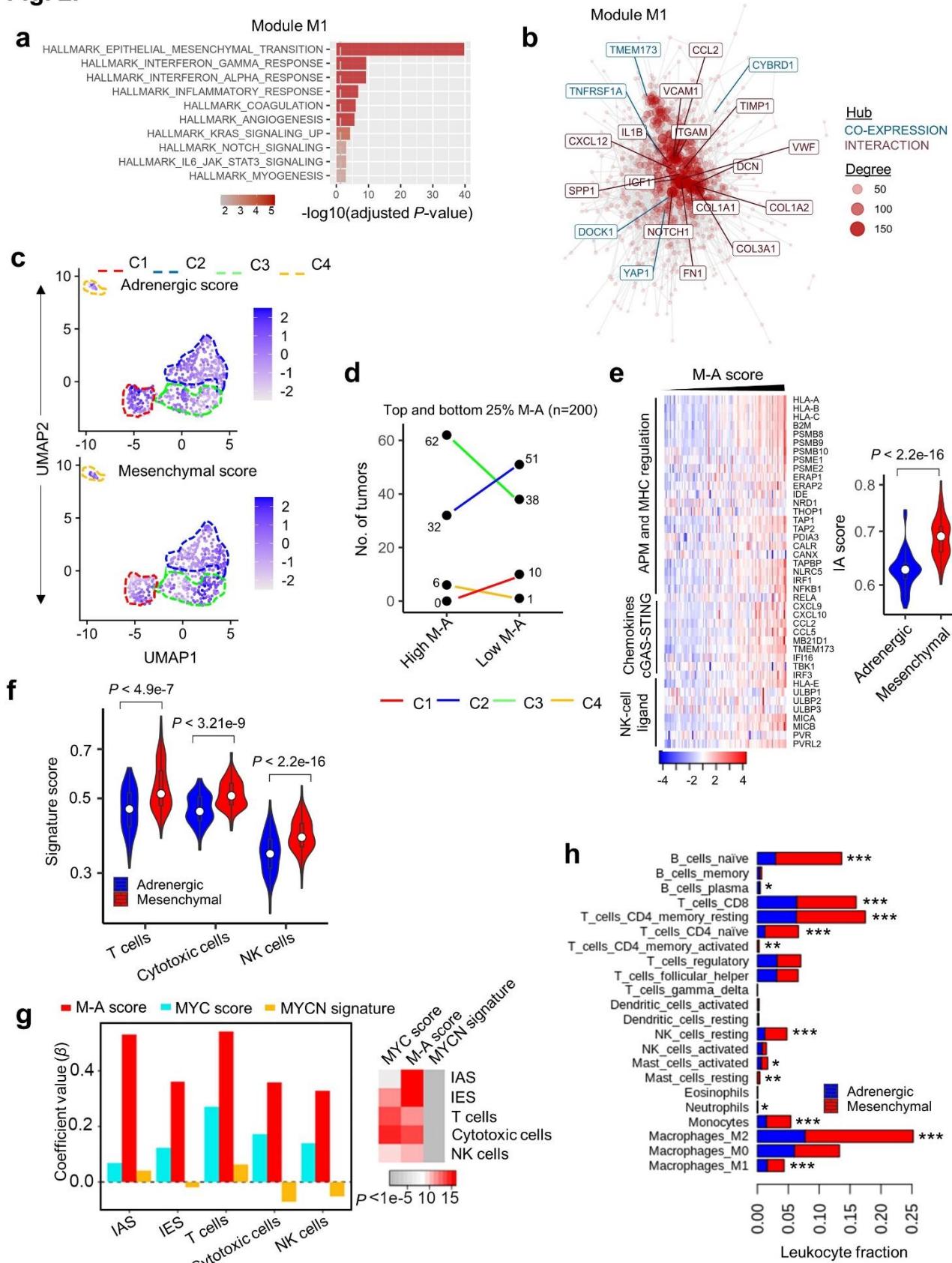
213 Having identified subsets of neuroblastomas with the potential for immunogenicity, we next sought
214 a biomarker that might consolidate the complex interactions between the immune system and the

215 tumor. To this end, we performed a modular gene co-expression analysis of the 140
216 transcriptomes within the immunogenic cluster in the SEQC-498 data set to identify biologically
217 relevant pathways based on similar gene expression patterns. Using the CEMiTTool (co-
218 expression modules identification tool) package (Russo et al., 2018), we identified five gene co-
219 expression modules (M1-M5) within the immunological cluster (**Supplementary Fig. 3a**). Among
220 these modules, M1, with the highest number of co-expressed genes, contained gene sets
221 enriched for epithelial to mesenchymal transition (EMT), inflammatory response, and interferon
222 signaling, suggesting an association between EMT and the preponderant representation of
223 immune marker genes within the immunogenic cluster (**Fig. 2a; Supplementary Fig. 3b**).
224 Furthermore, integration of the co-expression data in module M1 with protein–protein interaction
225 data from the STRING 11.0 database identified mesenchymal lineage and immune markers as
226 top regulatory hubs (**Fig. 2b**), leading us to hypothesize that in neuroblastoma, tumor
227 immunogenicity could be determined by cell state.

228

229 Two independent groups^{37,38} recently described two distinct cell states in neuroblastoma: a
230 differentiated sympathetic neuron-like adrenergic (ADR) phenotype, defined by lineage markers
231 including *PHOX2B*, *DBH*, and *TH*, and a mesenchymal (MES) phenotype, characterized as
232 “neural crest cell-like” (NCC), and expressing genes such as *PRRX1*, *FOSL1*, and *FOSL2*. To
233 test our prediction, we first quantified the adrenergic and mesenchymal identities of each tumor
234 in our cohort based on the expression levels of the lineage-specific genes in each cell state as
235 established by Groningen et al³⁸ (see Methods). We ensured that there was no overlap between
236 the 369-gene adrenergic signature and the genes that made up the IA data set and removed the
237 6 IA genes that were also present in the 485-gene mesenchymal signature. Next, we assigned
238 either an adrenergic (A-score) or a mesenchymal (M-score) score to each tumor within our four
239 previously identified clusters. This analysis revealed significant enrichment of the mesenchymal
240 cell state within the immunogenic and metabolic clusters (**Fig. 2c; Supplementary Fig. 3c**).

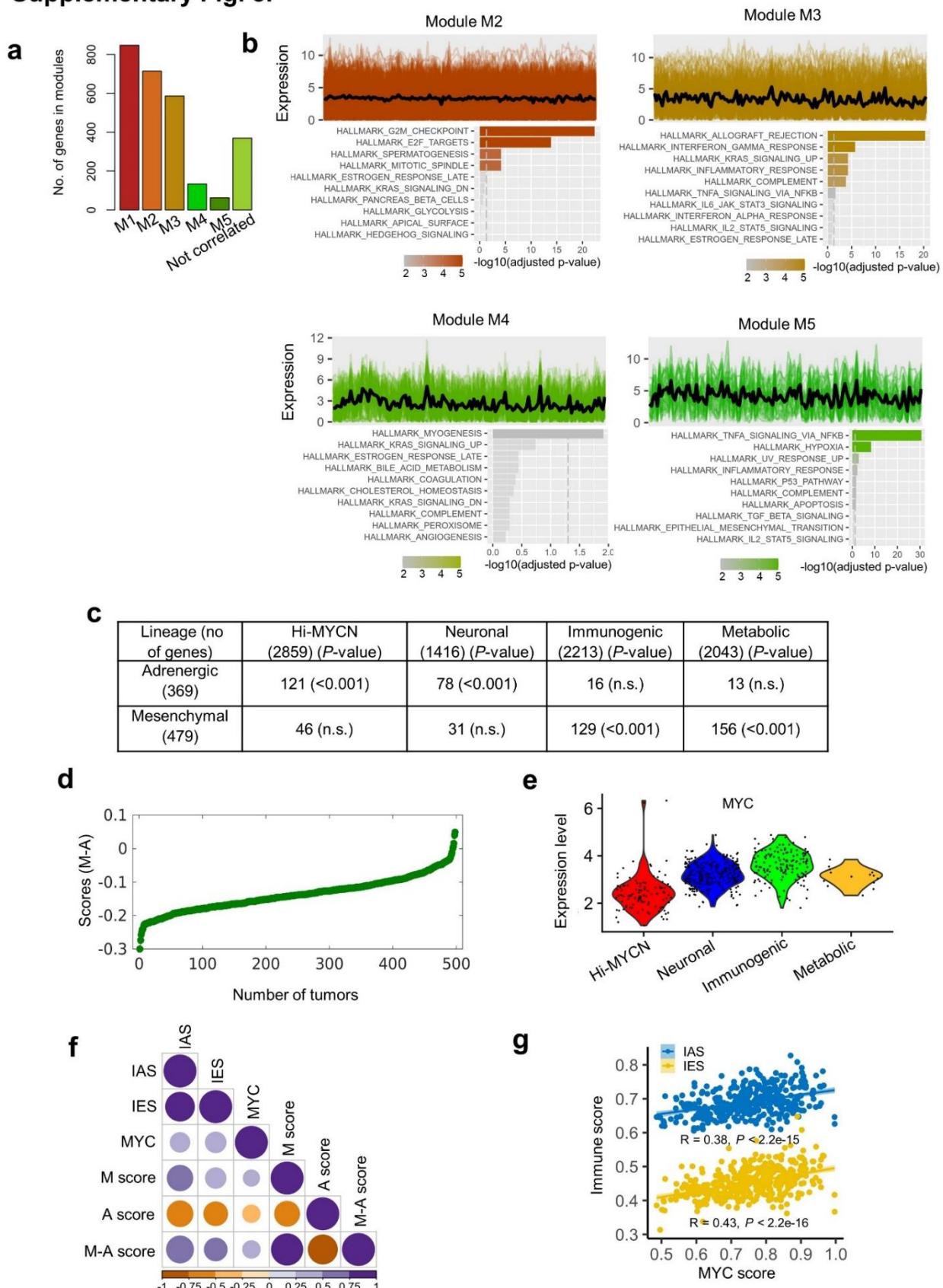
Fig. 2.



241 **Fig. 2. The mesenchymal cell state is associated with an immunogenic signature in NB. (a)**
242 GO analysis of co-expressed genes associated with module M1 using the KEGG (Kyoto
243 encyclopedia of genes and genomes) database. The vertical dashed line indicates the adjusted
244 *P*-value of 0.05. **(b)** Gene network representing all possible interactions in module M1. The
245 topmost connected genes (hubs) are indicated. Hubs derived from module M1 are colored blue
246 (co-expression) and those from the STRING database are indicated in red (interaction). The size
247 of each node corresponds to the degree of interaction. **(c)** UMAP visualization of the distribution
248 of adrenergic (top) and mesenchymal scores (bottom) among the four tumor clusters. Color bar
249 represents normalized z-scores. Values <2.5 and >2.5 were set to -2.5 and +2.5 respectively, to
250 reduce the effects of extreme outliers. **(d)** Dot plots showing the distribution of *MYCN*-
251 nonamplified tumors ($n = 400$) within each of the clusters based on ranked M-A scores. *Left*,
252 Tumors from the upper (high M-A) and lower (low M-A) M-A score quartiles are shown ($n = 200$;
253 $P < 0.01$ for C3). *Right*, Representations based on the median M-A scores of the entire tumor
254 cohort ($n = 400$; $P = 0.05$ for C3). Fisher's exact test was used for both calculations. **(e)** *Left*,
255 Heatmap representation of the expression of tumor cell-intrinsic immune activation genes in
256 *MYCN*-amplified tumors ($n=92$). Samples are ranked by increasing M-A score. Log₂ gene
257 expression values were z-score transformed for heatmap visualization. *Right*, Violin plots of the
258 distribution of immune activation scores in the tumors on the left, classified either as adrenergic
259 or mesenchymal, based on the median M-A score. The box plots within the violin plots are defined
260 by center lines (medians), box limits (25th and 75th percentiles), whiskers (minima and maxima;
261 1.5X the interquartile range). Significance was determined by the two-sided Kolmogorov-Smirnov
262 (KS) test. APM, antigen processing machinery. **(f)** Violin plots comparing the quantitative scores
263 of the indicated immune cell signatures in 100 tumors from the upper (mesenchymal) and lower
264 (adrenergic) quartiles of the tumor M-A scores using the two-sided KS test. The box plots within
265 the violin plots are defined as in (D). **(g)** *Left*, Bar diagram comparing regression coefficient (β)
266 values derived from multivariate multiple regression model analysis of *MYCN*-nonamplified

267 tumors. β -coefficient values were compared between three predictors: MYC score, M-A score and
268 MYCN signature. IA, IE, T cell, cytotoxic cell and NK cell scores were used as response variables
269 to generate the model³⁹. *Right*, Heat map of the *P*-values associated with the three predictors. **(h)**
270 Bar diagram comparing the CIBERSORT-estimated fractional content of the indicated tumor-
271 infiltrating leukocytes between *MYCN*-nonamplified adrenergic and mesenchymal tumors.
272 Adrenergic and mesenchymal tumors were assigned as in **(f)**. Data represent the means, n = 100
273 tumors, **P* < 0.05, ***P* < 0.01, ****P* < 0.001 two-tailed Welch's t-test.

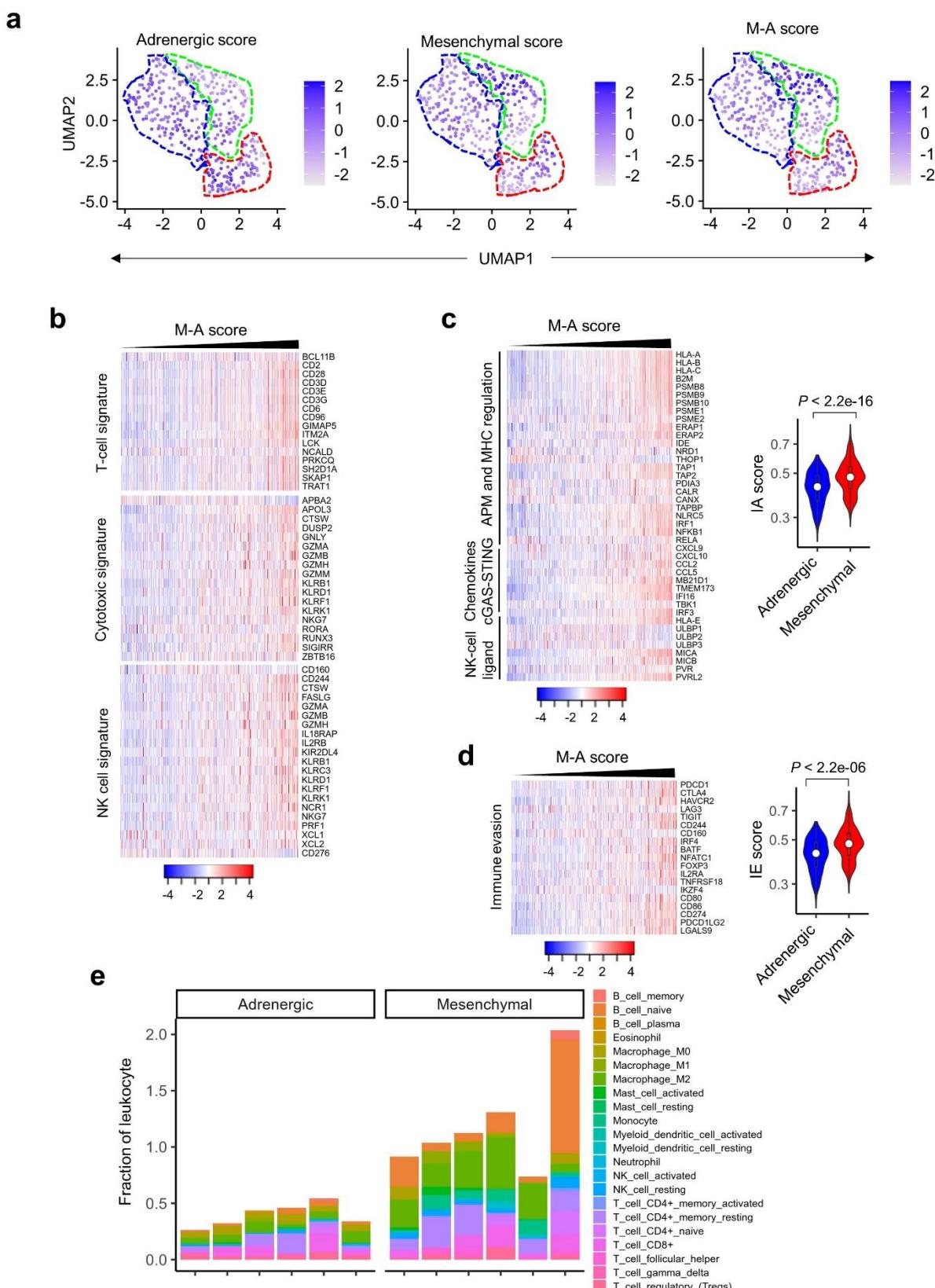
Supplementary Fig. 3.



274 **Supplementary Fig. 3. Cell lineage markers are significantly associated with immune gene**
275 **signatures in NB.** **(a)** Bar plots representing the numbers of co-expressed genes within each
276 module. **(b)** Profile plots depicting the expression levels (y-axis) of individual genes (colored lines)
277 and their mean expression (black line) in 140 tumors (x-axis) from modules M2, M3, M4 and M5.
278 GO analysis of co-expressed genes associated with each module using the KEGG database is
279 appended below the respective profile plot. The vertical dashed line indicates the adjusted *P*-
280 value of 0.05. **(c)** Summary of the overlap between the DEGs associated with the four tumor
281 clusters and the adrenergic or mesenchymal signature genes as per Groningen et al., 2017.
282 Significance was determined by Fisher's exact test. **(d)** Scatter plot of the 498 primary NB tumors
283 ranked based on increasing M-A score. **(e)** Violin plots of the distribution of normalized expression
284 levels of MYC in the four tumor clusters. **(f)** Pearson correlation matrix showing pairwise
285 correlation values among the indicated parameters. The colors and sizes of the circles indicate
286 the correlation coefficient values, with the least (smaller, orange circles) to the most (larger, blue
287 circles) degree of association between the parameters shown. **(g)** Scatter plot of the correlation
288 between MYC expression and immune activation (blue dots) or evasion (yellow dots) scores in
289 *MYCN*-nonamplified tumors.

290

Supplementary Fig. 4.



291 **Supplementary Fig. 4. The relative mesenchymal score (M-A score) is positively correlated**
292 **with an immunogenic signature. (a)** UMAP visualization of the distribution of adrenergic,
293 mesenchymal, and M-A scores among the three tumor clusters derived from 394 NBs in the
294 GSE120572 dataset. Color bar represents normalized z-scores. Values <2.5 and >2.5 were set
295 to -2.5 and +2.5 respectively, to reduce the effects of extreme outliers. **(b)** Heat maps of the
296 indicated immune cell signatures in *MYCN*-nonamplified tumors, ranked by increasing M-A
297 scores. Log₂ gene expression values were z-score transformed for heatmap visualization. **(c, d)**
298 Heatmaps depicting the immune activation **(c)** and evasion **(d)** signatures in *MYCN*-nonamplified
299 tumors, ranked by increasing M-A score. Log₂ gene expression values were z-score transformed
300 for visualization. Violin plots comparing the distribution of immune activation **(c)** and evasion **(d)**
301 signatures in 100 tumors from upper (mesenchymal) and lower (adrenergic) quartiles of the M-A
302 score are shown next to the heatmaps. Significance determined by the two-sided KS test. Box
303 plots within the violin plots are defined by center lines (medians), box limits (25th and 75th
304 percentiles), whiskers (minima and maxima; 1.5X the interquartile range). **(e)** Bar graph
305 comparing the CIBERSORT-estimated fractional content of the indicated tumor-infiltrating
306 leukocytes in six tumors with the uppermost (mesenchymal) and lowermost (adrenergic) M-A
307 scores.

308 By contrast, the Hi-MYCN and neuronal clusters were enriched for the adrenergic cell state (**Fig.**
309 **2c; Supplementary Fig. 3c).**

310 Next, to identify predictors of immunogenicity among the *MYCN*-nonamplified tumors, we
311 calculated the relative mesenchymal score for each tumor by subtracting the adrenergic from the
312 mesenchymal score (M-A score). This resulted in a continuum of low to high M-A scores,
313 corresponding to a less mesenchymal to a more mesenchymal tumor state (**Supplementary Fig.**
314 **3d**). To determine whether these cell states had any effect on immune response in the *MYCN*-
315 nonamplified tumors, we determined whether the mesenchymal, adrenergic, or M-A scores
316 correlated with our previously defined immune activation and evasion scores (**Fig. 1e, f**). We also
317 tested the effect of *MYC*, one of the top differentially expressed genes in the immunogenic cluster
318 (**Supplementary Fig. 3e**). *MYC* is overexpressed in approximately 10% of *MYCN*-nonamplified
319 neuroblastomas⁴⁰ and regulates the expression of cell-intrinsic immune evasion markers in
320 lymphoma⁴¹. Of these variables, the M-A score showed the strongest correlation with
321 immunogenicity, not only in terms of the immune activation score ($R = 0.71$), but also the immune
322 evasion score ($R = 0.51$) (**Supplementary Fig. 3f**). *MYC* expression was only modestly correlated
323 with immune evasion and activation scores ($R = 0.43$; $R = 0.38$, respectively) (**Supplementary**
324 **Fig. 3g**). With no overlap between the lineage marker and immune response gene sets, these
325 results suggest that the relative abundance of a mesenchymal signature (M-A score) is a better
326 predictor of immune response than individual adrenergic or mesenchymal signatures. In
327 agreement, tumors with high M-A scores were represented at a significantly higher proportion
328 within the immunogenic cluster compared to tumors with low M-A scores (**Fig. 2d**), a result that
329 was recapitulated in our second data set, (GSE120572) (**Supplementary Fig. 4a**). Finally, our
330 finding of the subset of tumors marked by the relatively high expression of immune activation and
331 evasion genes within the Hi-MYCN cluster (**Supplementary Fig. 2a, b**) prompted us to further
332 evaluate the cell states of these tumors. Ranking these tumors based on increasing M-A scores
333 revealed a positive relationship between cell-intrinsic immunogenicity and the mesenchymal state

334 (Fig. 2e), suggesting that similar to our results in *MYCN*-nonamplified tumors, the presence of
335 immune gene expression in *MYCN*-amplified tumors is significantly correlated with the
336 mesenchymal phenotype.

337

338 The preferential overexpression in the mesenchymal phenotype of tumor cell-intrinsic genes
339 that induce a positive immune response (IA score), as well as its correlation with transcripts that
340 suppress the immune response (IE score), suggested that these tumors may support increased
341 immune cell infiltration. To test this prediction, we used two orthogonal approaches. First, we
342 assessed whether established signatures of immune cell infiltration^{39,42} were present in the
343 tumors arranged according to increasing M-A scores, and observed enrichment for signatures of
344 infiltrating immune cells in tumors with mesenchymal phenotypes (**Supplementary Fig. 4b**).
345 Intriguingly, we also noted that adrenergic tumors were enriched for CD276 (B7-H3) expression,
346 an immune checkpoint marker that protects neuroblastoma cells from NK cell-mediated
347 cytotoxicity, which may partly account for the decreased immune response signatures in these
348 cells¹⁴ (**Supplementary Fig. 4b**). We next quantified the immune response signatures in a subset
349 of tumors (n = 100) from both the upper and lower quartiles of the M-A score that had significant
350 differences in their activation and evasion scores (**Supplementary Fig. 4c, d**). High M-A scoring
351 (mesenchymal) tumors had significantly higher expression levels of cytotoxic T and NK cell
352 signatures compared with those of low M-A scoring (adrenergic) tumors (**Fig. 2f**). Considering
353 that the M-A score and, to a lesser extent, higher *MYC* expression were positively associated with
354 IA and IE scores in pairwise testing (**Supplementary Fig. 3f**), we next assessed their relative
355 contributions as independent predictors of an immune response in a multivariate multiple
356 regression model consisting of immune activation and evasion scores and T and NK cell
357 signatures (**Fig. 2g**). Because *MYCN* amplification is linked to immune suppression, we also
358 included the 157-gene *MYCN* signature generated by Valentijn et al²⁷ and subsequently used by
359 Wei et al to identify immune predictors in *MYCN*-nonamplified neuroblastoma²⁵. In this analysis

360 also, the M-A score was a better predictor of tumor immunogenicity than *MYC* expression or
361 *MYCN* signature (**Fig. 2g**).

362

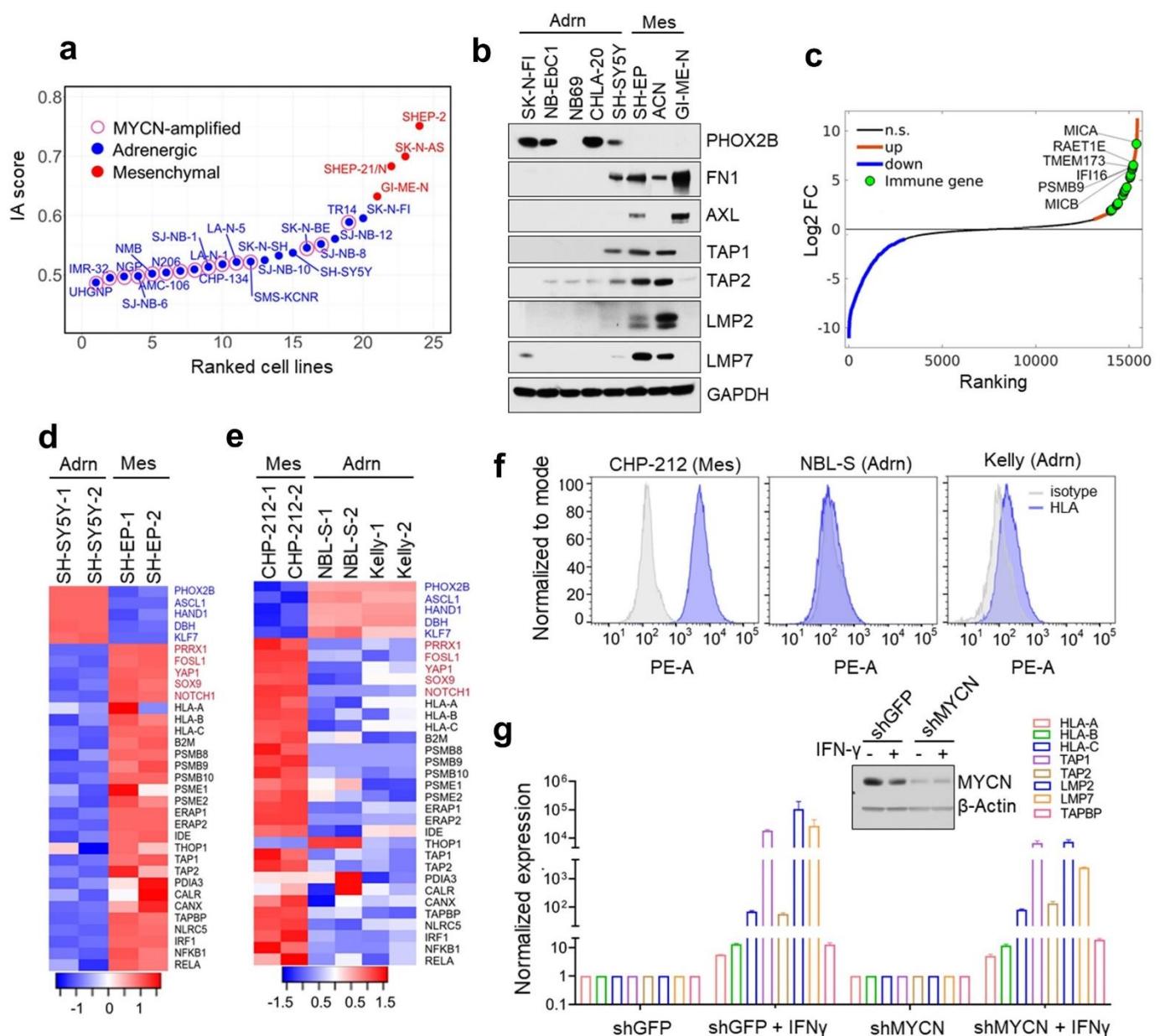
363 Second, we used CIBERSORT (Cell type Identification By Estimating Relative Subsets Of
364 known RNA Transcripts)⁴³, as a deconvolution approach to estimate the fraction of immune cell
365 infiltration associated with the adrenergic and mesenchymal lineage tumors. We first established
366 the fraction of tumor-infiltrating leukocytes (TILs) in the most mesenchymal versus the most
367 adrenergic tumors (n = 6 each), observing significantly higher fractions in mesenchymal tumors
368 (**Supplementary Fig. 4e**). Next, we quantified the average immune cell content in 100 tumors
369 from the upper and lower quartiles of the M-A score (**Fig. 2h**). Consistent with our previous results
370 (**Fig. 2f**), CIBERSORT analysis also showed significantly increased enrichment for cytotoxic CD8⁺
371 T cells in mesenchymal tumors, which was also associated with a concomitant increase in
372 regulatory T cells, including those expressing markers of T-cell exhaustion (**Fig. 2h**;
373 **Supplementary Fig. 4e**). Interestingly, we also observed that mesenchymal tumors comprised a
374 higher fraction of naïve B cells, which were recently shown to take part in antitumor immunity⁴⁴
375 (**Fig. 2h; Supplementary Fig. 4e**). Together, our findings indicate that the mesenchymal cell
376 state is a strong predictor of neuroblastoma immunogenicity.

377

378 **Tumor cell-intrinsic upregulation of immune pathways in mesenchymal neuroblastoma**

379 We next sought to understand the extent to which the presence or absence of an immunogenic
380 signature in the bulk RNA-sequencing data was intrinsic to tumor cells or was conferred by the
381 tumor microenvironment. Analysis of the lineage identities of a panel of 24 human neuroblastoma
382 cell lines (15 *MYCN*-amplified; 9 *MYCN*-nonamplified) from RNA-sequencing data (GSE28019)
383 revealed a gradient of adrenergic-to-mesenchymal scores (**Supplementary Fig. 5a**). Consistent

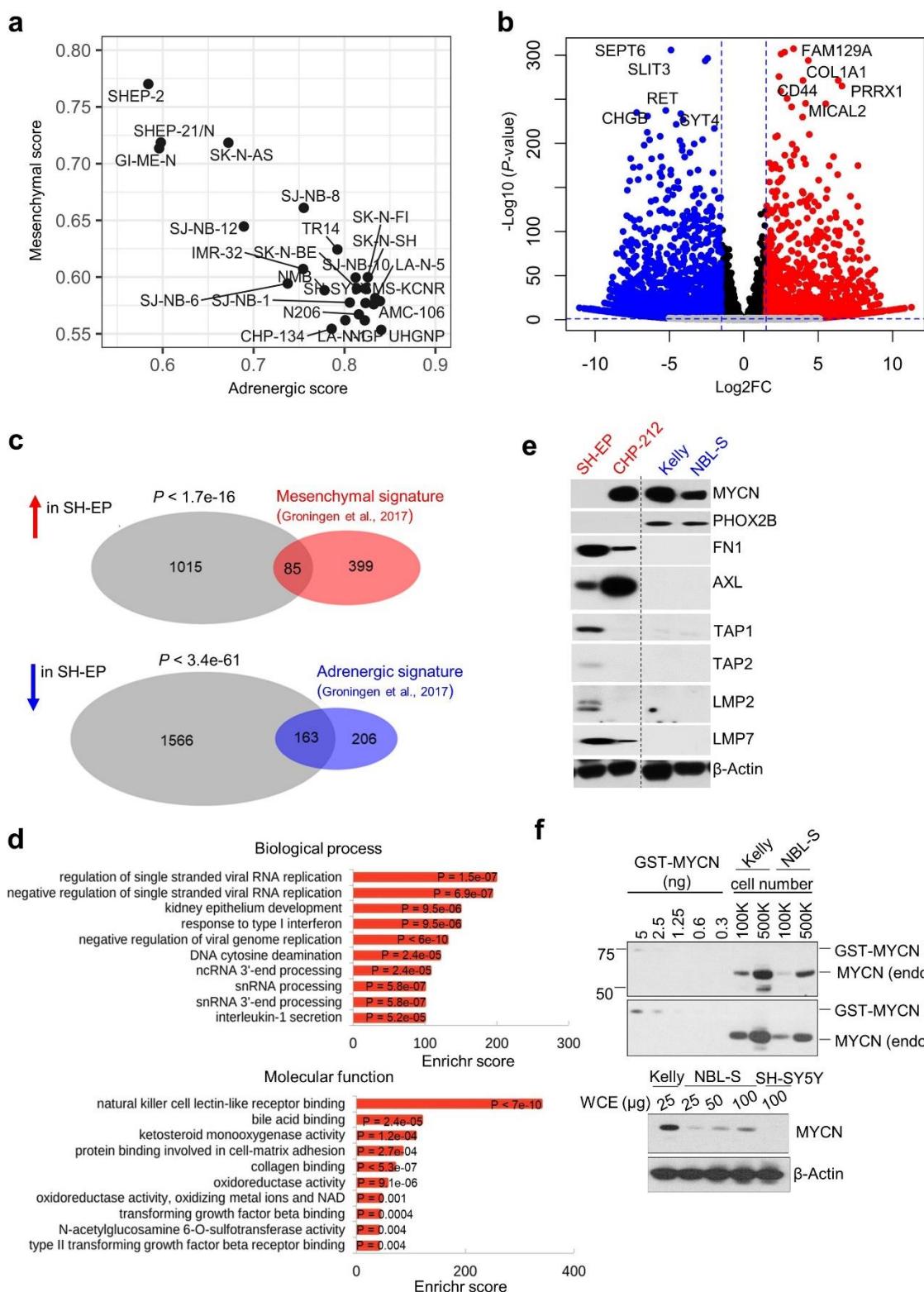
Fig. 3.



384 **Fig. 3. Tumor cell-intrinsic immune marker genes are upregulated in mesenchymal NBs.**

385 **(a)** Scatter plot of the immune activation (IA) scores of human neuroblastoma cell lines (RNA-seq
386 data; GSE28019). Cell lines are arranged based on increasing IA scores and designated as
387 adrenergic or mesenchymal based on lineage-specific gene expression. **(b)** Western blot (WB)
388 analysis of adrenergic (PHOX2B) and mesenchymal (FN1, AXL) cell lineage markers and antigen
389 processing genes (TAP1/2, LMP2/7) in *MYCN*-nonamplified NB cell lines. GAPDH was used as
390 the loading control. Adrn, adrenergic; Mes, mesenchymal. **(c)** Waterfall plot of the fold-change in
391 RNA expression levels of up- and downregulated genes in SH-EP compared to SH-SY5Y NB
392 cells; selected immune genes are highlighted in green. **(d, e)** Heat maps of lineage marker (*blue*,
393 adrenergic; *red*, mesenchymal) and MHC and antigen processing machinery gene (*black*)
394 expression in the indicated *MYCN*-nonamplified **(d)** and *MYCN*-amplified (CHP-212, Kelly) and
395 overexpressing (NBL-S) **(e)** adrenergic and mesenchymal cells ($n = 2$ biological replicates). Rows
396 are z-scores calculated for each transcript in each cell type. **(f)** Fluorescence activated cell sorting
397 (FACS) analysis of cell surface HLA expression in the cells depicted in **e**. Isotype controls are
398 depicted in gray. The X-axis denotes fluorescence intensity of indicated proteins using
399 phycoerythrin (PE-A) tagged antibodies. Results representative of 2 independent experiments.
400 **(g)** RT-qPCR analysis of antigen processing and presentation genes in *MYCN*-amplified Kelly NB
401 cells engineered to express sh*MYCN* or sh*GFP* (control) with or without IFN- γ induction (100
402 ng/mL for 24 hr.). Data are normalized to GAPDH and represent means \pm SD, $n = 2$ biological
403 replicates. Inset, WB analysis of *MYCN* in control and sh*MYCN* cells. Actin was used as a loading
404 control.

Supplementary Fig. 5.



405 **Supplementary Fig. 5. Mesenchymal lineage-specific marker and cell-intrinsic immune**
406 **gene expression are significantly correlated in NB.** (a) Scatter plot of adrenergic and
407 mesenchymal scores in NB cell lines (GSE28019). (b) Volcano plot showing the gene expression
408 changes between mesenchymal SH-EP and adrenergic SH-SY5Y cells. The top ten lineage
409 marker genes are highlighted. The fold changes are represented in \log_2 scale (X-axis) and the -
410 \log_{10} of the *P*-values depicted on Y-axis (FDR < 0.1 and $\log_2\text{FC} > 1$). (c) Venn diagram of the
411 overlap between DEGs in SH-EP cells (compared to SH-SY5Y cells) and the mesenchymal or
412 adrenergic signatures derived from Groningen et al³⁸. Statistical significance was determined
413 using Fisher's exact test. (d) GO analysis of differentially upregulated genes in mesenchymal SH-
414 EP compared to adrenergic SH-SY5Y cells. (e) WB analysis of cell lineage marker and antigen
415 processing gene expression in the indicated NB cells. Dotted line indicates the margin where gel
416 images have been cut. (f) *Upper*, WB analysis of MYCN levels in the indicated numbers of Kelly
417 and NBL-S NB cells titrated against known amounts of purified GST-MYCN protein. *Lower*, WB
418 analysis comparing MYCN levels in whole cell extracts (WCE) from Kelly cells to titrated levels
419 from WCE in NBL-S cells. SH-SY5Y cells that do not express MYCN serve as a negative control.
420 Actin was used as a loading control in (e) and (f).

421 with our observations in primary tumors, cell lines with higher mesenchymal gene signatures
422 grouped together and had significantly higher expression of tumor cell-intrinsic immune genes,
423 compared with the remainder, which had higher adrenergic scores and were mostly associated
424 with reduced immune marker gene expression (**Fig. 3a, b**). To further understand the association
425 of tumor cell-intrinsic immune pathways with lineage state, we focused on two neuroblastoma cell
426 lines –SH-SY5Y and SH-EP – subclones of the *MYCN*-nonamplified SK-N-SH cell line separated
427 on the basis of neuroblastic versus substrate-adherent morphology⁴⁵ and determined to be
428 adrenergic and mesenchymal³⁸, respectively. RNA sequencing showed that the differentially up-
429 and down-regulated genes in SH-EP compared with SH-SY5Y cells significantly overlapped with
430 established signatures of mesenchymal and adrenergic states, thus confirming their respective
431 phenotypes (**Supplementary Fig. 5b, c**). We noted that genes with roles in eliciting an immune
432 response were among the top differentially upregulated genes in mesenchymal SH-EP cells,
433 especially those involved in antigen processing and presentation and positive regulation of MHC
434 expression (**Fig. 3c, d**). Moreover, gene ontology (GO) analysis of the upregulated transcripts
435 revealed enrichment for innate and adaptive immune responses including type-I interferon
436 signaling and ligands for the NK cell receptor, NKG2D (NK cell lectin-like receptor, KLRK1)
437 (**Supplementary Fig. 5d**). By contrast, adrenergic SH-SY5Y cells that showed upregulation of
438 neuronal lineage markers did not show significant enrichment of immune function genes (**Fig.**
439 **3d**), providing further evidence that cell-intrinsic immunogenicity is associated with the
440 mesenchymal phenotype.

441 The absence of a productive immune response has often been described in *MYCN*-
442 amplified neuroblastoma tumors^{24,25}; indeed, the vast majority of such tumors in our cohort
443 exhibited similar findings (**Supplementary Fig. 2a, b**). Nonetheless, based on our intriguing
444 finding of upregulation of immune response genes in a small number of *MYCN*-amplified tumors
445 (**Supplementary Fig. 2a, b**) that possessed mesenchymal cell signatures (**Fig. 2e**), we sought
446 to understand the role of *MYCN* in mediating this immune response. We used Kelly and CHP-212

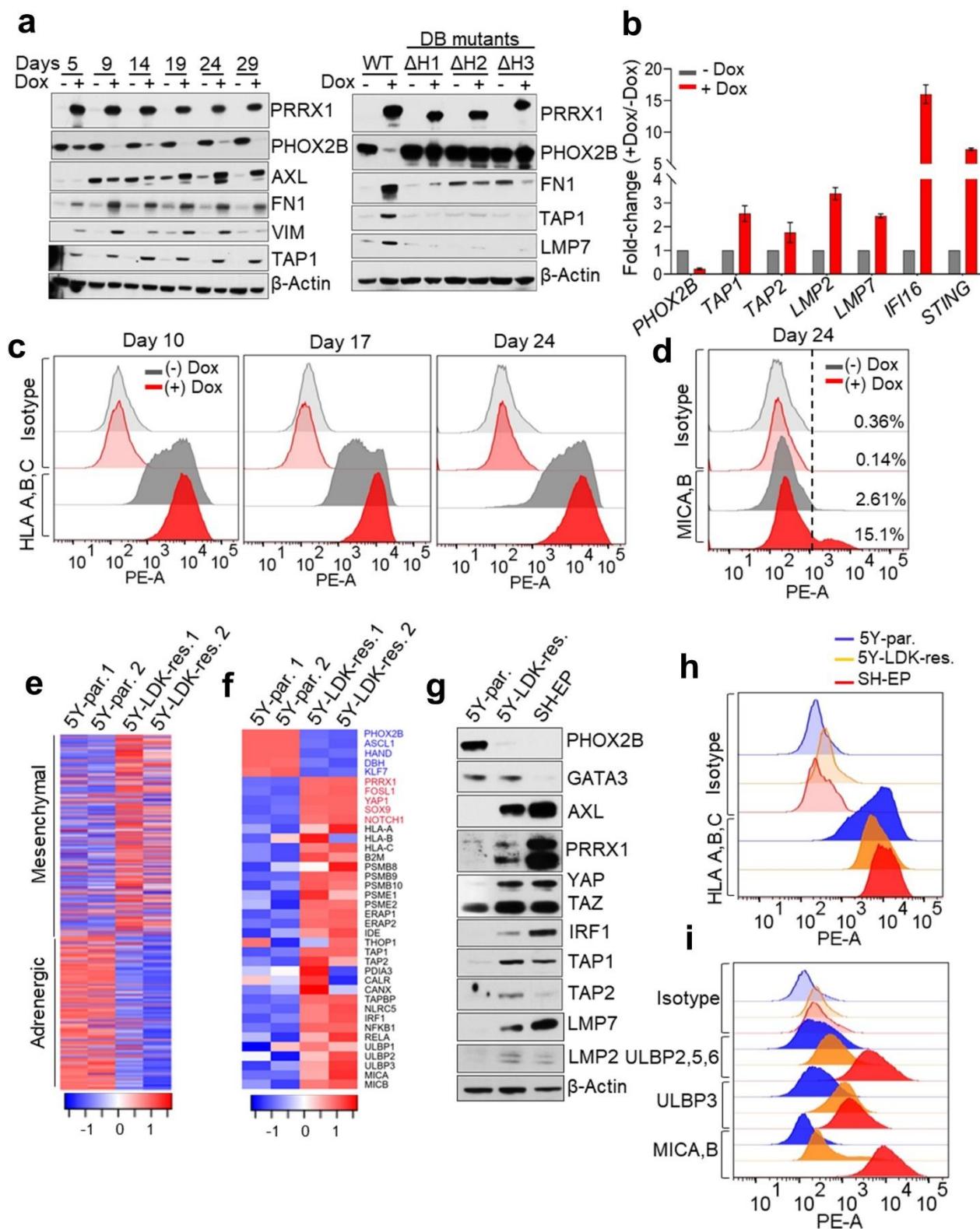
447 human neuroblastoma cells that expressed amplified *MYCN* but were of adrenergic and
448 mesenchymal phenotypes, respectively^{37,38}, and NBL-S cells that lacked *MYCN* amplification but
449 expressed moderate levels of *MYCN* RNA and protein⁴⁶ (**Supplementary Fig. 5e**) and were
450 classified as adrenergic (van Groningen et al., 2017 and this study). RNA-sequencing and flow
451 cytometry analysis suggested that tumor cell-intrinsic immune genes involved in antigen
452 processing and MHC regulation were highly expressed in mesenchymal CHP-212 compared to
453 adrenergic Kelly and NBL-S cells (**Fig. 3e, f; supplementary fig. 5e**). Importantly, although
454 *MYCN* expression in NBL-S cells was lower than in Kelly cells (**Supplementary Fig. 5f**), these
455 immune transcripts were expressed at lower levels in both cell lines, consistent with their
456 adrenergic status (**Fig. 3e; Supplementary Fig. 5f**). To further verify that cell state dictate tumor
457 cell-intrinsic immunogenicity, we depleted *MYCN* expression in Kelly cells and observed no
458 significant change to the IFN-γ-induced expression of HLA and antigen processing genes
459 compared to control cells (**Fig. 3g**). Thus, our findings suggest that the lineage state of
460 neuroblastoma cells specifies the expression of tumor cell-intrinsic immune marker genes.

461

462 **Cellular reprogramming to the mesenchymal state leads to increased immunogenicity**

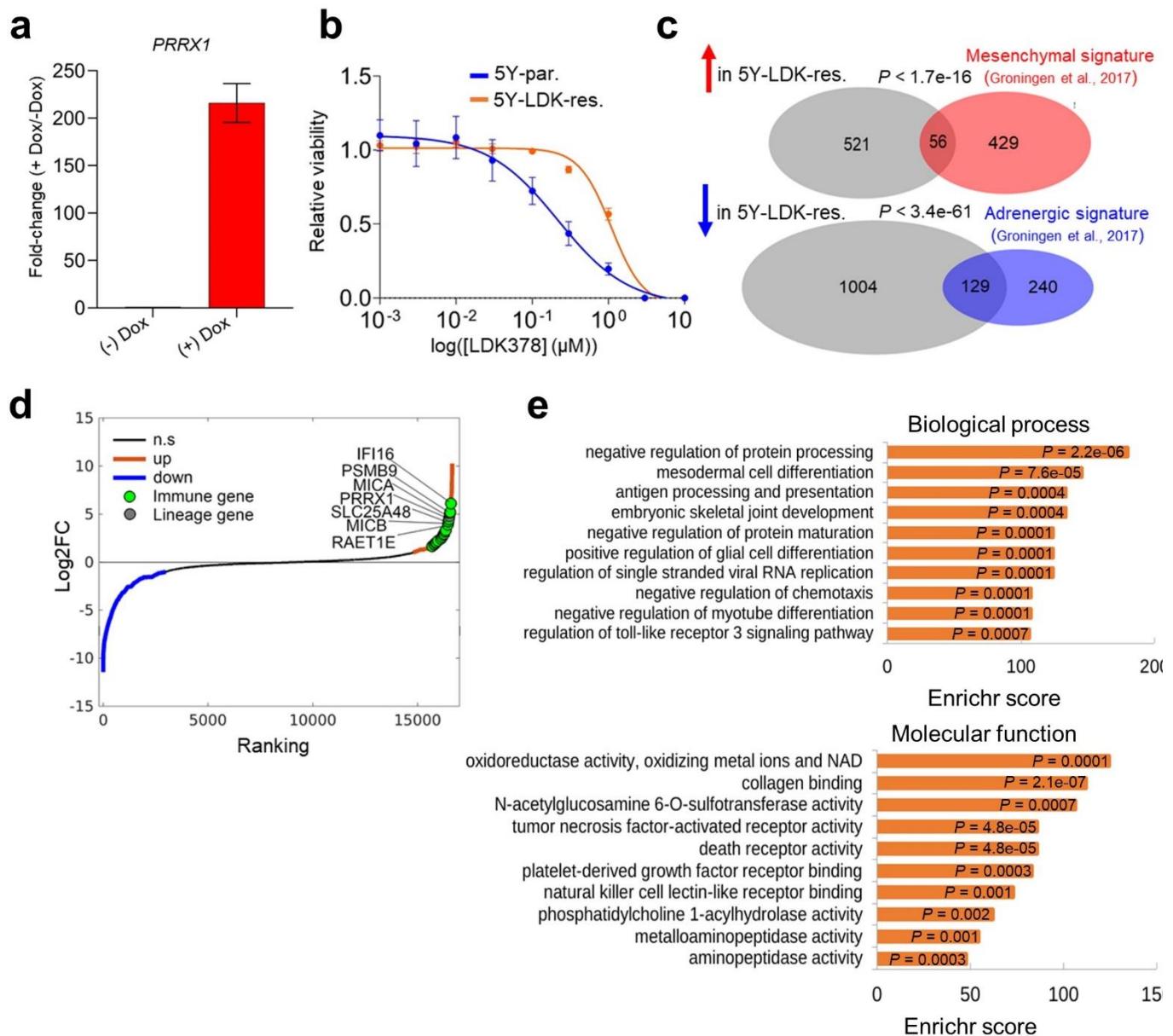
463 We next questioned whether acquisition of the mesenchymal phenotype would be sufficient to
464 render adrenergic neuroblastoma cells immunogenic. One of the top overexpressed genes in SH-
465 EP mesenchymal cells, *PRRX1*, encodes a core lineage-specific homeobox transcription factor
466 (TF), whose overexpression induces the mesenchymal state in neuroblastoma cells^{37,38}
467 (**Supplementary Fig. 5b**). We therefore overexpressed doxycycline-inducible *PRRX1* in
468 adrenergic SH-SY5Y cells and observed a gradual loss of the adrenergic lineage marker
469 PHOX2B, together with increased expression of the mesenchymal markers, fibronectin, vimentin
470 and AXL (**Fig. 4a; Supplementary Fig. 6a**). By contrast, overexpression of DNA-binding mutants

Fig. 4.



471 **Fig. 4. Reprogramming of adrenergic NB cells to the mesenchymal cell state leads to**
472 **increased expression of immune response genes.** **(a)** *Left*, WB analysis of PHOX2B and
473 antigen processing gene expression in adrenergic SH-SY5Y cells engineered to express
474 doxycycline (dox)-inducible PRRX1 in the presence or absence of dox (200 ng/mL) at the
475 indicated time points. *Right*, WB analysis of the indicated proteins in SH-SY5Y cells expressing
476 dox-inducible wild-type (WT) or DNA-binding mutants of PRRX1 at 10 days post dox-induction.
477 The DNA-binding (DB) mutants harbor individual deletions of the three α -helices (Δ H1, Δ H2 and
478 Δ H3) within the PRRX1 homeodomain. **(b)** RT-qPCR analysis of the indicated immune response
479 genes in the same cells as in **(a)**. Data represent the means \pm SD, $n = 2$ biological replicates. **(c)**
480 FACS analysis of cell surface HLA expression following dox-inducible expression of PRRX1 in
481 SH-SY5Y cells at the indicated time points. Data are representative of 2-3 independent
482 experiments. **(d)** FACS analysis of cell surface MICA/MICB expression after PRRX1 induction for
483 24 days in the same cells as in **(c)**. A logscale expression value of 10^3 was used as a threshold
484 (vertical line) to gate MICA/MICB negative ($<10^3$) and positive ($\geq 10^3$) populations. Numbers on
485 the right indicate the percentage of MICA/MICB-positive cells. Plots are representative of 2
486 independent experiments. **(e)** Heat map representation of adrenergic and mesenchymal gene
487 signatures in parental (5Y-par) and LDK-resistant (5Y-LDK-res) SH-SY5Y cells ($n = 2$ biological
488 replicates). Rows represent z-scores of \log_2 expression values for each gene in both cell types.
489 **(f)** Heat map depicting the expression of cell lineage markers (blue, adrenergic; red,
490 mesenchymal), antigen processing machinery genes and NKG2D ligands (black) in parental and
491 LDK-resistant SH-SY5Y cells ($n = 2$ biological replicates). Rows represent z-scores of \log_2
492 expression values. **(g)** WB analysis of lineage marker and antigen processing gene expression in
493 the indicated cells. Actin was used as a loading control in all immunoblots. **(h, i)** FACS analysis
494 of cell surface HLA **(h)** and NKG2D ligand **(i)** expression in the indicated cells.

Supplementary Fig. 6.



496 **Supplementary Fig. 6. Acquired resistance to ceritinib (LDK387) in adrenergic SH-SY5Y**
497 **cells is associated with reprogramming to the mesenchymal lineage and increased**
498 **expression of immune response genes. (a)** RT-qPCR analysis of PRRX1 expression in
499 adrenergic SH-SY5Y cells engineered to express doxycycline (dox)-inducible PRRX1 in the
500 presence or absence of dox (200 ng/mL) for 10 days. Data represent the means ± SD, n = 2
501 biological replicates. **(b)** Dose–response curves of ceritinib (LDK378)-sensitive (5Y-par.) and -
502 resistant (5Y-LDK-res.) SH-SY5Y cells treated with increasing concentrations of LDK378 for 72
503 h. Data represent means ± SD, n = 2 biological replicates. **(c)** Venn diagrams depicting the overlap
504 between the DEGs in LDK-resistant SH-SY5Y cells (compared to parental SH-SY5Y cells) and
505 the mesenchymal or adrenergic signatures derived from Groningen et al³⁸. P-values were
506 determined by Fisher's exact test. **(d)** Waterfall plot of the fold-change in RNA expression levels
507 of up- and down-regulated genes in LDK-resistant SH-SY5Y cells compared to parental SH-SY5Y
508 cells; selected immune genes are highlighted in green. **(e)** GO analysis of differentially
509 upregulated genes in LDK-resistant SH-SY5Y compared to parental SH-SY5Y cells.

510 of *PRRX1* that contained homeodomain deletions had no effect on mesenchymal marker
511 expression, indicating that the lineage switch was a direct consequence of *PRRX1*-mediated
512 transcriptional control (**Fig. 4a**). Next, to determine whether the phenotypic switch had any effect
513 on tumor cell-intrinsic pro-inflammatory pathways, we analyzed the expression of genes involved
514 in antigen processing (*TAP1*, *TAP2*, *LMP2*, *LMP7*) as well as *IFI16* and *STING* (*TMEM173*),
515 innate immune regulators that were differentially upregulated in mesenchymal NB cells (**Fig. 3c**).
516 Induction of wild-type (WT) *PRRX1* led to increased RNA expression of these genes (**Fig. 4b**).
517 Moreover, WT *PRRX1* but not its DNA-binding mutants led to increased *TAP1* and *LMP7* protein
518 expression, which was accompanied by a sustained increase in cell surface MHC expression
519 (**Fig. 4a, c**). Additionally, *PRRX1* induction led to increased cell surface expression of *MICA* and
520 *MICB*, ligands for the activating NK cell receptor *NKG2D*, in a minor population of cells (**Fig. 4d**),
521 in agreement with elevated expression of these proteins in mesenchymal neuroblastoma cells
522 (**Fig. 3c**). These results suggest that conversion from the adrenergic to mesenchymal cell state
523 may be adequate to reprogram immune-insensitive cells toward immunocompetency.

524 Transition from the adrenergic to the mesenchymal state in neuroblastoma is
525 accompanied by resistance to chemotherapy³⁸. Whether this transition in the face of treatment
526 pressure might include the acquisition of a pro-inflammatory signature is unclear, leading us to
527 compare adrenergic neuroblastoma cells that had gained mesenchymal features during the
528 development of treatment resistance with their sensitive, adrenergic counterparts. For this
529 purpose, we used an isogenic pair of cell lines comprising adrenergic SH-SY5Y neuroblastoma
530 cells that express the *ALK*^{F1174L} mutation and are sensitive to the small molecule inhibitor ceritinib
531 (LDK378) (parental SH-SY5Y, IC₅₀ = 150 nM), and their ceritinib-resistant derivatives (LDK-
532 resistant SH-SY5Y, IC₅₀ = 1101 nM) (**Supplementary Fig. 6b**)⁴⁷. Comparison of the gene
533 expression signatures of these cell lines revealed significant downregulation of adrenergic
534 transcripts in LDK-resistant SH-SY5Y cells with concomitant upregulation of the mesenchymal

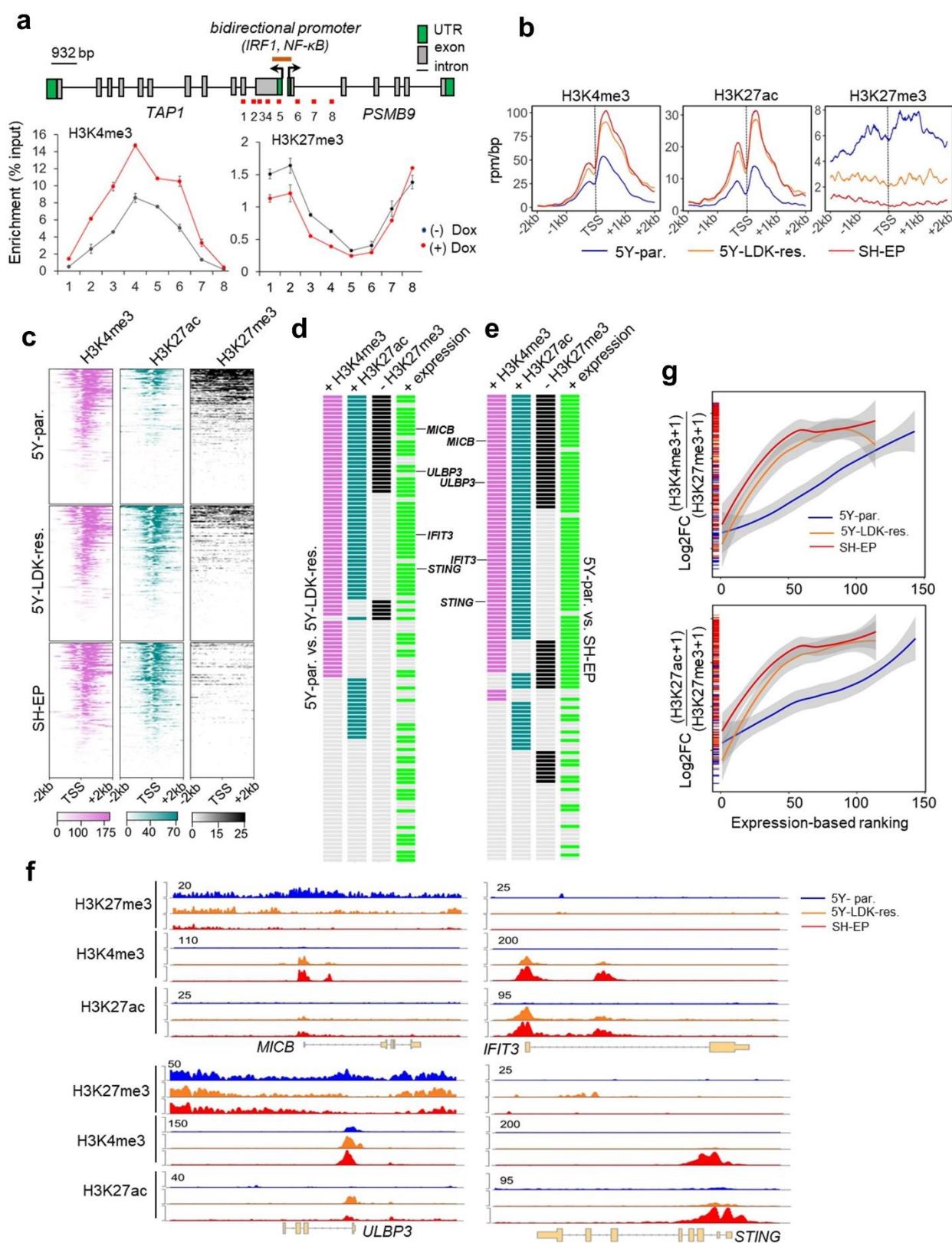
535 signature (**Fig. 4e**). Moreover, a significant overlap was noted between the differentially up- or
536 downregulated transcripts in the LDK-resistant SH-SY5Y cells and established signatures of
537 mesenchymal and adrenergic states, respectively (**Supplementary Fig. 6c**), suggesting that
538 these cells had acquired features of the mesenchymal phenotype with resistance. Consistent with
539 the key role of PRRX1 in triggering the conversion from an adrenergic to mesenchymal cell state,
540 we observed that this TF was among the top upregulated genes in LDK-resistant SH-SY5Y cells
541 (**Supplementary Fig. 6d**). The mesenchymal state of the LDK-resistant SH-SY5Y cells was
542 further supported by the loss of the pivotal adrenergic marker, PHOX2B, and increased
543 expression of additional mesenchymal markers AXL, YAP, TAZ, and IRF1, although these
544 changes were not as pronounced as those in SH-EP mesenchymal cells that served as a positive
545 control (**Fig. 4f, g**). Further evidence supporting the conversion to the mesenchymal state came
546 from the differential upregulation in LDK-resistant SH-SY5Y cells of cell-intrinsic immune markers
547 engaged in antigen processing and presentation and NK cell activating receptor ligands (PSMB9,
548 MICA, MICB); in fact, these were among the top differentially upregulated genes in LDK-resistant
549 SH-SY5Y cells (**Supplementary Fig. 6d, e; Fig. 4f, g**). These changes in immune genes
550 coincided with increases in cell surface expression of MHC receptors to levels comparable to
551 those in mesenchymal SH-EP cells (**Fig. 4h**), as well as the increased expression of ligands for
552 the NK cell-activating receptor NKG2D (**Fig. 4i**). Thus, the genetic reprogramming from the
553 adrenergic to the mesenchymal state that occurred with therapy resistance also led to the
554 upregulation of tumor cell-intrinsic pro-inflammatory pathway genes suggesting that such
555 conversion could render the tumor cells susceptible to recognition by T and NK cells.

556

557 **Immune response gene expression during cell state transition is epigenetically regulated**

558 As lineage plasticity in neuroblastoma is epigenetically driven^{37,38,48,49}, we next questioned
559 whether the altered expression of immune response genes observed in the individual cell states
560 could be the result of changes in chromatin organization. To this end, we analyzed the chromatin
561 occupancies of active and repressive histone marks at immune genes that were upregulated in
562 adrenergic SH-SY5Y cells upon induction of PRRX1 (**Fig. 4b**). Indeed, PRRX1 induction resulted
563 in increased binding of the active H3K4me3 mark as well as loss of repressive H3K27me3 binding
564 at several candidate immune genes, including the APM genes *TAP1* and *PSMB9* (**Fig. 5a**;
565 **Supplementary Fig. 7a**). To understand epigenetic modifications that occur during the
566 spontaneous transition between the two lineage states (as compared with forced expression of
567 *PRRX1*) on a genome-wide basis, we compared histone occupancies between adrenergic
568 (parental SH-SY5Y) cells and those that had acquired mesenchymal characteristics with drug
569 resistance (LDK-resistant SH-SY5Y) (**Fig. 4e**), using SH-EP cells as a typical example of the
570 mesenchymal state. ChIP-seq analysis of active H3K27ac binding identified that the super-
571 enhancers (SEs) in LDK-resistant SH-SY5Y cells were associated with genes that conferred
572 mesenchymal identity while parental SH-SY5Y cells retained SEs at genes that conferred
573 adrenergic identity (**Supplementary Fig. 7b**), consistent with evidence that lineage plasticity is
574 driven by cell type-specific SEs^{37,38}. To determine whether the SE-mediated regulation of lineage
575 genes also extended to genes associated with immune responsiveness, we analyzed the genes
576 in our 41-gene immune activation signature (**Supplementary Table 3;Fig. 1e**) as well as those
577 associated with an IFN-response signature (n = 91) in primary tumors and cell lines (**Fig. 1b**;
578 **Supplementary Fig. 5d**, see Methods). Despite the higher expression of these genes in
579 mesenchymal cells (LDK-resistant SH-SY5Y and SH-EP), none was associated with an SE,
580 prompting us to focus on the promoter regions. We observed significantly higher enrichment of
581 H3K27ac and H3K4me3 binding at regions spanning the transcription start sites (TSS ± 2 kb) at
582 cell-intrinsic immune genes in LDK-resistant SH-SY5Y and SHEP compared to parental SH-SY5Y

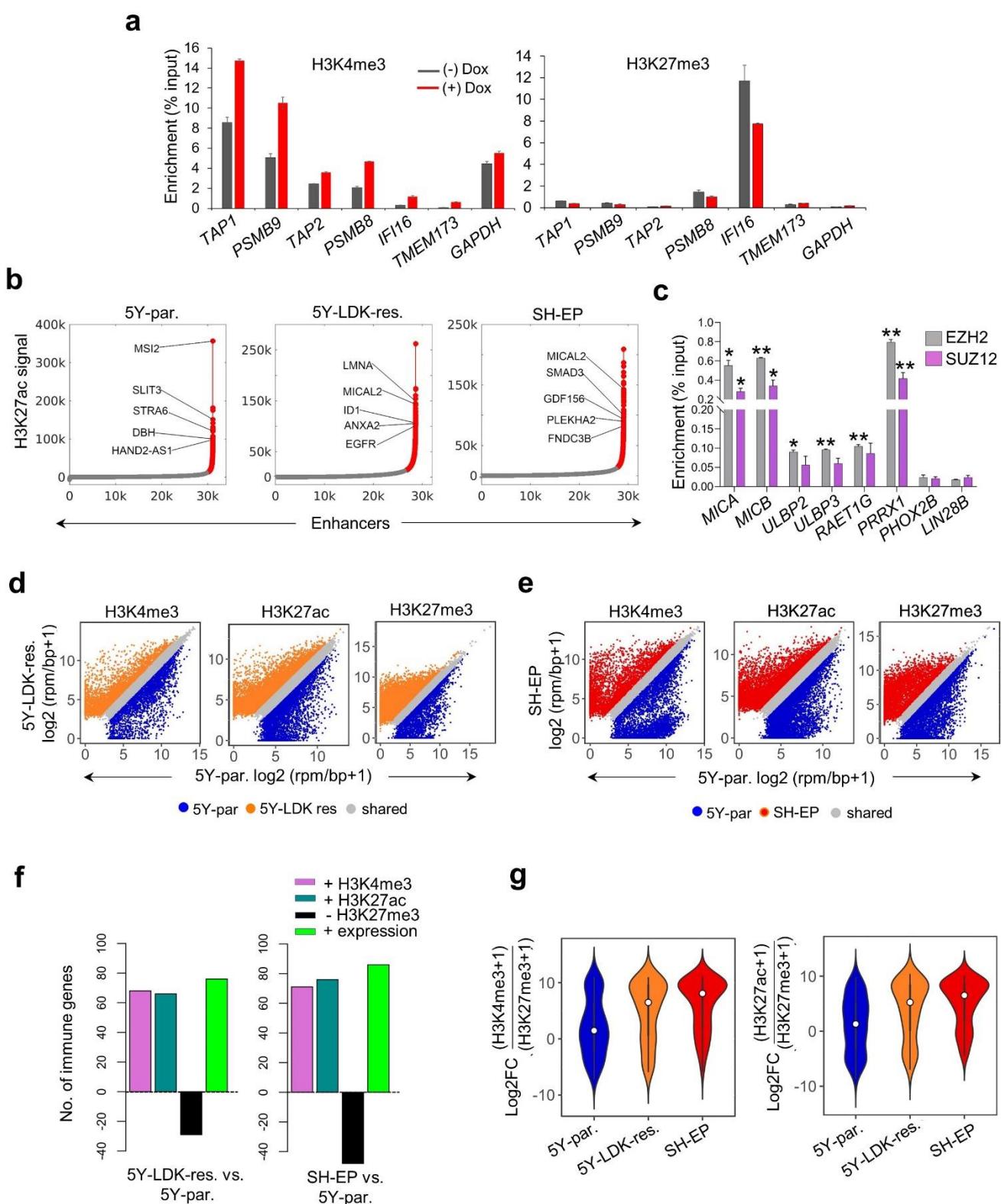
Fig. 5



583 **Fig. 5. Activation of immune gene expression associated with cell state transition is**
584 **epigenetically regulated. (a)** *Upper*, Linear representation of TAP1 and PSMB9 gene loci
585 showing the locations of the bidirectional promoter and IRF1 and NF- κ B binding sites. The
586 amplicons (1-8) analyzed for histone mark occupancy are shown in red. *Lower*, ChIP-qPCR
587 analysis of H3K4me3 and H3K27me3 enrichment at the indicated amplicons along the
588 TAP1/PSMB9 locus in adrenergic SH-SY5Y cells expressing dox-inducible PRRX1 in the
589 presence or absence of dox (200 ng/mL) for 10 days. Data represent the means \pm SD, $n = 2$
590 biological replicates. **(b)** Metagene representations of average ChIP-seq occupancies of the
591 indicated histone marks at the promoters of tumor cell-intrinsic immune response genes (TSS \pm
592 2 kb; $n = 134$) in parental (5Y-par.), LDK-resistant (5Y-LDK-res.) SH-SY5Y and SH-EP NB cells.
593 **(c)** Heat map representation of histone enrichment at the same immune gene promoters as in
594 **(b)**, ranked in decreasing order of occupancy in the indicated cells. Each row represents the
595 normalized densities of histone marks within a \pm 2 kb window centered on the TSS. **(d, e)**
596 Representation of pairwise comparisons between parental SH-SY5Y and LDK-resistant SH-SY5Y
597 **(d)**, and parental SH-SY5Y and SH-EP cells **(e)**. The changes (+, gained; -, lost) in occupancies
598 of the active (H3K4me3, H3K27ac) and repressive (H3K27me3) histone marks (\log_2 FC ≥ 0.75 ,
599 TSS \pm 2 kb), together with the corresponding changes in RNA expression (+, overexpressed; \log_2
600 FC ≥ 1) of each of the 134 tumor cell-intrinsic immune genes analyzed in **(b)** are shown.
601 Representative genes showing either a switch from repressive to active chromatin (*MICB*, *ULBP3*)
602 or associated only with a gain of active chromatin (*IFIT3*, *STING*) are shown. **(f)** ChIP-seq tracks
603 depicting the gain of active histone binding together with the loss of repressive histone binding
604 (*left*) or gain of active marks without changes in repressive mark occupancy (*right*) at the indicated
605 immune gene loci. Signal intensity is given at the top left corner for each track. **(g)** Loess
606 regression analysis of the correlation between the ratios of active to repressive histone binding at
607 the promoters (TSS \pm 2kb) of immune response genes and their RNA expression (*Upper*,

608 H3K4me3:H3K27me3; Lower, H3K27ac:H3K27me3). Genes are ranked based on increasing
609 expression. Shaded regions represent 95% confidence intervals.

Supplementary Fig. 7.



611 **Supplementary Fig. 7. Immune gene activation associated with the mesenchymal cell state**
612 **is epigenetically regulated.** **(a)** ChIP-qPCR analysis of H3K4me3 and H3K27me3 enrichment
613 at the promoters of the indicated immune genes in SH-SY5Y cells expressing doxycycline-
614 inducible PRRX1 in the presence or absence of dox (200 ng/mL) for 10 days. Enrichments at
615 TAP1 and PSMB9 loci correspond to amplicons 4 and 6 respectively, as described in **Fig. 5a**.
616 Data represent the means \pm SD, $n = 2$ biological replicates. **(b)** Identification of enhancer regions
617 in parental SH-SY5Y, LDK-resistant SH-SY5Y and SH-EP cells. H3K27ac bound regions
618 identified as significant peaks were stitched together if they were within 12.5 kb of each other and
619 termed typical enhancers (plotted in grey). Super enhancers (SEs) were defined as stitched
620 enhancers surpassing the threshold signal based on the inclination point in all cell types (plotted
621 in red). In parental SH-SY5Y, LDK-resistant and SH-EP cells, 2.94% (915/31116), 6.56%
622 (1880/28635) and 4.18% (1215/29057) of the enhancers were classified as SEs respectively. The
623 top five SE-associated lineage-specific genes are highlighted. **(c)** ChIP-qPCR analysis of EZH2
624 and SUZ12 enrichment at the indicated genes in adrenergic 5Y-par. cells. Data represent the
625 means \pm SD, $n = 2$ biological replicates, $^*P < 0.05$; $^{**}P < 0.01$ two-tailed Student's t-test. P -values
626 were calculated in comparison to enrichment observed at the Lin28B TSS (negative control
627 locus). **(d, e)** Scatter plots representing the differential binding of the indicated histone marks at
628 the promoter regions (TSS \pm 2 kb) of all protein coding genes between parental SH-SY5Y (5Y-
629 par.) and LDK-resistant SH-SY5Y (5Y-LDK res.) **(d)**, and parental SH-SY5Y and SH-EP cells **(e)**.
630 rpm/bp, reads per million mapped reads per base pair. A $\geq 0.75 \log_2$ FC threshold was used to
631 identify unique peaks for each individual histone mark. Unique and shared peaks are shown in
632 different colors. **(f)** Bar plots representing the numbers of immune genes with increased
633 deposition of H3K4me3 and H3K27ac (\log_2 FC ≥ 0.75 , TSS \pm 2 kb) and loss of H3K27me3 (\log_2
634 FC ≥ 0.75 , TSS \pm 2 kb) histone marks, together with increased RNA expression (\log_2 FC ≥ 1) in
635 mesenchymal LDK-resistant SH-SY5Y (*left*) or SH-EP (*right*) as compared to adrenergic parental
636 SH-SY5Y cells. **(g)** Violin plots of the ratios of active to repressive histone marks (*left*,

637 H3K4me3:H3K27me3; *right*, H3K27ac:H3K27me3) surrounding immune gene promoters (TSS ±
638 2kb) in parental SH-SY5Y, LDK-resistant SH-SY5Y and SH-EP cells. Significance was
639 determined by the two-sided Wilcoxon rank-sum test.

640 cells (**Fig. 5b, c**). On the other hand, the adrenergic parental SH-SY5Y cells showed significantly
641 higher occupancies of the H3K27me3 repressor mark at these immune gene promoters (**Fig. 5b,**
642 **c**). Analysis of the polycomb repressive complex 2 (PRC2) that promotes H3K27me3 deposition
643 at repressed chromatin⁵⁰ revealed that immune response genes enriched for H3K27me3 binding,
644 such as those encoding the NKG2D ligands *MICA/B*, *ULBP2/3* and *RAET1G* had significantly
645 higher occupancies for PRC2 subunits, EZH2 and SUZ12 in adrenergic parental SH-SY5Y cells
646 compared to negative control regions that lacked H3K27me3 binding (*PHOX2B* and *LIN28B*)
647 (**Supplementary Fig. 7c**), suggesting active immune gene repression in these cells.

648

649 We next sought to understand whether the activation of immune response genes observed
650 during the cell state transition from sensitivity to resistance represented a *switch* from repressive
651 to active chromatin or a *gain* of active chromatin marks. To this end, we quantified the changes
652 in histone binding occupancies between adrenergic parental SH-SY5Y and mesenchymal LDK-
653 resistant SH-SY5Y or SH-EP cells using pair-wise comparisons (**Supplementary Fig. 7d, e**).
654 Compared to parental SH-SY5Y, LDK-resistant SH-SY5Y and SH-EP cells gained significant
655 H3K4me3 binding at the promoters of 60% and 62% immune genes (68 and 71 of 114)
656 respectively, which corresponded with their increased expression (**Fig. 5d, e; Supplementary**
657 **Fig. 7f**). A similar significant enrichment of the H3K27ac histone mark was observed at the
658 promoters of these immune genes [LDK-resistant SH-SY5Y, 58% (66/114); SH-EP, 67%
659 (76/114)]. Interestingly, gain of these active marks was accompanied by a concomitant loss of
660 H3K27me3 repressive histone binding at the promoters of 25% and 35% of (29 and 40 of 114)
661 immune genes in LDK-resistant SH-SY5Y and SH-EP cells respectively, as represented by the
662 NKG2D ligands, *MICB* and *ULBP3* (**Fig. 5d-f; Supplementary Fig. 7f**). On the other hand, in
663 LDK-resistant SH-SY5Y and SH-EP mesenchymal cells, 48% and 41% (55 and 47 of 114)
664 immune genes, such as the IFN-regulated factors *IFIT3* and *STING*, gained either one or both
665 active marks without changes in occupancy of the repressive mark (**Fig. 5d-f; Supplementary**

666 **Fig. 7f).** Furthermore, the mesenchymal cells showed a significantly higher ratio of active to
667 repressor histone binding at the TSSs of immune-related genes (H3K4me3 or
668 H3K27ac/H3K27me3) (**Supplementary Fig. 7g**), which importantly, also correlated with the
669 increased expression of these genes in this cell state (**Fig. 5g**). Therefore, our results suggest
670 that the immune gene activation observed with the transition from the adrenergic to the
671 mesenchymal cell state represents either a switch from repressive to active chromatin or a gain
672 of active chromatin at promoter regions.

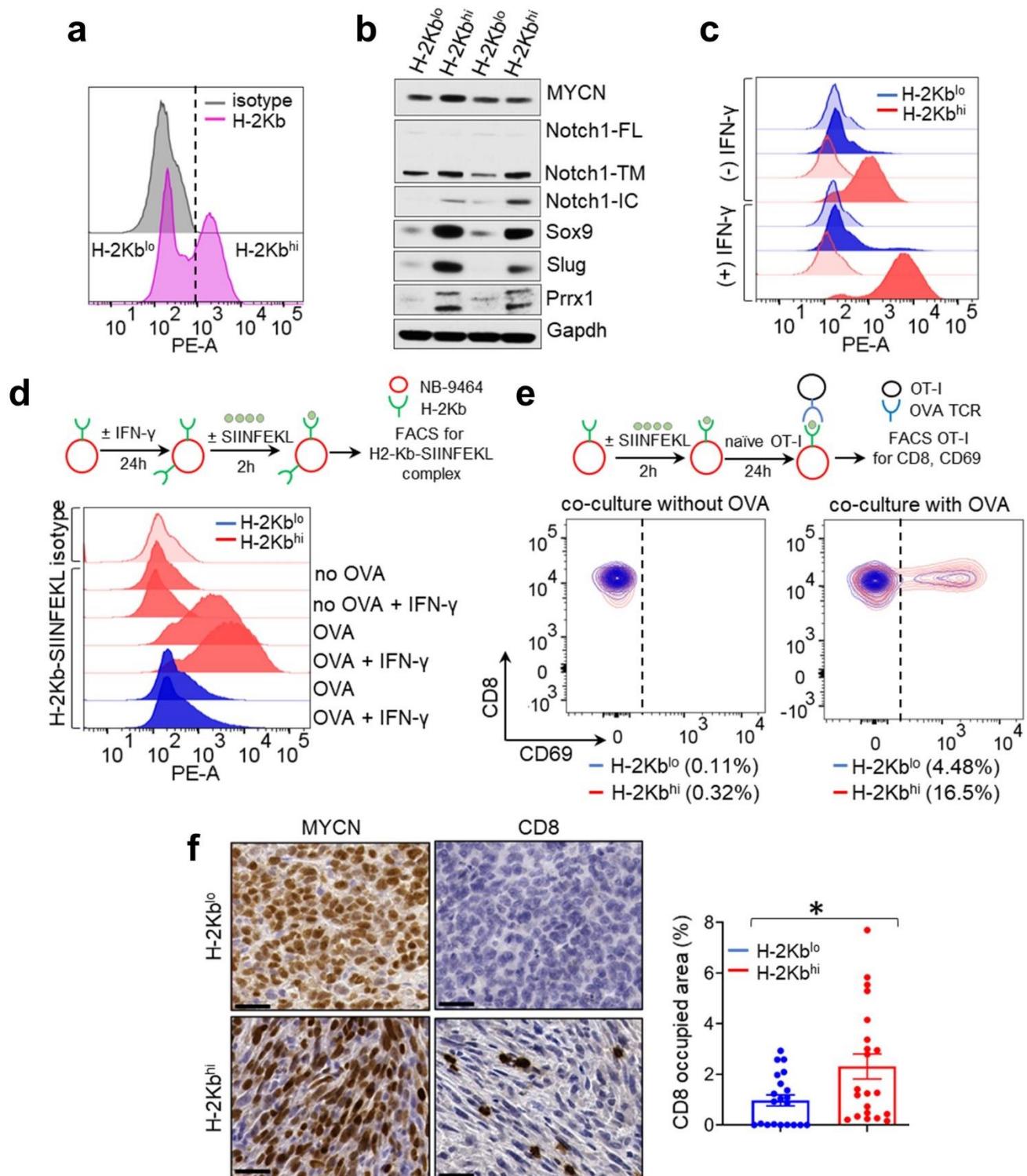
673

674 **Mesenchymal neuroblastoma cells functionally engage cytotoxic T cells**

675 To assess the functional consequences of the increased immunogenicity associated with a
676 mesenchymal phenotype, we utilized the murine neuroblastoma cell line NB-9464, which was
677 derived from tumors arising in the Th-MYCN genetically engineered mouse model (GEMM). This
678 model was generated in immunocompetent C57BL/6 mice and the tumors recapitulate the genetic
679 and immunological features of human neuroblastoma^{51,52}. We observed that NB-9464 cells
680 consisted of distinct populations that could be sorted on the basis of surface MHC class I H-2Kb
681 expression into high (H-2Kb^{hi})- or low (H-2Kb^{lo})-expressing populations (**Supplementary Fig. 8a**;
682 **Fig. 6a**), both of which expressed transgenic human MYCN (**Fig. 6b**). Consistent with our
683 hypothesis, H-2Kb^{hi} cells were enriched for *bona fide* mesenchymal markers - Prrx1, Sox9,
684 Notch1, and Snai2 (Slug) (**Fig. 6b**), and showed enhanced migration and invasion, as might be
685 expected from their neural crest cell-like state (**Supplementary Fig. 8b, c**). Importantly, the
686 mesenchymal H-2Kb^{hi} NB-9464 cells, but not their adrenergic H-2Kb^{lo} counterparts showed
687 augmented expression of cell surface class I MHC H-2Kb in response to IFN-γ, suggesting that
688 the mesenchymal cells had the potential for inducing a T cell-driven antitumor immune response
689 (**Fig. 6c**).

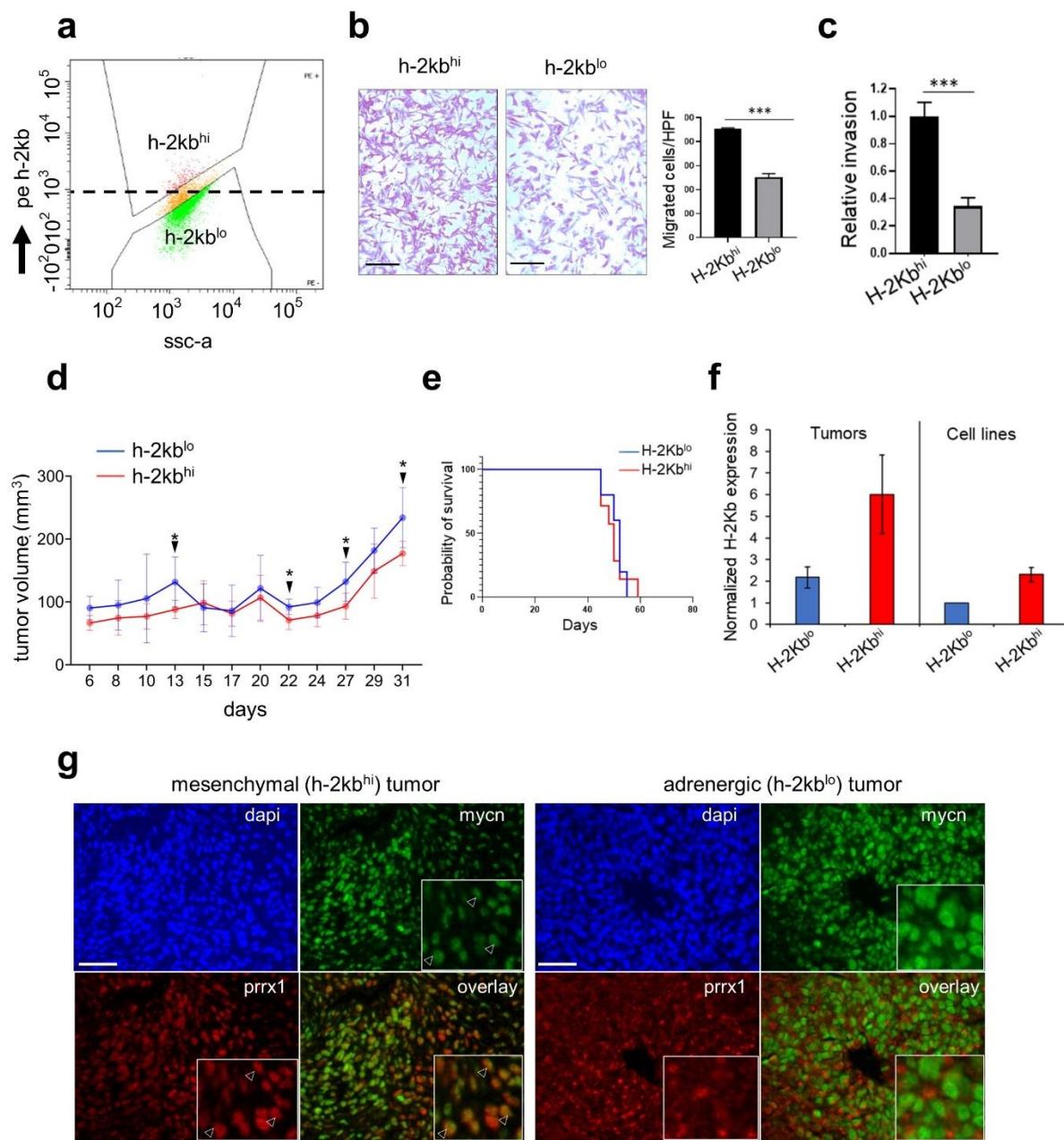
Hence, to determine whether the increased MHC class I expression of mesenchymal versus adrenergic cells translated into T cell activation, we first asked whether mesenchymal H-2K^{bhi} NB-9464 cells were capable of exogenous antigen presentation. Using the well-characterized chicken ovalbumin-derived peptide (OVA₂₅₇₋₂₆₄ or SIINFEKL) antigen that binds to H-2K^b and can be recognized by specific T cell receptors (TCRs) on CD8⁺ T cells⁵³, we found that in comparison to H-2K^{blo} cells, H-2K^{bhi} cells expressed significantly higher levels of the H-2K^b-SIINFEKL complex (**Fig. 6d**). Next, we determined whether antigen presentation through H-2K^b enables mesenchymal tumor cells to be recognized by antigen-specific T cell receptors (TCRs) on CD8⁺ T cells, the first step towards a cytotoxic response. For this purpose, we used OT-I CD8⁺ T cells from C57BL/6 mice expressing a transgenic TCR that specifically recognizes the H-2K^b-SIINFEKL complex⁵³. H-2K^{bhi} or H-2K^{blo} cells loaded with the SIINFEKL peptide were cocultured with naïve OT-I cells, after which OT-I activation was measured through cell surface CD69 expression, an early marker of T-cell activation⁵⁴. Mesenchymal H-2K^{bhi} cells led to significantly higher OT-I activation in comparison with adrenergic H-2K^{blo} cells, indicating specific recognition of the H-2K^b-SIINFEKL complex by the TCR on OT-I cells (**Fig. 6e**). By contrast, co-cultures of OT-I cells and either H-2K^{blo} or H-2K^{bhi} NB-9464 neuroblastoma cells without the SIINFEKL peptide did not lead to T-cell recognition, confirming the specificity of the TCR-antigen interaction (**Fig. 6e**). Finally, we investigated whether the differential MHC-I expression between adrenergic and mesenchymal neuroblastoma cells influenced tumor growth *in vivo* through subcutaneous injection of H-2K^{bhi} or H-2K^{blo} cells into syngeneic C57BL/6 (H-2K^b haplotype) mice (**Supplementary Fig. 8d**). We noted an earlier onset of tumor formation with H-2K^{blo} cells compared to H-2K^{bhi} cells (**Supplementary Fig. 8d**). However, once consistent tumor growth was established, growth or survival rates did not change substantially between the two groups (**Supplementary Fig. 8e**), despite the persistence of higher H-2K^b and Prrx1 expression in the H-2K^{bhi} tumors compared with the H-2K^{blo} tumors (**Supplementary Fig. 8f,g**). While both types of tumor cells had MYCN expression, histologically, in keeping with their adrenergic phenotype

Fig. 6.



716 **Fig. 6. Mesenchymal NB cells functionally engage cytotoxic T cells.** **(a)** FACS analysis of
717 H-2K^b expression in unsorted NB-9464 cells. Vertical dashed line denotes the logscale
718 expression value used as a threshold to gate H-2K^b^{lo} and H-2K^b^{hi} cell populations. **(b)** WB
719 analysis of the indicated lineage markers in H-2K^b^{lo} and H-2K^b^{hi} cell populations. Notch1-FL, full
720 length; -TM, transmembrane; -IC, intracellular. GAPDH is used as a loading control. **(c)** FACS
721 analyses of basal and IFN- γ -induced (100 ng/mL for 24 hr.) surface H-2K^b expression (darker
722 colored histograms) in H-2K^b^{lo} and H-2K^b^{hi} cells compared to isotype controls (lighter colored
723 histograms). Plots representative of 2 independent experiments. **(d)** *Upper*, Schematic of OVA
724 binding assay. *Lower*, FACS analysis of surface H-2K^b-bound SIINFEKL OVA peptide in H-2K^b^{hi}
725 and H-2K^b^{lo} cells under basal or IFN- γ -induced conditions as in **(c)** and in the absence or
726 presence of the OVA peptide. Plots representative of 2 independent experiments. **(e)** *Upper*,
727 Schematic of NB-9464-OT-I co-culture assay. *Lower*, Contour plots showing the percentage of
728 naïve OT-I cells that were activated (CD8⁺ CD69⁺) following co-culture with H-2K^b^{lo} and H-2K^b^{hi}
729 cells for 24 hr. with or without the OVA peptide. OT-I activation was measured by FACS analysis
730 of cell surface CD69. **(f)** *Left*, Immunohistochemical (IHC) staining for MYCN and CD8 expression
731 in representative murine NB xenograft tumors derived from NB-9464 H-2K^b^{lo} (adrenergic) and H-
732 2K^b^{hi} (mesenchymal) cells in immunocompetent syngeneic (C57BL/6) mice. Scale bars, 100 μ m.
733 *Right*, Bar graphs showing the percentage of area occupied by CD8⁺ T cells in H-2K^b^{lo} ($0.9\% \pm$
734 0.2%) vs. H-2K^b^{hi} ($2.3\% \pm 0.5\%$) tumors; * $P < 0.05$, two-tailed Welch's t-test. Each dot represents
735 one of three independent measurements for each tumor. Data represent mean \pm SEM.

Supplementary Fig. 8.



736 **Supplementary Fig. 8. Tumors that arise from NB-9464 H-2K^{hi} (mesenchymal) cells show**
737 **cytotoxic T cell infiltration.** **(a)** FACS scatter plot showing gating conditions used for sorting
738 NB-9464 cells into H-2K^{hi} and H-2K^{lo} populations. X axis represents side scatter (SSC-A); Y
739 axis denotes fluorescence intensity of surface H-2K^b detected using phycoerythrin (PE)-
740 conjugated antibody against H-2K^b. A logscale expression value of 10^3 was used as the threshold
741 (horizontal dashed line) to gate H-2K^{hi} ($\geq 10^3$) and H-2K^{lo} ($< 10^3$) populations. **(b)** *Left*, Bright
742 field images of crystal violet-stained H-2K^{hi} and H-2K^{lo} cells in transwell migration assays. Scale
743 bars, 100 μ m. *Right*, Quantification of migrating cells per high-power field (HPF). Data represent
744 the means \pm SD, $n = 2$ biological replicates, *** $P < 0.001$; two-tailed Student's t-test. **(c)**
745 Quantification of the relative invasiveness of H-2K^{hi} and H-2K^{lo} cells. Data represent the means
746 \pm SD, $n = 2$ biological replicates, *** $P < 0.001$; two-tailed Student's t-test. **(d)** Tumor volumes in
747 immunocompetent C57BL/6 mice injected subcutaneously with 1×10^6 H-2K^{lo} or H-2K^{hi} NB-
748 9464 cells. Measurements were started on day 3 after injection and continued three times weekly
749 for up to 50 days or until euthanized due to tumor growth. Graphs represent changes in tumor
750 volume until day 31 (means \pm SD; $n = 7$ per group at all time points) to highlight an earlier onset
751 of tumor formation with H-2K^{lo} cells compared to H-2K^{hi} cells. Tumors were considered to be
752 established upon reaching a volume of ~ 250 mm³ (observed between days 31-34 for both H-2K^{lo}
753 and H-2K^{hi} tumors), following which both tumor types displayed equal increases in tumor growth
754 (data not shown). Tumor onset was defined as the day following which tumor volumes showed a
755 consistent increase (24.3 ± 2.2 days for H-2K^{lo} and 27.4 ± 2.1 days for H-2K^{hi} cells, $P < 0.05$).
756 Closed arrows refer to the indicated days (13, 22, 27 and 31) on which there were significant
757 differences in tumor volumes (day 13, $P = 0.02$; day 22, $P = 0.01$; day 27, $P = 0.02$ and day 31,
758 $P = 0.02$; $n = 7$ per group at all time points). All P -values calculated using the two-tailed Student's
759 t-test. **(e)** Kaplan-Meier survival analysis of immunocompetent C57BL/6 mice bearing NB tumor
760 xenografts derived from H-2K^{lo} (adrenergic) and H-2K^{hi} (mesenchymal) cells (50.8 ± 3.7 vs.
761 49.8 ± 4.8 days; $P = 0.7$; $n = 7$ per group; log-rank test. **(f)** RT-qPCR analysis of H-2K^b expression

762 in H-2K^b^{lo} and H-2K^b^{hi} NB tumor xenografts and the cell lines used to generate the xenografts.
763 Data represent the means ± SD, n = 3 biological replicates. (g) Immunofluorescence images of
764 MYCN (green) and Prrx1 (red) expression in representative murine NB xenograft tumors derived
765 from NB-9464 H-2K^b^{hi} (mesenchymal) and H-2K^b^{lo} (adrenergic) cells in immunocompetent
766 syngeneic (C57BL/6) mice. Nuclei are counterstained with DAPI (blue). Insets depict cells with
767 nuclear co-staining of MYCN and Prrx1 (arrowheads) and are exclusively present in the H-2K^b^{hi}
768 mesenchymal tumor. Scale bars, 100 µm, insets 33.3 µm.

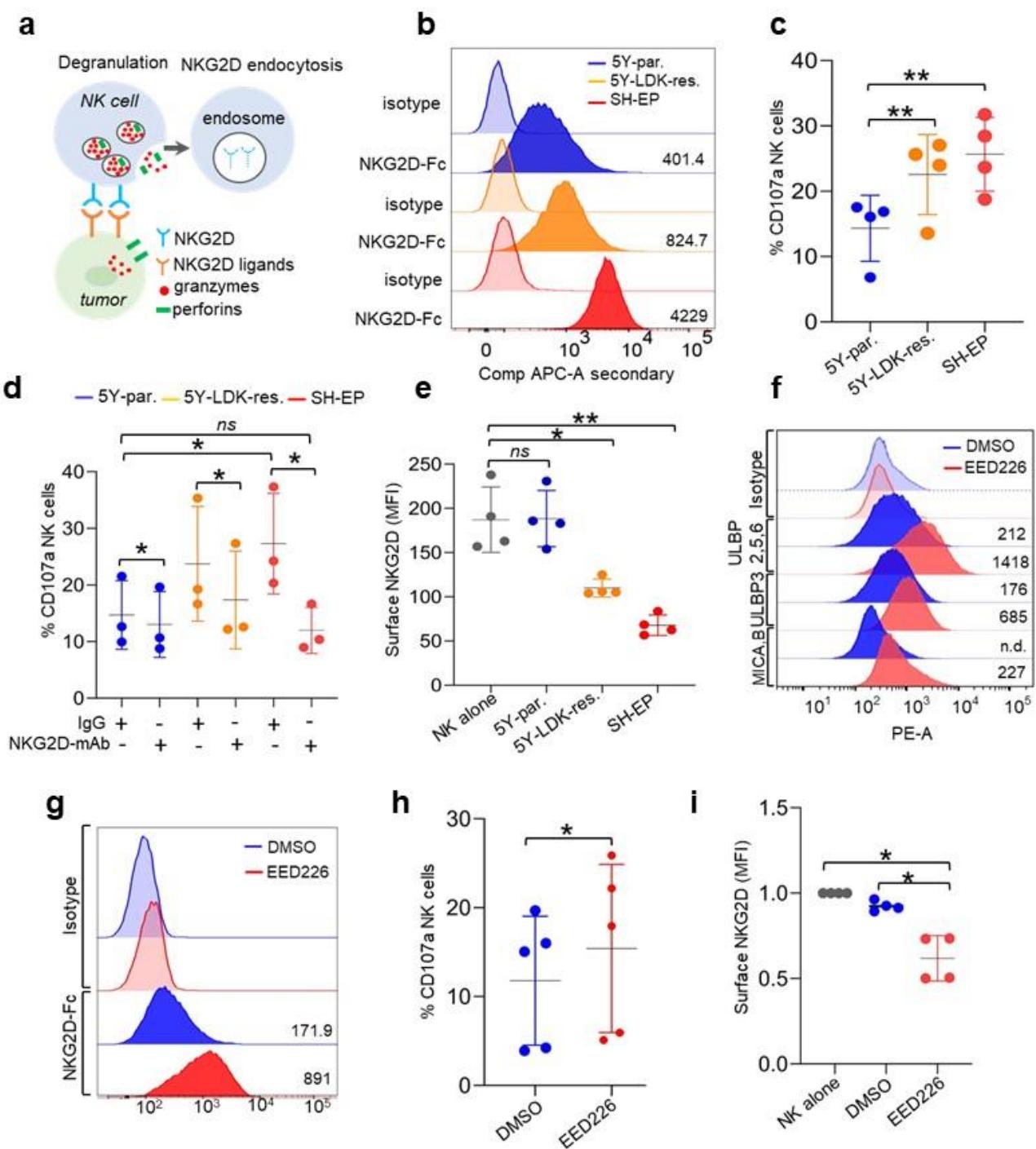
769 H-2K b^{lo} tumors were composed of densely arranged small round blue cells, whereas H-2K b^{hi}
770 tumors predominantly comprised elongated, spindle-like cells interspersed with clusters of small
771 round blue cells (**Fig. 6f**). We next analyzed the immune status of these tumors, reasoning that
772 tumors arising from immunogenic H-2K b^{hi} cells would be infiltrated by T cells. Indeed, H-2K b^{hi}
773 tumors showed significantly higher CD8 $^{+}$ T cell infiltration compared with H-2K b^{lo} tumors (**Fig. 6f**).
774 Taken together, these results suggest that the immunogenic traits of mesenchymal
775 neuroblastoma cells translate into the recruitment of cytotoxic T cells into the tumor
776 microenvironment.

777

778 **Mesenchymal neuroblastoma cells induce NK cell degranulation**

779 We next analyzed the functional relevance of the increased expression of the NKG2D NK cell
780 receptor ligands (ULBP1-3, MICA, MICB) seen in mesenchymal neuroblastoma tumors
781 (**Supplementary Fig. 4c; Fig. 4i**). Since the interaction of the receptor with its cognate ligands
782 on target cells is the first step towards a cytotoxic response (**Fig. 7a**), we compared the ability of
783 adrenergic (parental SH-SY5Y) and mesenchymal (LDK-resistant SH-SY5Y and SH-EP) cells to
784 bind to the recombinant NKG2D receptor fusion protein in an *in vitro* binding assay. Both
785 mesenchymal cell types showed increased binding to the NKG2D receptor, in keeping with their
786 increased cell surface expression of the ULBP2/3/5/6, MICA and MICB ligands (**Fig. 7b**). NK cell
787 cytotoxicity is mediated by granzyme proteases and the pore-forming protein perforin, which in
788 resting cells are stored in secretory lysosomes or lytic granules marked by lysosome-associated
789 membrane protein-1 (LAMP-1 or CD107a)⁵⁵. Upon target recognition, NK cells undergo
790 degranulation, or exocytosis of the lytic granules, which is associated with relocation of the
791 CD107a antigen to the cell membrane. Using cell-surface CD107a as a specific marker of
792 degranulation, we measured degranulation of peripheral blood NK cells harvested from healthy
793 human donors in the presence of parental SH-SY5Y, LDK-resistant SH-SY5Y and SH-EP targets.

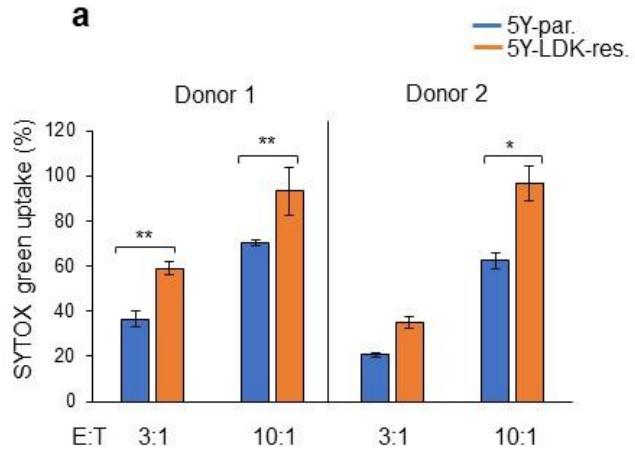
Fig. 7.



794 **Fig. 7. Mesenchymal NB cells induce NK cell degranulation.** **(a)** Schematic representation of
795 the interaction between NKG2D receptors on NK cells and cognate ligands on tumor cells, leading
796 to NK cell degranulation and receptor endocytosis. **(b)** FACS analysis of purified human NKG2D-
797 Fc protein binding (darker histograms) to adrenergic parental SH-SY5Y, and mesenchymal LDK-
798 resistant SH-SY5Y and SH-EP cells. Comp Alexa-647, compensated fluorescence intensity of
799 NKG2D-Fc protein detected using Alexa 647-conjugated anti-human IgG. Lighter histograms
800 indicate staining with Alexa 647-conjugated anti-human IgG only. Numbers indicate median
801 fluorescence intensity (MFI). Plots representative of 2 independent experiments. **(c)** X-Y plot
802 showing the percentage of degranulating NK cells following co-culture with the same cells as in
803 **(b)** for 4 hr. at an effector: target (E: T) cell ratio of 1:2. Degranulation was measured by FACS
804 analysis of cell-surface CD107a. Data represent the means \pm SD, $n = 4$ biological replicates. **(d)**
805 X-Y plot showing the effect of a control IgG1 or an NKG2D blocking antibody on NK cell
806 degranulation following co-culture with the indicated cells for 4 hr. NK cell degranulation was
807 measured as in **(c)**. Data represent the means \pm SD, $n = 4$ biological replicates. **(e)** X-Y plot
808 depicting the MFI of NKG2D expression measured by FACS on naïve NK cells (NK alone) or
809 following co-culture with parental SH-SY5Y, LDK-resistant SH-SY5Y and SH-EP cells for 4 h.
810 Data represent the means \pm SD, $n = 4$ biological replicates. **(f)** FACS analysis of surface
811 ULBP2/5/6, ULBP3, and MICA/MICB in adrenergic parental SH-SY5Y cells treated with DMSO
812 (vehicle control) or EED226 ($5 \mu\text{M}$ for 8 days). **(g)** FACS analysis of purified human NKG2D-Fc
813 protein binding to parental SH-SY5Y cells treated with either DMSO or EED226 as in **(f)**. Light
814 gray histograms indicate human IgG1 isotype control. Numbers on the right represent MFI values
815 for FACS plots. Plots in **(f)** and **(g)** are representative of 2 independent experiments. **(h, i)** X-Y
816 plots showing NK cell degranulation **(h)** and MFI of surface-NKG2D **(i)** following co-culture of
817 naïve NK cells with parental SH-SY5Y cells treated with DMSO or EED226 as in **(f)**. NK cell
818 granulation and NKG2D MFI were measured as in **(c)** and **(e)**. Data represent means \pm SD, $n =$

819 4 (**h**) and 5 (**i**) biological replicates. Significance for all results was calculated using the paired
820 two-tailed Student's t-test (* $P < 0.05$; ** $P < 0.01$); *ns*, not significant

Supplementary Fig. 9.



Supplementary Fig. 9. LDK-resistant SH-SY5Y cells are more susceptible to NK-induced cell death.

(a) Bar graphs showing NK-induced cell death in parental SH-SY5Y and LDK-resistant SH-SY5Y cells assessed by SYTOX green uptake following 1 hour of co-culture at indicated effector-to-target (E:T) ratios. Experiments were performed in two biological replicates using NK cells harvested from two independent donors. Data represent means \pm SD, n = 3 technical replicates. Significance for all results was calculated using the paired two-tailed Student's t-test (* P < 0.05; ** P < 0.01).

821 Co-culture of NK cells with LDK-resistant SH-SY5Y and SH-EP cells, both of which express
822 ligands for the NKG2D receptor, resulted in increased NK cell degranulation compared to parental
823 SH-SY5Y cells that did not express these ligands (**Fig. 7c**). To confirm that the increased NK cell
824 degranulation in LDK-resistant SH-SY5Y and SH-EP cells was specific to the NKG2D receptor,
825 we blocked its function with an anti-NKG2D monoclonal antibody. Compared to the isotype
826 control, blockade of NKG2D receptor activity completely abrogated the increased NK cell
827 degranulation in LDK-resistant SH-SY5Y and SH-EP cells, but had no effects on parental SH-
828 SY5Y cells, signifying that the modest but robust increase in degranulation in the presence of
829 mesenchymal cells was specific to the NKG2D receptor on NK cells (**Fig. 7d**). Upon interaction
830 with their cognate ligands on target cells, NKG2D receptors are internalized via ubiquitin-
831 dependent endocytosis leading to their lysosomal degradation, rendering the loss of surface
832 NKG2D receptor expression a robust readout for ligand-receptor engagement⁵⁶ (**Fig. 7a**). In line
833 with the presence of functional NKG2D ligands on mesenchymal cells, co-cultures with LDK-
834 resistant SH-SY5Y and SH-EP cells led to significant downregulation of NK cell surface-
835 associated NKG2D expression, whereas co-culture with the adrenergic parental SH-SY5Y cells
836 did not alter the abundance of surface NKG2D expression (**Fig. 7e**). Moreover, consistent with
837 the increased levels of NK degranulation upon co-culture with mesenchymal cells, LDK-resistant
838 SH-SY5Y were more susceptible to NK-induced cell death compared to parental SH-SY5Y cells
839 (**Supplementary Fig. 9a**)

840
841 The observation that genes encoding NKG2D ligands are repressed by the PRC2 complex in
842 adrenergic parental SH-SY5Y cells (**Supplementary Fig. 7c**) prompted us to examine whether
843 PRC2 inhibitors could induce the expression of these transcripts and influence NK cell function.
844 Indeed, treatment of parental SH-SY5Y cells with EED226, an allosteric inhibitor of the PRC2
845 complex⁵⁷, led to increased expression and surface localization of ULBP2/3 and MICA/B NKG2D
846 ligands (**Fig. 7f**). Moreover, such increased ligand expression led to their increased binding to the

847 NKG2D receptor fusion protein in EED226-treated cells compared to cells treated with DMSO
848 alone (**Fig. 7g**). Consequently, PRC2 inhibition resulted in an ~20% increase in NK cell
849 degranulation (**Fig. 7h**). Finally, co-culture of primary NK cells with adrenergic parental SH-SY5Y
850 cells treated with EED226 led to a significant loss of surface NKG2D receptor expression (**Fig.**
851 **7i**), suggesting that the increased degranulation resulting from PRC2 inhibition was driven by the
852 NKG2D receptor. Overall, these results suggest that the lineage-specific expression of NK cell
853 ligands in mesenchymal neuroblastoma cells has a functional impact on NK cell activity, and that
854 pretreatment of adrenergic neuroblastoma cells could potentially render these cells susceptible
855 to NK-cell mediated immunotherapy by upregulating ligand expression.

856 **DISCUSSION**

857 Despite the relatively poor track record of immunotherapy for neuroblastoma, growing evidence
858 suggests that subsets of these tumors have the potential to induce a productive immune
859 response⁹. Here, we demonstrate that the mesenchymal cell state, characterized by neural crest
860 cell (NCC)-like phenotypes, is a strong predictor of an antitumor immune response in
861 neuroblastoma. Induction of this state was accompanied by the expression of tumor cell-intrinsic
862 immune response-inducing genes that were epigenetically repressed in the more differentiated
863 (adrenergic) tumor cells. Importantly, inhibition of the PRC2 complex relieved such repression of
864 ligands for the activating NK cell receptor NKG2D, and led to NK cell degranulation, suggesting
865 that this strategy could be explored as a potential measure to improve the response of patients
866 with adrenergic neuroblastomas to NK cell-mediated therapy.

867

868 We sought to identify neuroblastomas capable of eliciting an immune response as those
869 characterized by the differential expression of immune gene signatures while remaining agnostic
870 to any of the established parameters that predict disease aggressiveness. Using UMAP
871 dimension reduction to analyze gene expression data from 498 primary neuroblastoma tumors,
872 we identified four clusters that were separated on the basis of differential activation of gene
873 networks that regulate the antitumor immune response, neuronal differentiation, MYCN-driven
874 processes and lipid metabolism. Intriguingly, the immunogenic cluster comprised almost equal
875 proportions of high- and low-risk tumors, raising the possibility that the molecular mechanisms
876 underlying immunogenicity in neuroblastoma are independent of disease aggressiveness. Thus,
877 our analysis using a cluster-based approach enabled the identification of antitumor immune
878 signatures as shared transcriptional programs between high- and low-risk neuroblastoma tumors,
879 a bridging feature that would have been missed in studies based solely on differential gene
880 expression between prognostically distinct groups of tumors.

881

882 A major finding of our study is the intimate link between tumor cell lineage and the propensity
883 of eliciting an immune response. Neuroblastoma tumors show lineage plasticity, underscored by
884 a phenotypic switch between undifferentiated NCC-like and more differentiated cells, two
885 divergent cell states driven by distinct transcriptional programs^{37,38}. Our analysis revealed that
886 neuroblastomas enriched in NCC-derived signatures showed significantly higher antitumor
887 immunity featuring T and NK cells compared to tumors enriched for signatures of the adrenergic
888 lineage. Our finding is substantiated by studies showing that diverse cellular states such as
889 stemness, senescence and metastasis strongly influence the engagement of innate and adaptive
890 immune pathways⁵⁸⁻⁶⁰. Importantly, while the NCC-like/mesenchymal state promoted immune
891 response mechanisms such as upregulation of MHC class I and infiltration of cytotoxic
892 lymphocytes, these tumors were also characterized by the activation of immune checkpoints such
893 as regulatory T cells and exhaustion markers linked to immune suppression, similar to those
894 observed in chronic virally infected states⁶¹. The presence of such seemingly contradictory gene
895 signatures has important therapeutic implications for selecting patients who are likely to benefit
896 from T cell-based immunotherapies, as agents that target negative regulatory immune
897 checkpoints are likely to be most effective in those with a pre-existing but damped antitumor
898 immune response³⁵. Moreover, inhibition of cancer cell-intrinsic transcriptional programs that
899 promote T cell exclusion have been shown to effect changes in cell state and could potentially
900 employed to sensitize tumors to immunotherapy (Jerby-Arnon et al., 2018; Koyama et al., 2016;
901 Spranger et al., 2015).

902

903 In contrast to other MYC-driven cancers where high MYC levels restrain inflammatory
904 signaling and anti-tumor immune pathways^{41,62}, tumors within our immunogenic cluster, enriched
905 largely for mesenchymal/NCC signatures, had relatively high MYC expression that positively
906 correlated with immune cell infiltration. These diametrically opposite roles of MYC in regulating
907 immune response suggest that its transcriptional functions are likely to be very different in cancers

908 in which aberrant MYC expression is the main oncogenic driver compared to *MYCN*-nonamplified
909 neuroblastomas that are not dependent on MYC overexpression. This notion is supported by the
910 observation that inhibiting endogenous MYC function in non MYC-driven pancreatic cancer
911 models leads to decreased recruitment and retention of inflammatory cells⁶³. As MYC is an
912 essential TF in NCCs^{64,65}, it is plausible that MYC promotes an immunogenic state by driving a
913 NCC-specific transcriptional program.

914

915 We also extended the analysis between cell state and immunogenicity to *MYCN*-amplified
916 tumors and identified a subset that incorporates both mesenchymal and immunogenic features.
917 This finding was substantiated by our data showing that expression of functional MHC class I is
918 retained in some *MYCN*-amplified mesenchymal neuroblastoma cells and is not perturbed by
919 changes in *MYCN* levels. Indeed, a robust antitumor immune response is observed during the
920 early stages of tumor development in Th-*MYCN* mice and in mouse-human chimeric tumors
921 derived from human NCCs expressing *MYCN* and oncogenic *ALK*¹⁷. Considering that these
922 mouse models are driven by gain of *MYCN* (~4-8 copies of the *MYCN* transgene)^{17,52} rather than
923 the amplification seen in human tumors, and that absolute levels of *MYCN* protein dictate
924 transcriptional output⁶⁶, the relatively low *MYCN* dosage in these tumors may account for their
925 immunogenicity. These findings, if confirmed in additional data sets, should encourage us to
926 reconsider the notion that all *MYCN*-amplified tumors are intrinsically immune tolerant and that a
927 subset may in fact, be capable of inducing an immune response by virtue of their cell state.

928

929 The mutual exclusivity of the neuronal and immunogenic clusters suggests that
930 neuroblastomas with characteristics of neuronal differentiation (i.e. adrenergic phenotype) are
931 weakly immunogenic and thus incapable of inducing an effective immune response. Moreover,
932 the presence of NCC-derived gene expression signatures among immunogenic tumors indicates
933 that phenotypic reversal of sympathetic neuronal cells to an NCC-like state could contribute

934 significantly to the antitumor immune response. Such cell state-dependent immunogenic
935 switching could be mediated by the lineage-specific core regulatory circuitry (CRC) that drives
936 distinct transcriptomic states in neuroblastoma. Indeed, several TFs that constitute the NCC-like
937 (mesenchymal) CRC, including interferon regulatory factors 1-3 and IFI16, function as major
938 drivers of tumor cell-intrinsic innate and adaptive immune responses^{67,68}. Furthermore, our data
939 identify PRRX1, another component of the mesenchymal CRC, as a regulator of MHC class I and
940 antigen-processing gene expression in neuroblastoma. Interestingly, PRRX1 was not identified
941 as a candidate regulator of MHC-I expression in genome-wide CRISPR knock-out screens to
942 identify NF-κB-dependent MHC-I suppressors in neuroblastoma⁶⁹, suggesting that the
943 mechanisms employed by PRRX1 could be independent of NF-κB activation and may involve
944 direct transcriptional activation of these genes. In addition, the upregulation of DNA damage
945 sensor proteins such as IFI16 and STING in mesenchymal neuroblastomas could also contribute
946 to the increased tumor-infiltrating lymphocyte abundance in these tumors, as suggested by results
947 in small cell lung cancer⁷⁰.

948

949 We have also established that the changes in immune gene expression accompanying the
950 adrenergic to mesenchymal transition are epigenetically regulated. Unlike lineage identity genes
951 that are regulated by super-enhancers, tumor cell-intrinsic immune genes involved in diverse
952 immune functions such as the inflammatory response, IFN-γ signaling and NK cell recognition,
953 are governed through changes in promoter structure, achieved by either *de novo* acquisition of
954 permissive chromatin or an epigenetic switch from PRC2-mediated repression to a permissive
955 chromatin landscape. Our findings support a role for the PRC2 complex in repressing genes that
956 encode ligands for the activating NK cell receptor NKG2D in adrenergic neuroblastoma cells and
957 the use of PRC2 inhibitors to augment the NK cell response against these cells. This approach is
958 justified on several grounds: our results add to the growing body of evidence for tumor-cell
959 autonomous function of PRC2 as a barrier to anti-tumor immunity, achieved through inhibition of

960 processes such as MHC expression^{11,71}, antigen processing and presentation and inflammatory
961 cytokine production^{72,73}. Moreover, our observation that NKG2D ligands are enriched in
962 mesenchymal neuroblastomas coupled with the demonstration of the critical effector role of NK
963 cells in the antitumor immune response against this tumor⁵¹ and the promising responses of
964 patients with other solid tumors to NKG2D-directed CAR NK cell therapy⁷⁴ strengthen this
965 premise. Considered together, the results of our analysis identify cell lineage as an important
966 determinant of the immune responsiveness of neuroblastoma and suggest rationales for the use
967 of immune-based therapies, either alone or in combination with epigenetic inhibitors, against the
968 two divergent phenotypes that define the lineage state of this pediatric tumor.

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988

989 **Author contributions**

990 Sa.S. and R.E.G. conceived the study. Sa.S., A.C., B.C.M., J.L., and R.E.G. designed the
991 experiments. Sa.S. performed the molecular, cellular and genomic studies. S.D. conceived and
992 performed the genomic and computational analysis with inputs from R.D., Sa.S. and R.E.G. Sa.S.,
993 A.C., and B.C.M. performed the T and NK cell studies. B.S. performed the animal and cloning
994 experiments. S.Z. performed quantitative analysis of IHC images. H.H. performed the cell
995 migration assays. M.K., D.N.D., and L.S. contributed to FACS analysis, generation of LDK378-
996 resistant SH-SY5Y cells, and compound testing, respectively. M.C., R.V., R.J., and St.S.
997 contributed ideas towards regulation of immune function and cell lineage state. Sa.S., S.D., and
998 R.E.G. wrote the manuscript with input from all authors.

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1003 **Competing Interests**

1004 R.J. is a cofounder of Fate Therapeutics, Fulcrum Therapeutics, and Omega Therapeutics and
1005 an advisor to Dewpoint Therapeutics.

1006 **References**

- 1007 1. Yu, A.L. *et al.* Anti-GD2 antibody with GM-CSF, interleukin-2, and isotretinoin for
1008 neuroblastoma. *N Engl J Med* **363**, 1324-34 (2010).
- 1009 2. Cheung, N.K. & Dyer, M.A. Neuroblastoma: developmental biology, cancer genomics and
1010 immunotherapy. *Nat Rev Cancer* **13**, 397-411 (2013).
- 1011 3. Singh, N. *et al.* T cells targeting NY-ESO-1 demonstrate efficacy against disseminated
1012 neuroblastoma. *Oncoimmunology* **5**, e1040216 (2016).
- 1013 4. Park, J.R. *et al.* Adoptive transfer of chimeric antigen receptor re-directed cytolytic T
1014 lymphocyte clones in patients with neuroblastoma. *Mol Ther* **15**, 825-33 (2007).
- 1015 5. Pule, M.A. *et al.* Virus-specific T cells engineered to coexpress tumor-specific receptors:
1016 persistence and antitumor activity in individuals with neuroblastoma. *Nat Med* **14**, 1264-
1017 70 (2008).
- 1018 6. Louis, C.U. *et al.* Antitumor activity and long-term fate of chimeric antigen receptor-
1019 positive T cells in patients with neuroblastoma. *Blood* **118**, 6050-6 (2011).
- 1020 7. Merchant, M.S. *et al.* Phase I Clinical Trial of Ipilimumab in Pediatric Patients with
1021 Advanced Solid Tumors. *Clin Cancer Res* **22**, 1364-70 (2016).
- 1022 8. Davis, K.L. *et al.* Nivolumab in children and young adults with relapsed or refractory solid
1023 tumours or lymphoma (ADVL1412): a multicentre, open-label, single-arm, phase 1-2 trial.
Lancet Oncol **21**, 541-550 (2020).
- 1025 9. Richards, R.M., Sotillo, E. & Majzner, R.G. CAR T Cell Therapy for Neuroblastoma. *Front
1026 Immunol* **9**, 2380 (2018).
- 1027 10. Bernards, R., Dessain, S.K. & Weinberg, R.A. N-myc amplification causes down-
1028 modulation of MHC class I antigen expression in neuroblastoma. *Cell* **47**, 667-74 (1986).
- 1029 11. Burr, M.L. *et al.* An Evolutionarily Conserved Function of Polycomb Silences the MHC Class
1030 I Antigen Presentation Pathway and Enables Immune Evasion in Cancer. *Cancer Cell* **36**,
1031 385-401 e8 (2019).
- 1032 12. Raffaghello, L. *et al.* Multiple defects of the antigen-processing machinery components in
1033 human neuroblastoma: immunotherapeutic implications. *Oncogene* **24**, 4634-44 (2005).
- 1034 13. Raffaghello, L. *et al.* Downregulation and/or release of NKG2D ligands as immune evasion
1035 strategy of human neuroblastoma. *Neoplasia* **6**, 558-68 (2004).
- 1036 14. Castriconi, R. *et al.* Identification of 4Ig-B7-H3 as a neuroblastoma-associated molecule
1037 that exerts a protective role from an NK cell-mediated lysis. *Proc Natl Acad Sci U S A* **101**,
1038 12640-5 (2004).
- 1039 15. Coughlin, C.M. *et al.* Immunosurveillance and survivin-specific T-cell immunity in children
1040 with high-risk neuroblastoma. *J Clin Oncol* **24**, 5725-34 (2006).
- 1041 16. Betancur, P.A. *et al.* A CD47-associated super-enhancer links pro-inflammatory signalling
1042 to CD47 upregulation in breast cancer. *Nat Commun* **8**, 14802 (2017).
- 1043 17. Cohen, M.A. *et al.* Formation of Human Neuroblastoma in Mouse-Human Neural Crest
1044 Chimeras. *Cell Stem Cell* **26**, 579-592 e6 (2020).
- 1045 18. Asgharzadeh, S. *et al.* Clinical significance of tumor-associated inflammatory cells in
1046 metastatic neuroblastoma. *J Clin Oncol* **30**, 3525-32 (2012).
- 1047 19. Song, L. *et al.* Valpha24-invariant NKT cells mediate antitumor activity via killing of tumor-
1048 associated macrophages. *J Clin Invest* **119**, 1524-36 (2009).

- 1049 20. Mao, Y. *et al.* Targeting Suppressive Myeloid Cells Potentiates Checkpoint Inhibitors to
1050 Control Spontaneous Neuroblastoma. *Clin Cancer Res* **22**, 3849-59 (2016).
- 1051 21. Tran, H.C. *et al.* TGFbetaR1 Blockade with Galunisertib (LY2157299) Enhances Anti-
1052 Neuroblastoma Activity of the Anti-GD2 Antibody Dinutuximab (ch14.18) with Natural
1053 Killer Cells. *Clin Cancer Res* **23**, 804-813 (2017).
- 1054 22. Brodeur, G.M., Seeger, R.C., Schwab, M., Varmus, H.E. & Bishop, J.M. Amplification of N-
1055 myc in untreated human neuroblastomas correlates with advanced disease stage. *Science*
1056 **224**, 1121-4 (1984).
- 1057 23. Seeger, R.C. *et al.* Association of multiple copies of the N-myc oncogene with rapid
1058 progression of neuroblastomas. *N Engl J Med* **313**, 1111-6 (1985).
- 1059 24. Layer, J.P. *et al.* Amplification of N-Myc is associated with a T-cell-poor microenvironment
1060 in metastatic neuroblastoma restraining interferon pathway activity and chemokine
1061 expression. *Oncoimmunology* **6**, e1320626 (2017).
- 1062 25. Wei, J.S. *et al.* Clinically Relevant Cytotoxic Immune Cell Signatures and Clonal Expansion
1063 of T-Cell Receptors in High-Risk MYCN-Not-Amplified Human Neuroblastoma. *Clin Cancer*
1064 *Res* **24**, 5673-5684 (2018).
- 1065 26. Brandetti, E. *et al.* MYCN is an immunosuppressive oncogene dampening the expression
1066 of ligands for NK-cell-activating receptors in human high-risk neuroblastoma.
1067 *Oncoimmunology* **6**, e1316439 (2017).
- 1068 27. Valentijn, L.J. *et al.* Functional MYCN signature predicts outcome of neuroblastoma
1069 irrespective of MYCN amplification. *Proc Natl Acad Sci U S A* **109**, 19190-5 (2012).
- 1070 28. Durbin, B.P., Hardin, J.S., Hawkins, D.M. & Rocke, D.M. A variance-stabilizing
1071 transformation for gene-expression microarray data. *Bioinformatics* **18 Suppl 1**, S105-10
1072 (2002).
- 1073 29. Lin, S.M., Du, P., Huber, W. & Kibbe, W.A. Model-based variance-stabilizing
1074 transformation for Illumina microarray data. *Nucleic Acids Res* **36**, e11 (2008).
- 1075 30. McInnes L, H.J. UMAP: Uniform manifold approximation and projection for dimension
1076 reduction. *arXiv preprint arXiv:1802.03426*. (2018).
- 1077 31. Becht, E. *et al.* Dimensionality reduction for visualizing single-cell data using UMAP. *Nat*
1078 *Biotechnol* (2018).
- 1079 32. Brodeur, G.M. *et al.* Revisions of the international criteria for neuroblastoma diagnosis,
1080 staging, and response to treatment. *Journal of Clinical Oncology* **11**, 1466-1477 (1993).
- 1081 33. Cohn, S.L. *et al.* The International Neuroblastoma Risk Group (INRG) classification system:
1082 an INRG Task Force report. *J Clin Oncol* **27**, 289-97 (2009).
- 1083 34. Satija, R., Farrell, J.A., Gennert, D., Schier, A.F. & Regev, A. Spatial reconstruction of single-
1084 cell gene expression data. *Nat Biotechnol* **33**, 495-502 (2015).
- 1085 35. Spranger, S. *et al.* Up-regulation of PD-L1, IDO, and T(regs) in the melanoma tumor
1086 microenvironment is driven by CD8(+) T cells. *Sci Transl Med* **5**, 200ra116 (2013).
- 1087 36. Wherry, E.J. & Kurachi, M. Molecular and cellular insights into T cell exhaustion. *Nat Rev*
1088 *Immunol* **15**, 486-99 (2015).
- 1089 37. Boeva, V. *et al.* Heterogeneity of neuroblastoma cell identity defined by transcriptional
1090 circuitries. *Nat Genet* **49**, 1408-1413 (2017).
- 1091 38. van Groningen, T. *et al.* Neuroblastoma is composed of two super-enhancer-associated
1092 differentiation states. *Nat Genet* **49**, 1261-1266 (2017).

- 1093 39. Bindea, G. *et al.* Spatiotemporal dynamics of intratumoral immune cells reveal the
1094 immune landscape in human cancer. *Immunity* **39**, 782-95 (2013).
- 1095 40. Wang, L.L. *et al.* Augmented expression of MYC and/or MYCN protein defines highly
1096 aggressive MYC-driven neuroblastoma: a Children's Oncology Group study. *Br J Cancer*
1097 **113**, 57-63 (2015).
- 1098 41. Casey, S.C. *et al.* MYC regulates the antitumor immune response through CD47 and PD-
1099 L1. *Science* **352**, 227-31 (2016).
- 1100 42. Cursons, J. *et al.* A Gene Signature Predicting Natural Killer Cell Infiltration and Improved
1101 Survival in Melanoma Patients. *Cancer Immunol Res* **7**, 1162-1174 (2019).
- 1102 43. Newman, A.M. *et al.* Robust enumeration of cell subsets from tissue expression profiles.
1103 *Nat Methods* **12**, 453-7 (2015).
- 1104 44. Hu, X. *et al.* Landscape of B cell immunity and related immune evasion in human cancers.
1105 *Nat Genet* **51**, 560-567 (2019).
- 1106 45. Ross, R.A., Spengler, B.A. & Biedler, J.L. Coordinate morphological and biochemical
1107 interconversion of human neuroblastoma cells. *J Natl Cancer Inst* **71**, 741-7 (1983).
- 1108 46. Cohn, S.L. *et al.* Prolonged N-myc protein half-life in a neuroblastoma cell line lacking N-
1109 myc amplification. *Oncogene* **5**, 1821-7 (1990).
- 1110 47. Debruyne, D.N. *et al.* ALK inhibitor resistance in ALK(F1174L)-driven neuroblastoma is
1111 associated with AXL activation and induction of EMT. *Oncogene* **35**, 3681-91 (2016).
- 1112 48. Chipumuro, E. *et al.* CDK7 inhibition suppresses super-enhancer-linked oncogenic
1113 transcription in MYCN-driven cancer. *Cell* **159**, 1126-1139 (2014).
- 1114 49. Durbin, A.D. *et al.* Selective gene dependencies in MYCN-amplified neuroblastoma
1115 include the core transcriptional regulatory circuitry. *Nat Genet* **50**, 1240-1246 (2018).
- 1116 50. Margueron, R. & Reinberg, D. The Polycomb complex PRC2 and its mark in life. *Nature*
1117 **469**, 343-9 (2011).
- 1118 51. Kroesen, M. *et al.* A transplantable TH-MYCN transgenic tumor model in C57Bl/6 mice for
1119 preclinical immunological studies in neuroblastoma. *Int J Cancer* **134**, 1335-45 (2014).
- 1120 52. Weiss, W.A., Aldape, K., Mohapatra, G., Feuerstein, B.G. & Bishop, J.M. Targeted
1121 expression of MYCN causes neuroblastoma in transgenic mice. *EMBO J* **16**, 2985-95
1122 (1997).
- 1123 53. Clarke, S.R. *et al.* Characterization of the ovalbumin-specific TCR transgenic line OT-I: MHC
1124 elements for positive and negative selection. *Immunol Cell Biol* **78**, 110-7 (2000).
- 1125 54. Cibrian, D. & Sanchez-Madrid, F. CD69: from activation marker to metabolic gatekeeper.
1126 *Eur J Immunol* **47**, 946-953 (2017).
- 1127 55. Uhrberg, M. The CD107 mobilization assay: viable isolation and immunotherapeutic
1128 potential of tumor-cytolytic NK cells. *Leukemia* **19**, 707-9 (2005).
- 1129 56. Molfetta, R. *et al.* Regulation of NKG2D Expression and Signaling by Endocytosis. *Trends*
1130 *Immunol* **37**, 790-802 (2016).
- 1131 57. Qi, W. *et al.* An allosteric PRC2 inhibitor targeting the H3K27me3 binding pocket of EED.
1132 *Nat Chem Biol* **13**, 381-388 (2017).
- 1133 58. Dongre, A. & Weinberg, R.A. New insights into the mechanisms of epithelial-mesenchymal
1134 transition and implications for cancer. *Nat Rev Mol Cell Biol* **20**, 69-84 (2019).
- 1135 59. Malladi, S. *et al.* Metastatic Latency and Immune Evasion through Autocrine Inhibition of
1136 WNT. *Cell* **165**, 45-60 (2016).

- 1137 60. Pereira, B.I. *et al.* Senescent cells evade immune clearance via HLA-E-mediated NK and
1138 CD8(+) T cell inhibition. *Nat Commun* **10**, 2387 (2019).
- 1139 61. Shin, H. & Wherry, E.J. CD8 T cell dysfunction during chronic viral infection. *Curr Opin*
1140 *Immunol* **19**, 408-15 (2007).
- 1141 62. Kortlever, R.M. *et al.* Myc Cooperates with Ras by Programming Inflammation and
1142 Immune Suppression. *Cell* **171**, 1301-1315 e14 (2017).
- 1143 63. Sodir, N.M. *et al.* Endogenous Myc maintains the tumor microenvironment. *Genes Dev*
1144 **25**, 907-16 (2011).
- 1145 64. Bellmeyer, A., Kruse, J., Lindgren, J. & LaBonne, C. The protooncogene c-myc is an
1146 essential regulator of neural crest formation in xenopus. *Dev Cell* **4**, 827-39 (2003).
- 1147 65. Rada-Iglesias, A. *et al.* Epigenomic annotation of enhancers predicts transcriptional
1148 regulators of human neural crest. *Cell Stem Cell* **11**, 633-48 (2012).
- 1149 66. Zeid, R. *et al.* Enhancer invasion shapes MYCN-dependent transcriptional amplification in
1150 neuroblastoma. *Nat Genet* **50**, 515-523 (2018).
- 1151 67. Chang, C.H., Hammer, J., Loh, J.E., Fodor, W.L. & Flavell, R.A. The activation of major
1152 histocompatibility complex class I genes by interferon regulatory factor-1 (IRF-1).
Immunogenetics **35**, 378-84 (1992).
- 1153 68. Unterholzner, L. *et al.* IFI16 is an innate immune sensor for intracellular DNA. *Nat*
1154 *Immunol* **11**, 997-1004 (2010).
- 1155 69. Spel, L. *et al.* Nedd4-Binding Protein 1 and TNFAIP3-Interacting Protein 1 Control MHC-1
1156 Display in Neuroblastoma. *Cancer Res* **78**, 6621-6631 (2018).
- 1157 70. Sen, T. *et al.* Targeting DNA Damage Response Promotes Antitumor Immunity through
1158 STING-Mediated T-cell Activation in Small Cell Lung Cancer. *Cancer Discov* **9**, 646-661
1159 (2019).
- 1160 71. Ennishi, D. *et al.* Molecular and Genetic Characterization of MHC Deficiency Identifies
1161 EZH2 as Therapeutic Target for Enhancing Immune Recognition. *Cancer Discov* **9**, 546-563
1162 (2019).
- 1163 72. Zingg, D. *et al.* The Histone Methyltransferase Ezh2 Controls Mechanisms of Adaptive
1164 Resistance to Tumor Immunotherapy. *Cell Rep* **20**, 854-867 (2017).
- 1165 73. Peng, D. *et al.* Epigenetic silencing of TH1-type chemokines shapes tumour immunity and
1166 immunotherapy. *Nature* **527**, 249-53 (2015).
- 1167 74. Xiao, L. *et al.* Adoptive Transfer of NKG2D CAR mRNA-Engineered Natural Killer Cells in
1168 Colorectal Cancer Patients. *Mol Ther* **27**, 1114-1125 (2019).
- 1169 75. Schindelin, J. *et al.* Fiji: an open-source platform for biological-image analysis. *Nat*
1170 *Methods* **9**, 676-82 (2012).
- 1171 76. Kamentsky, L. *et al.* Improved structure, function and compatibility for CellProfiler:
1172 modular high-throughput image analysis software. *Bioinformatics* **27**, 1179-80 (2011).
- 1173 77. Love, M.I., Huber, W. & Anders, S. Moderated estimation of fold change and dispersion
1174 for RNA-seq data with DESeq2. *Genome Biol* **15**, 550 (2014).
- 1175 78. Zhu, X. *et al.* Single-Cell Clustering Based on Shared Nearest Neighbor and Graph
1176 Partitioning. *Interdiscip Sci* **12**, 117-130 (2020).
- 1177 79. Russo, P.S.T. *et al.* CEMiTool: a Bioconductor package for performing comprehensive
1178 modular co-expression analyses. *BMC Bioinformatics* **19**, 56 (2018).
- 1179

- 1180 80. Yu, G., Wang, L.G., Han, Y. & He, Q.Y. clusterProfiler: an R package for comparing biological
1181 themes among gene clusters. *OMICS* **16**, 284-7 (2012).
- 1182 81. Alexopoulos, E.C. Introduction to multivariate regression analysis. *Hippokratia* **14**, 23-8
1183 (2010).
- 1184 82. Das, S. & Bansal, M. Variation of gene expression in plants is influenced by gene
1185 architecture and structural properties of promoters. *PLoS One* **14**, e0212678 (2019).
- 1186 83. Debruyne, D.N. *et al.* BORIS promotes chromatin regulatory interactions in treatment-
1187 resistant cancer cells. *Nature* **572**, 676-680 (2019).
- 1188
- 1189

1190 **Methods**

1191 **Cell culture**

1192 Human neuroblastoma (NB) cell lines (Kelly, NBL-S, CHP-212, SH-SY5Y, SH-EP, CHLA-20,
1193 NB69, SK-N-FI) were obtained from the Children's Oncology Group cell line bank. ACN, GI-ME-
1194 N, NB-EbC1 were kind gifts from A. Thomas Look and Kimberly Stegmaier at Dana Farber Cancer
1195 Institute (DFCI). NB-9464 cells were provided by To-Ha Thai at Beth Israel Deaconess Medical
1196 Center, Boston, MA. The cell lines were authenticated through STR analyses at the DFCI Core
1197 facility and were routinely tested for mycoplasma. All NB cells were grown in RPMI-1640 medium
1198 (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and 1%
1199 penicillin/streptomycin (Life Technologies). HEK293T cells obtained from the American Type
1200 Culture Collection (ATCC) were grown in DMEM (Invitrogen) supplemented with 10% FBS and
1201 1% penicillin/streptomycin (Life Technologies). SH-SY5Y cells resistant to the ALK inhibitor
1202 ceritinib (LDK378) were described previously (Debruyne et al., 2016) and were grown in complete
1203 RPMI-1640 in the presence of 1.5 µM ceritinib.

1204

1205 **Generation of PRRX1-inducible cell lines**

1206 Lentiviral vectors containing wild type and DNA-binding mutants of PRRX1 were generated by
1207 cloning cDNAs encoding full length or homeodomain deletions of the human PRRX1A sequence
1208 into the pLinducer20 lentiviral plasmid (gift from Stephen Elledge, Addgene plasmid #44012). The
1209 DNA-binding mutants harbor individual deletions of the three α -helices (Δ H1, Δ H2 and Δ H3) within
1210 the PRRX1 homeodomain (amino acids (aa) 94-153). Amino acid boundaries of the deleted
1211 regions are as follows: Δ H1 (aa 103-116); Δ H2 (aa 121-131); Δ H3 (aa 135-151). The lentivirus
1212 was packaged by co-transfection of pLinducer20 plasmid with the helper plasmids, pCMV-
1213 deltaR8.91 and pMD2.G-VSV-G into HEK293T cells using the TransIT-LT1 Transfection Reagent
1214 (Mirus Bio LLC). Virus-containing supernatants were collected 48 hr after transfection. SH-SY5Y
1215 cells were transduced with the viral supernatant in the presence of 8 µg/ml polybrene (Sigma-

1216 Aldrich) and 24 hr later selected using neomycin (G418) (5 µg/ml). Induction of gene expression
1217 was achieved by treating cells every 2–3 days with doxycycline (dox; 200 ng/ml) in RPMI-1640
1218 medium supplemented with 10% tetracycline-negative fetal bovine serum (tet-free FBS) (Gemini
1219 Bio-Products) and 1% penicillin/streptomycin.

1220

1221 **MYCN shRNA knockdown and IFN- γ treatment**

1222 The pLKO.1 shRNA construct targeting MYCN (TRCN0000020694) was purchased from Sigma-
1223 Aldrich and the pLKO.1 GFP shRNA was a gift from D. Sabatini (Addgene plasmid #30323).
1224 Lentiviral packaging was performed in HEK293T cells as described above and viral supernatant
1225 was collected on days 2 and 3 after transfection. Kelly human NB cells were transduced with the
1226 viral supernatant in the presence of 8 µg/ml polybrene (Sigma-Aldrich) and 24 h later selected
1227 using puromycin (1 µg/ml) for 2 days. Puromycin-resistant Kelly cells were cultured for an
1228 additional 6 days. Cells were then treated with 100 ng/ml of recombinant human IFN γ (Biolegend)
1229 for 24h, following which they were harvested for analysis of RNA and protein expression.

1230

1231 **FACS analysis for cell surface protein staining**

1232 For each staining reaction 1×10^6 live cells were placed in a 12 x 75 mm polystyrene round bottom
1233 tube (Falcon), resuspended in 100 µl 1x PBS and stained with the Zombie near-infrared (Zombie
1234 NIR) viability dye (BioLegend) at a 1:1,000 dilution for 15 minutes at RT. Cells were then washed
1235 once in FACS buffer (0.5% BSA in 1x PBS), resuspended in 100 µl of FACS buffer and incubated
1236 in 5 µl of Human TruStain FcX™ (Fc receptor blocking solution, BioLegend) for 10 minutes at RT.
1237 Next, appropriate volumes of conjugated fluorescent primary antibodies at predetermined
1238 optimum concentrations were added and incubated on ice for 20 minutes in the dark. Cells were
1239 then washed once in 2 ml of FACS buffer by centrifugation at 1500 rpm for 5 minutes. All FACS
1240 samples were analyzed on a FACSCalibur flow cytometer (Becton Dickinson) using Cell Quest
1241 software (Becton Dickinson). A minimum of 50,000 events was counted per sample and used for

1242 further analysis. Data were analyzed using FlowJo v10 software (Becton Dickinson). The following
1243 primary antibodies were used: PE-HLA (Biolegend; clone W6/32), PE-MICA/B (Biolegend clone
1244 6D4), PE-ULBP2 (R&D Systems; clone 165903), PE-ULBP3 (R&D Systems; clone 166510), PE-
1245 H-2K^b (Biolegend AF6-88.5), PE-H-2K^b SIINFEKL (Biolegend; clone 25-D1.16), PE-mouse
1246 IgG2a κ isotype control (Biolegend MOPC-173), PE- mouse IgG1 κ isotype control (MOPC-21).

1247

1248 **Cell viability assay**

1249 SH-SY5Y parental and SH-SY5Y LDK-resistant cells were seeded in 96-well plates at a density
1250 of 2×10^3 cells/well. After 24 h, cells were treated with increasing concentrations of LDK378
1251 (ranging from 1 nM to 10 μM) dissolved in Dimethyl Sulfoxide (DMSO). DMSO solvent without the
1252 drug served as a negative control. Following 72 h incubation, cells were analyzed for viability
1253 using the CellTiter-Glo Luminescent Cell Viability Assay (Promega) according to the
1254 manufacturer's instructions. Drug concentrations that inhibited cell growth by 50% (IC_{50}) were
1255 determined using a non-linear regression curve fit with GraphPad Prism 8 software.

1256

1257 **Cell migration and invasion assays**

1258 Cell migration was measured using transwell chambers (Falcon). NB9464-H-2K^b^{lo} or
1259 NB9464-H-2K^b^{hi} cells in serum-free medium (0.5×10^6 cells/ml) were added to the upper
1260 chamber and inserts (8 μm pore size) were placed in the lower chamber containing medium
1261 with 10% FBS. Following incubation at 37° C for 8 h, cells that migrated to the lower chamber
1262 were fixed with methanol and stained with crystal violet (Sigma-Aldrich). The stained cells
1263 were photographed with a light microscope at 100X magnification and migration was
1264 quantified as the number of cells per high power field. Cell invasion was measured using the
1265 fluorometric QCM™ ECMatrix™ Cell Invasion Assay (Millipore). NB9464-H-2K^b^{lo} or NB9464-
1266 H-2K^b^{hi} cells in serum-free medium (0.5×10^6 cells/ml) were added to the upper chamber

1267 and inserts (8 µm pore size) placed in the lower chamber containing medium with 10% FBS.

1268 Following incubation at 37° C for 24 h, cell invasion was measured according to
1269 manufacturer's instructions.

1270

1271 **EED226 treatment**

1272 5 x10⁵ SH-SY5Y cells were seeded into 10 cm plates and treated with either 5 µM EED226
1273 (Selleck Chemicals) or DMSO (vehicle control) for 6-8 days, following which samples were
1274 harvested for downstream analyses. Cells were replenished with fresh media containing DMSO
1275 or EED226 every 2 days.

1276

1277 **Compounds**

1278 Ceritinib (LDK378) and EED226 were purchased from Selleck Chemicals. Doxycycline and
1279 dimethyl Sulfoxide (DMSO) was purchased from Sigma-Aldrich.

1280

1281 **RNA extraction and q-PCR**

1282 Total RNA was isolated using the RNAeasy Mini kit (Qiagen). Purified RNA was reverse
1283 transcribed to cDNA using Superscript IV VILO master mix (Thermo Fisher Scientific) following
1284 the manufacturer's protocol. Quantitative PCR was performed using 1 µl cDNA, 1x PowerTrack
1285 SYBR Green PCR master mix (Thermo Fisher Scientific) and PCR primers (200 nM) in a total
1286 volume of 25 µl and analyzed on a ViiA 7 Real-Time PCR system (Thermo Fisher Scientific). Each
1287 individual biological sample was amplified in technical duplicate and normalized to GAPDH as an
1288 internal control. Relative expression was calculated according to the 2^{-ΔΔCT} quantification method
1289 (Livak and Schmittgen, 2001). PCR primer sequences are shown in Table S5.

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1293 **Synthetic RNA spike-in and RNA-sequencing**

1294 RNA-sequencing was performed on the following human NB cell lines: Kelly, NBL-S, CHP-212,
1295 SH-SY5Y, SH-SY5Y LDK-resistant and SH-EP. Biological duplicates (5×10^6 cells per replicate)
1296 were homogenized in 1 ml of TRIzol Reagent (Invitrogen) and purified using the mirVANA miRNA
1297 isolation kit (Ambion) following the manufacturer's instructions. Total RNA was treated with DNA-
1298 free™ DNase I (Ambion), spiked-in with ERCC RNA Spike-In Mix (Ambion) and analyzed on an
1299 Agilent 2100 Bioanalyzer (Agilent Technologies) for integrity. Sequencing libraries were prepared
1300 using LP-KAPA mRNA Hyper Prep and sequenced using Illumina HiSeq for 40 bases.

1301

1302 **Western blotting**

1303 Cells were homogenized in NP40 lysis buffer (Life Technologies) containing 1x cOmplete EDTA-
1304 free protease inhibitor cocktail and 1x PhosSTOP (Roche). Protein concentration was measured
1305 using the DC Protein Assay (Bio-Rad). 100 µg total protein was denatured in LDS sample buffer
1306 (Invitrogen), separated on pre-cast 4-12% Bis-Tris gels (Invitrogen) and transferred to
1307 nitrocellulose membranes (Bio-Rad). Membranes were blocked using 5% dry milk (Sigma-Aldrich)
1308 in Tris-buffered saline (TBS) supplemented with 0.2% Tween-20 (TBS-T) for 1 hr, and incubated
1309 overnight with primary antibody in blocking buffer at 4 °C. Chemiluminescent detection was
1310 performed with appropriate HRP-conjugated secondary antibodies and enhanced
1311 chemiluminescence reagents (Thermo Scientific). Images were developed using Genemate Blue
1312 ultra-autoradiography film (VWR).

1313

1314 **Antibodies**

1315 The following primary antibodies were used: MYCN (Cat #51705), MYC (13987), GATA3 (5852),
1316 TAP1 (12341), TAP2 (12259), LMP7 (13635), NOTCH1 (3608), cleaved NOTCH1 (4147), SOX9
1317 (82630), AXL (8661), GAPDH (2118), β-actin (3700), IRF1 (8478), VIM (5741), YAP1 (4912),

1318 TAZ1 (4883) (Cell Signalling Technologies (CST)); PHOX2B (Abcam;183741), FN1 (RnD
1319 systems; AF1918), LMP2 (Santa Cruz; 271354) and PRRX1 (Santa Cruz; 293386).

1320

1321 **Chromatin immunoprecipitation-quantitative PCR (ChIP-qPCR)**

1322 Soluble chromatin was prepared as above from SH-SY5Y cells without or with dox-inducible
1323 PRRX1 expression (200 ng/ml dox for 10 days). ChIP was performed as described in the
1324 preceding section using the following antibodies: H3K4me3 (Abcam 8580), H3K27me3 (Millipore
1325 07-729), EZH2 (CST 5246), SUZ12 (CST 3737), EED (Millipore 17-10034), rabbit IgG (CST
1326 2729). Purified ChIP DNA was dissolved in 60 µl of 1x TE. Quantitative PCR was performed on a
1327 ViiA 7 Real-Time PCR system (Thermo Fisher Scientific) with 1 µl purified DNA, 1x PowerTrack
1328 SYBR Green PCR master mix (Thermo Fisher Scientific) and PCR primers (200 nM) against the
1329 genomic regions of interest. Each individual biological sample was amplified in technical
1330 duplicate. Relative enrichment was quantified using the percent input method. PCR primer
1331 sequences are shown in Table S5.

1332

1333 **Chromatin immunoprecipitation-sequencing (ChIP-seq)**

1334 Approximately 10-12 × 10⁷ cells were crosslinked with 1% formaldehyde (Thermo Scientific) for
1335 10 min at room temperature (RT) followed by quenching with 0.125 M glycine for 5 min. The cells
1336 were then washed twice in ice-cold 1x Phosphate Buffered Saline (PBS), and the cell pellet
1337 equivalent of 4 × 10⁷ cells were flash frozen and stored at -80°C. Crosslinked cells were lysed in
1338 lysis buffer 1 (50 mM HEPES-KOH pH7.5, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP40,
1339 0.25% Triton X-100). The resultant nuclear pellet was washed once in lysis buffer 2 (10 mM Tris-
1340 HCl pH 8, 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA) and then resuspended in sonication buffer
1341 (50 mM HEPES-KOH pH 7.5, 140 mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100, 0.1%
1342 sodium deoxycholate, 0.2% SDS). Chromatin was sheared using a Misonix 3000 sonicator
1343 (Misonix) and at the following settings: 10 cycles, each for 30 s on, followed by 1 min off, at a

1344 power of approximately 20 W. The lysates were then centrifuged for 15 min at 4 °C, supernatants
1345 collected and diluted with an equal amount of sonication buffer without SDS to reach a final
1346 concentration of 0.1% SDS. For each ChIP, the chromatin equivalent of 1×10^7 cells was used.
1347 50 µl of Protein G Dynabeads per sample (Invitrogen) were blocked with 0.5% BSA (w/v) in 1x
1348 PBS. Magnetic beads were loaded with the following antibodies: 10 µg of H3K27me3 (Millipore
1349 07-729); 3 µg of H3K27ac (Abcam 4729), and 3 µg of H3K4me3 (Abcam 8580) and incubated
1350 overnight at 4°C. The sonicated lysates were then incubated overnight at 4°C with the antibody-
1351 bound magnetic beads, washed with low-salt buffer (50 mM HEPES-KOH (pH 7.5), 0.1% SDS,
1352 1% Triton X-100, 0.1% sodium deoxycholate, 1 mM EGTA, 1 mM EDTA, 140 mM NaCl and 1x
1353 complete protease inhibitor), high-salt buffer (50 mM HEPES-KOH (pH 7.5), 0.1% SDS, 1% Triton
1354 X-100, 0.1% sodium deoxycholate, 1 mM EGTA, 1 mM EDTA, 500 mM NaCl and 1x complete
1355 protease inhibitor), LiCl buffer (20 mM Tris-HCl (pH 8), 0.5% NP-40, 0.5% sodium deoxycholate,
1356 1 mM EDTA, 250 mM LiCl and 1x complete protease inhibitor) and Tris-EDTA (TE) buffer. DNA
1357 was then eluted in elution buffer (50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 1% SDS), and high-
1358 speed centrifugation performed to pellet the magnetic beads and collect the supernatants. The
1359 crosslinking was reversed overnight at 65° C in the presence of 300 mM NaCl. RNA and protein
1360 were digested using RNase A and proteinase K, respectively, and DNA was purified with phenol-
1361 chloroform extraction and ethanol precipitation. Purified ChIP DNA was used to prepare Illumina
1362 multiplexed sequencing libraries using the NEBNext Ultra II DNA Library Prep kit and the
1363 NEBNext Multiplex Oligos for Illumina (New England Biolabs) according to the manufacturer's
1364 protocol. Libraries with distinct indices were multiplexed and run together on the Illumina NextSeq
1365 500 (SY-415-1001, Illumina) for 75 base pairs.

1366

1367 **IFN-gamma induction and antigen presentation in NB9464 cells**

1368 Approximately 1×10^6 cells were seeded onto 10 cm plates. 24 hr later, adherent cells were
1369 treated with recombinant mouse IFN-γ (Biologend) for 24h and harvested for H-2Kb analysis

1370 using FACS as described above. For antigen presentation assays, cells treated with IFN- γ were
1371 pulsed with SIINFEKL (OVA peptide) at 37°C. Cells were subsequently washed with 1x PBS to
1372 remove unbound peptide and processed for analysis using FACS.

1373

1374 **T cell activation assays**

1375 OT-I T cell receptor (TCR) transgenic mice were purchased from Jackson Laboratories (Bar
1376 Harbor, ME). Splenocytes were harvested and T cells were subsequently isolated from the
1377 mononuclear layer using Ficoll separation and directly used in co-culture assays. Successful
1378 enrichment of CD8 $^{+}$ T cells was confirmed by FACS analysis using the FITC-CD8 antibody. Cells
1379 were pulsed with SIINFEKL (OVA peptide) at 37 °C, to bind to cell surface H-2Kb. Cells were
1380 subsequently washed with 1x PBS to remove unbound peptide and then co-cultured with
1381 unstimulated OT-1 T cells for 24 h. OT-I cells were then harvested, and sequentially stained with
1382 the Zombie NIR viability dye and FITC-CD8, PE-CD69 antibodies, followed by fixation with 1%
1383 paraformaldehyde (Polysciences, Inc). OT-1 cells were analyzed by flow cytometry using a
1384 FACSCanto II cell analyzer (Becton Dickinson) and FlowJo V10 software (Becton Dickinson).

1385

1386 **In vitro assays for NKG2D binding**

1387 SH-SY5Y parental, SH-SY5Y LDK-resistant, and SH-EP cells were incubated with recombinant
1388 human NKG2D-Fc chimeric protein or an equivalent concentration of human IgG , following which
1389 cells were washed, and sequentially stained with the Zombie NIR viability dye as described above
1390 followed by incubation with an Alexa 647-conjugated anti-human IgG antibody for 30 minutes.
1391 Cells were washed in FACS buffer and analyzed by flow cytometry using a FACSCanto II cell
1392 analyzer (Becton Dickinson) and FlowJo V10 software (Becton Dickinson).

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1396 **NK cell degranulation assays**

1397 Human peripheral blood NK cells were isolated from blood collars using a RosetteSep™ human
1398 NK cell enrichment cocktail (STEMCELL Technologies). NK cells were then co-cultured for 4 h
1399 with confluent monolayers of SH-SY5Y parental, SH-SY5Y LDK-resistant, and SH-EP cells at in
1400 the presence of CD107a antibody (Biolegend). Additionally, co-cultures of NK cells and 721.221
1401 B cells were included as positive controls for degranulation. At the endpoint, NK cells were
1402 harvested, stained with Zombie Yellow (Biolegend) and CD56 FITC (or NKp46 AlexaFluor 647™)
1403 and NKG2D PE (or mouse IgG1 PE) antibodies (Biolegend), followed by fixation with 1%
1404 paraformaldehyde (Polysciences, Inc). For the NKG2D blocking assay, NK cells were incubated
1405 with purified anti-NKG2D antibody or mouse IgG1 isotype control at 37 °C, following which the
1406 degranulation assay was performed as detailed above. NK cells were analyzed by flow cytometry
1407 using a FACS Canto II (Becton Dickinson) and FlowJo V10 software (Becton Dickinson).

1408

1409 **Data sets**

1410 Publicly available RNA-seq data (GEO accession number GSE49711/GSE62564) from a cohort
1411 of 498 primary human neuroblastoma tumors, microarray expression data from 394
1412 neuroblastoma tumors (GSE120572) and 24 human neuroblastoma cell lines (GSE28019) were
1413 accessed through the R2 genomics analysis and visualization platform
1414 (<https://hgserver1.amc.nl/cgi-bin/r2/>). Clinical annotations for tumors were obtained from
1415 GSE49711/GSE62564 regarding MYCN status (MYCN-nonamplified vs. MYCN-amplified, INSS
1416 stage [high (stage 4) vs. low (1, 2, 3 and 4s], risk status (high vs. low) and age (< 18 months vs.
1417 ≥ 18 months).

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1422 **Analysis of RNA-sequencing data**

1423 **RNA-seq data processing and identification of differentially expressed genes**

1424 Single-end RNA-seq samples with 40 base pair (bp) read lengths were mapped to the human
1425 genome (GRCh38) and ERCC spike-in sequences. Reads were mapped to the genome using
1426 Bowtie2 (version 2.3.4.3) and default parameters. Reads that overlapped with the genomic
1427 location for exonic regions were used to calculate gene counts with the FeatureCounts (Subread
1428 package of version 1.6.3) package. Further, spike-in read counts were used for each sample to
1429 normalize the library sizes. These read counts were used to calculate the sample-specific size
1430 factor by using the function estimateSizeFactors (DESeq2) available in R. Normalized sample
1431 coverage profiles were then created from previously determined size factors by using
1432 bamCoverage (DeepTools v3.0.2) and parameters “--scaleFactor --skipNonCoveredRegions”. To
1433 check the reproducibility of biological replicates for each condition, principal component analysis
1434 (PCA) and correlation (Spearman’s rank coefficient) were assessed from the sample coverage
1435 profiles at genome-wide scale and visualized using scatterplots and heatmaps. Because these
1436 analyses showed a high correlation of sample coverage profiles between replicates, replicates
1437 were merged using samtools merge and processed again as described for the individual
1438 replicates. Next, differential gene expression analysis was performed using the DESeq2 in R. To
1439 detect differentially expressed genes (DEGs) in each sample, raw read counts from RNA-seq
1440 data were imported to the DESeq2 and the size factors calculated using the estimateSizeFactors
1441 function. A transcript with an absolute log₂ fold-change ≥ 1.5 and an adjusted *P*-value ≤ 0.01 was
1442 considered significant.

1443

1444 **Enrichment analysis**

1445 Gene ontology enrichment for selected gene sets was performed by the Enrichr program
1446 (<https://amp.pharm.mssm.edu/Enrichr/>). All GO terms were ranked based on the Enrichr
1447 combined score, calculated by multiplying the adjusted *P*-value with the z-score using the Fisher’s

1448 exact test. The Fisher's exact test was used to determine significant overlaps between the queried
1449 gene sets and other publicly available datasets. Enrichment of gene sets was considered
1450 significant for an adjusted P-value ≤ 0.01 , unless stated otherwise.

1451

1452 **Estimation of immune cell content in neuroblastoma tumors**

1453 Cell type identification by estimating relative subsets (CIBERSORT)⁴³, a deconvolution method
1454 was used to evaluate immune cell fractions from gene expression data using the R package
1455 'immunedeconv'. RNA transcript estimations were generated for all 498 neuroblastoma tumors
1456 using the LM22 signature matrix available for 22 immune cell types. CIBERSORT was run in
1457 "Absolute mode" with disabled quantile normalization as recommended for tumor RNA-seq data
1458 and the overall immune content produced by the algorithm compared among tumors.

1459

1460 **ChIP-seq analysis**

1461 **Data processing-** All ChIP-seq raw datasets were processed as previously described⁸³. The raw
1462 read quality of the samples was accessed using the Fastqc tool (v0.11.7) to identify possible
1463 sequencing errors and biases. Reads were aligned to the human genome (build hg19,
1464 GRCh37.75) using the mapper Bowtie (v2.3.4.3) with default parameters. Unique and non-
1465 duplicate reads that mapped to the reference genome were further processed using Samtools
1466 (v1.9) and the MarkDuplicates (v2.1.1) command of Picard tools. Next, antibody enrichment in
1467 each replicate as compared to input samples was verified using the PlotFingerprint command of
1468 deepTools (v3.1.1). Peak caller MACS2 (2.1.1) was used to identity narrow peaks (H3K4me3 and
1469 H3K27ac) with the parameters "--q 0.01--call-summits" and broad peaks (H3K27ac and
1470 H3K27me3) with the parameters "--broad-cutoff 0.01". Peaks that overlapped with black-listed
1471 regions (<http://mitra.stanford.edu/kundaje/akundaje/release/blacklists/>) of the reference genome
1472 (mostly comprised of major satellite repeats of telomeric and pericentromeric regions) were
1473 filtered out. Command "bamCompare" from the deepTools was used with the parameters "--

1474 scaleFactorsMethod readCount --binSize 40 --operation subtract --smoothLength 80 --
1475 extendReads 200" to create the input normalized bedgraph tracks for each replicate and
1476 afterwards negative values were set to zero and counts were scaled to reads per million/base pair
1477 (rpm/bp) to account for differences in the library size. Bigwig files were created for visualization
1478 with bedGraphToBigWig. Subsequently, correlations among the ChIP-seq replicates were
1479 accessed using bigwigs with the command "multiBigwigSummary" from deepTools and highly
1480 correlated replicates merged at the BAM level. Peak identifications were then repeated in the
1481 same manner for these merged BAM files.

1482

1483 **Identification of super-enhancer regions-** Super-enhancers (SEs) were identified using the
1484 ROSE algorithm (https://bitbucket.org/young_computation/rose/src/master/). Briefly, H3K27ac
1485 binding regions identified by MACS2 as significant peaks, termed typical enhancers, were stitched
1486 together if they were within 12.5 kb of each other. These stitched enhancers were ranked by
1487 comparing the H3K27ac signal (density * length) with the input signal. The ROSE algorithm was
1488 used to determine the inclination point for all stitched H3K27ac signals and to segregate regular
1489 enhancers from SEs. To compare SEs in 5Y-parental, 5Y-LDK-resistant and SH-EP cells, the
1490 same maximum threshold was used between the conditions.

1491

1492 **Analysis of histone binding changes between lineage states-** To analyze the changes in
1493 occupancies of active (H3K27ac and H3K4me3) and repressive (H3K27me3) histone marks
1494 during the transition from adrenergic (5Y-par.) to mesenchymal (5Y-LDK-res., SHEP) states, we
1495 compared the peaks of histone marks identified by MACS2 at the promoter regions. For this
1496 purpose, we first extracted the promoter regions \pm 2 kb with respect to the TSS (-2kb upstream
1497 to +2kb downstream) of all annotated protein coding genes and subsequently, retrieved the peaks
1498 of H3K27ac, H3K4me3 and H3K27me3 from 5Y-par., 5Y-LDK-res. and SH-EP cells. Now, to
1499 determine the differential binding of each histone mark between 5Y-par. and 5Y-LDK-res. cells,

1500 we first combined all significant peaks called by MACS2 at the promoter regions and merged the
1501 peak regions that overlapped by at least 50%. This 50% threshold was used to avoid merging
1502 peaks that had clear and distinct summits. Next, the normalized active or repressive histone
1503 marks read densities were calculated for each region and a ratio of [\log_2 (5Y-LDK-res./ 5Y-par.)]
1504 was calculated. Shared peaks had similar enrichment of either active or repressive histone marks
1505 in both the cell types. Similar comparisons were made for active or repressive histone marks
1506 between 5Y-par. and SH-EP cells. To further compare changes in all histone marks at the
1507 promoters of immune genes, the gain of significant H3K27ac, H3K4me3 binding and loss of
1508 H3K27me3 signals were listed in mesenchymal (5Y-LDK-res., SHEP) cells as compared to
1509 adrenergic (5Y-par.) cells.

1510

1511 **Integrated analysis of histone binding and gene expression-** Cell-type specific differential
1512 enrichment of H3K27me3 and H3K4me3 binding in 5Y-par., 5Y-LDK-res. and SH-EP cells was
1513 determined by calculating the \log_2 (H3K4me3+1/H3K27me3+1) ratios in the promoter regions
1514 (TSS \pm 2 kb) of immune genes. Next, to examine the association between gene expression and
1515 differential enrichment of H3K27me3 and H3K4me3 binding in immune genes in 5Y-par., 5Y-LDK-
1516 res. and SH-EP cells, genes were ranked based on their expression values and plotted against
1517 the calculated ratios between the histone marks.