



Published in final edited form as:

*Nat Rev Cancer*. 2013 June ; 13(6): 397–411. doi:10.1038/nrc3526.

## Neuroblastoma: Developmental Biology, Cancer Genomics, and Immunotherapy

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### Abstract

Neuroblastoma is a solid tumor that arises from the developing sympathetic nervous system. Over the past decade, our understanding of this disease has advanced tremendously. The future challenge is to apply the knowledge gained toward developing risk-based therapies and ultimately improving outcome. Here we review the key discoveries in the developmental biology, molecular genetics, and immunology of neuroblastoma, as well as new translational tools to bring these promising scientific advances into the clinic.

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Neuroblastoma (NB) is a rare childhood cancer affecting 10.2 per million children under 15 years of age, and the most common malignancy diagnosed before the first birthday<sup>1</sup>. As a complex and heterogeneous disease,<sup>2</sup> many factors, such as age at diagnosis, stage of disease at diagnosis, and the molecular, cellular, and genetic features of the tumor determine whether it will spontaneously regress or metastasize and become refractory to therapy. Over the past decade, major advances in the clinical staging of NB have improved risk stratification<sup>3</sup>. However, not enough is known about how these disease features relate to its underlying biology and how this can be exploited to improve outcome. Our challenge is to bridge the gap between characterizing the molecular and genetic properties of NB and understanding the precursor cells that give rise to NB, focusing on those features that make the cells susceptible to malignant transformation.

In the past decade the major effort has been focused on discovering somatic mutations in human tumors. Targeting therapy at tumor-specific mutations holds promise of precision

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#### Conflicts of Interest

MSKCC has a patent application on hu3F8 and NKC was named as one of the inventors. MSKCC has licensed the patent on beta-glucan to Biotec Pharmacon, and the patent on antibody 8H9 to United Therapeutics, and NKC was named as one of the inventors for both agents. Clinical trials of hu3F8 are funded by the NCI (NKC) and the DOD (NKC).

and effectiveness in eradicating cancer, while sparing patients the acute and long term toxicities of chemo-radiotherapy. However, genome-wide searches are uncovering striking differences in the prevalence of mutations among tumor types, from very frequent among melanomas to rare among pediatric cancers such as NB<sup>4-5</sup>. The infrequency of mutations<sup>4-6</sup> is a major disappointment for those looking for actionable targets from gene mutations and an increasingly apparent hurdle for others hunting for tumor-specific immunity. In adult cancers like melanoma, the rich epitope landscape<sup>7</sup>, or mutanome<sup>8</sup>, has been successfully exploited for T-cell based therapy. But in NB with a small mutanome, the classic immunotherapy model may be difficult to apply. Antibody-based instead of T-cell-based therapy directed at oncofetal differentiation antigens has provided a viable alternative.

Despite this paucity of recurrent somatic mutations, NB is a complex, heterogeneous disease<sup>2</sup>. As the search for druggable targets continues, a better understanding of the developmental biology of this tumor may offer new insights. Many cellular processes that guide tissue morphogenesis and differentiation have parallel functions in cancer. For example, tumor cells from the same patient can be remarkably heterogeneous and change dramatically during disease progression. This is reminiscent of progenitor cell heterogeneity and unidirectional changes in progenitor competence in developing tissues and organs. As in normal developing cells, tumor cells are sensitive to non-cell autonomous influences and require a precise balance between differentiation and proliferation for growth and homeostasis. Also, like rapidly growing embryonic tissues and organs, tumors are metabolically tuned for biosynthesis and often evade cell death machinery to proliferate massively. Thus, developmental biology and cancer biology are natural partners, though integrating the two fields for therapeutic applications can be daunting.

In this review we will update our current understanding of the neural crest and cellular origins of NB. We will review the normal differentiation and physiology of the sympathetic neurons, highlighting potential actionable targets unique to NB. The clinical success of anti-ganglioside GD2 [G] antibody therapy in the face of an immunosuppressive tumor microenvironment is analyzed. Looking ahead, we propose a comprehensive translational research roadmap that takes advantage of high throughput drug screening, new generations of animal models, and study designs to mimic real clinical settings. We will not discuss modern evolutions of chemotherapy including those in the myeloablative[G] setting, which have been summarized extensively by other investigators<sup>9</sup>.

## Neural crest origin of neuroblastoma

Most NBs are diagnosed in the abdomen, associated with the adrenal gland [G] or sympathetic ganglia [G]<sup>1-2</sup>. Based on these common sites of primary disease and the cellular and neurochemical features of NBs, it is widely accepted that the cell origin for NB arises from the sympathoadrenal lineage of the neural crest during development (Figure 1)<sup>10</sup>.

The neural crest is a remarkable structure that is present only during embryogenesis and gives rise to diverse cell types including peripheral neurons, enteric neurons and glia, melanocytes, Schwann cells, and cells of the craniofacial skeleton and adrenal medulla<sup>11</sup>.

Cells arising in the adrenal medulla are postganglionic neurons that have lost their dendrites and axons. Preganglionic neurons from the central nervous system (CNS) connect directly to adrenal medulla cells and stimulate the release of catecholamines (i.e., epinephrine, norepinephrine, and dopamine). Thus, the adrenal medulla is a ganglion of the sympathetic nervous system. Most NBs arise in the adrenal medulla (65%). The rest is distributed among the chest (20%), neck (5%), and pelvis (5%), a pattern similar to that of normal sympathetic ganglia in the thoracic/lumbar (16%), cervical (3%), and sacral (4%) regions. A small subset of patients presents with bilateral adrenal NB<sup>12</sup>, suggesting that transformation can be initiated in the neural crest, before the cells migrate. Alternatively, it is possible that patients with bilateral NB had a predisposing genetic lesion and the bilateral tumors result from two independent genetic lesions in the cells of the left and right sympathoadrenal lineage as predicted by Knudson and Strong<sup>13</sup>. Whole genome sequencing of paired bilateral NB tumors could distinguish between these two possibilities.

### Familial neuroblastoma

Familial NB is rare (<2% of all NBs)<sup>14</sup>. Mutations in some of the signalling pathways (Figure 1) important for the development of the sympathoadrenal lineage are associated with familial genetic syndromes characterized by defects in development and predisposition to NB<sup>15</sup>. The first predisposition mutation identified in NB was in *PHOX2B*<sup>16-17</sup>, a gene encoding a paired homeodomain transcription factor that promotes cell cycle exit and neuronal differentiation<sup>18-19</sup>, playing a critical role in the development of neural crest-derived autonomic neurons. *PHOX2B* has two polyalanine repeat sequences. Expression of the second polyalanine repeat is associated with congenital central hypoventilation syndrome (CCHS)[G]<sup>20</sup>, while non-polyalanine repeat expansion mutations (NPARMs) accompany the NB-HSCR (Hirschsprung disease)[G]-CCHS association. Thus, perturbations in the *PHOX2B*-regulated differentiation pathway in the sympathoadrenal lineage of the neural crest may contribute to NB tumorigenesis, and subsequent gene expression studies support that hypothesis<sup>21</sup>. Yet, when NPARMS were introduced into the endogenous *Phox2b* allele of the mouse, even though most clinical features of HSCR and CCHS were recapitulated, those of NB were not<sup>22</sup>.

A more common lesion associated with familial NB is in the *ALK* receptor tyrosine kinase gene<sup>14, 23-25</sup> (Figure 2). Its known natural ligands include pleiotrophin and midkine. *ALK* is expressed in the developing sympathoadrenal lineage of the neural crest<sup>26-27</sup>, and it may regulate the balance between proliferation and differentiation through multiple cellular pathways, including the mitogen activated protein kinase (MAPK) and Ras-related protein 1 (RAP1) signal transduction pathways<sup>28-30</sup>. In addition there is evidence to suggest that *PHOX2B* can directly regulate *ALK* gene expression<sup>31</sup> providing a connection between these two pathways that are mutated in familial NB. Furthermore, *ALK* signaling through midkine may be important for proliferation of the sympathoadrenal lineage during development<sup>32</sup>. The *ALK*-activating mutation F1174L found in some cases of familial NB contributes to NB tumorigenesis in mice<sup>33-35</sup>.

Ongoing genome-wide association studies (GWAS) have identified additional pathways that contribute to NB initiation and/or progression. Several predisposing single nucleotide

polymorphisms (SNPs) have been identified: *LINC00340* and *LOC729177 (FLJ44180)*, *BARD1*<sup>36</sup>, *LMO1*<sup>37</sup>, *DUSP12*, *HSD17B12*, *DDX4-IL31RA*<sup>38</sup>, *HACE1*<sup>39</sup>, and *LIN28B*<sup>39–40</sup>. The *BARD1* SNP effect has been confirmed in African-American children<sup>41</sup>, while *BARD1*β isoform<sup>42</sup> and *LIN28*<sup>43</sup> are oncogenic in NB.

### Sporadic neuroblastoma

While mutations of *PHOX2B* and the GWAS predisposing loci are relatively rare in sporadic NB, approximately 6–10% of NBs carry somatic *ALK* activating mutations, and an additional 3–4% carry high level *ALK* gene amplifications<sup>4, 14, 23–25</sup>. These findings in familial and sporadic NB suggest that *ALK* is a major oncogenic driver in NB, and activating *ALK* mutations or amplifications are associated with lethal disease.<sup>33, 44</sup> *ALK* is a natural target for molecular therapy in preclinical studies and clinical trials for NB<sup>45</sup>.

However, the most common focal genetic lesion in sporadic NB is the amplification of *MYCN* ( $\geq 10$  copies for diploid genome or  $>4$  fold signal relative to chromosome 2), which occurs in approximately 22% of tumors and is associated with poor outcome<sup>2</sup>. *MYCN* regulates the proliferation, growth, differentiation, and survival of cells in the developing CNS. It is expressed in the developing neural crest, and several signalling pathways regulate its expression (e.g., Hh and Wnt)<sup>46</sup>. Ectopic expression of *MYCN* in the sympathoadrenal lineage, under the regulation of the tyrosine hydroxylase promoter, is sufficient to drive NB tumorigenesis in zebrafish<sup>47</sup> and in the mouse<sup>48</sup>. Although *MYCN* is a major oncogenic driver in NB and it has been extensively studied for nearly 3 decades<sup>49</sup>, there are currently no clinical trials targeting the *MYCN* protein directly in NB because of the difficulties inherent to developing molecular targeted therapies to transcription factors. However, recent efforts have focused on targeting signaling pathways that are deregulated as a result of elevated *MYCN* activity such as aurora kinase A<sup>50</sup> and bromodomain/extra-terminal (BET) family of proteins<sup>51</sup>.

*ATRX* mutations are among the most common lesions in sporadic NB. *ATRX* encodes a SWI/SNF chromatin-remodelling ATP-dependent helicase (Figure 2)<sup>5, 52</sup> and *ATRX* mutations are associated with X-linked mental retardation (XLMR) and  $\alpha$ -thalassemia, suggesting that *ATRX* functions in various developmental processes. However, little is known about how *ATRX* contributes to the development or differentiation of the sympathoadrenal lineage. Children with XLMR do not have an increased incidence of NB, suggesting that *ATRX* mutations alone are not sufficient to promote tumorigenesis. However, there is an important association between *ATRX* mutations and age at diagnosis of NB<sup>52</sup>. Very young children ( $<18$  months of age) with stage 4 disease tend to have a better prognosis than their older counterparts, and no *ATRX* mutations have been identified in this age group. *ATRX* mutations occur in 17% of children aged 18 months to 12 years with stage 4 disease, and in 44% of patients older than 12 years who uniformly have a very poor prognosis. The relationship between age at diagnosis and *ATRX* mutations is statistically significant<sup>52</sup>, but analysis of the prognostic significance of *ATRX* mutations will require a much larger study. To date, *ATRX* mutations have not been identified in tumors with *MYCN* amplification<sup>5, 52</sup>.

The relationship of *ATRX* mutation and abnormal telomere highlights the importance of telomere content in NB. Cancer cells must maintain telomeres for survival<sup>53</sup>. One mechanism of maintaining telomeres in NB is through increased expression of telomerase. Telomerase, composed of an RNA template (hTR) and a catalytic subunit (hTERT), elongates telomeric repeats at chromosomal ends<sup>53</sup>. Telomerase activity is strongly associated with hTERT and hTR expression<sup>54</sup>. High telomerase activity is found in 30% of NB at diagnosis and predicts reduced event-free survival[G] and overall survival (OS) in multivariate analyses<sup>55–56</sup>. However, telomere length does not necessarily correlate with telomerase activity<sup>57</sup>. In some tumor cells, a homologous recombination based mechanism of telomere maintenance and elongation is activated. This alternative lengthening of telomere (ALT) pathway is often identified by the presence of longer telomeres and large ultrabright telomere signal in tumor cells using telomere fluorescence in situ hybridization. Most of the tumors with *ATRX* mutations have evidence of ALT and it is possible that this is a direct effect of defects of histone H3.3 deposition at telomeres. *ATRX* loss in somatic cell hybrids segregate with ALT activation<sup>58</sup>, but whether *ATRX* functions in epigenetic processes that are important for sympathoadrenal lineage development or NB differentiation remains unknown. While both males and females were found to have *ATRX* mutations in the original analyses, additional studies will be required to determine if there is any gender bias for ALT mediated through *ATRX* mutations in NB. *ATRX* mutations have not yet been modelled in the mouse, and no molecular therapies have yet targeted this pathway.

By whole-genome sequencing (WGS), recurrent genetic lesions have been reported in the Rac/Rho pathway<sup>4–5</sup> as well as chromatin-remodelling genes *ARID1A* and *ARRID1B*<sup>59</sup>. Additional studies will be required to elucidate the role of these genes in NB initiation and/or progression and the prognostic significance of disruptions in these pathways. Taken together, these genomic studies have identified few recurrent genetic lesions in druggable pathways. The major new challenge we face is to further elucidate the underlying biology of the tumors to identify deregulated developmental, epigenetic or metabolic pathways that can be exploited therapeutically for patients with high-risk or recurrent NB.

### Neuronal differentiation in neuroblastoma

Analyses of familial NB, polymorphisms by GWAS, and recurrent somatic mutations by WGS have greatly enriched our understanding of NB, and in some cases, identified valuable targets for therapy. Another bridge is the unique physiology associated with the neuronal differentiation of the sympathoadrenal lineage. A valuable connection between cellular differentiation and tumorigenesis has come from the Shimada histology-grading system for NB, where the degree of differentiation, the Schwannian stromal content[G], and mitotic-karyorrhexis index[G] help stratify patients into risk groups<sup>60</sup>. The seminal observation that neuronal differentiation is driven by retinoids in vitro<sup>61</sup> has led to isotretinoin (13-*cis*-retinoic acid) becoming the standard of care for high-risk NB<sup>62</sup>. Another connection between neuronal differentiation and tumorigenesis is highlighted by the identification of GD2 on neuronal stem cells<sup>63</sup>, the neuroblastic but not the glial lineage<sup>64</sup>, and on NB tumors<sup>65–66</sup>, which can provide further insight into using oncofoetal differentiation antigens as targets for antibody-based therapy (see below).

Another important clue about the relationship between normal differentiation and NB origins came from secreted catecholamine metabolites in patients' urine. Catecholamines are found in the cells of the adrenal medulla and paraspinal ganglia<sup>67</sup>, and NB cells often have dense core vesicles (DCVs) wherein catecholamines are stored. Most catecholamine metabolism occurs in these cells as a result of leakage from the DCVs<sup>67–68</sup>. Meta-iodobenzylguanidine (MIBG), an analogue of norepinephrine, is readily taken up by NB cells and a fraction of it is stored in DCVs<sup>69</sup>. Thus, radioiodinated MIBG is used for diagnostic (<sup>123</sup>I-MIBG) and therapeutic (<sup>131</sup>I-MIBG) purposes<sup>70</sup>.

The metabolism of catecholamines in the adrenal medulla differs from that in the sympathetic ganglia. In sympathetic neurons 90% of the dopamine is converted to norepinephrine by dopamine β-hydroxylase and stored in DCVs<sup>67</sup>. The remaining dopamine is oxidized by monoamine oxidase (MAO) to DOPAL, a toxic catecholaldehyde. DOPAL is rapidly detoxified to DOPAC by aldehyde dehydrogenase. When norepinephrine or epinephrine is leaked from DCVs into cytoplasm, it gets metabolized by MAO to DOPEGAL, another toxic aldehyde. In contrast, the adrenal medulla cells express catechol-O-methyltransferase (COMT) in addition to MAO. Here, the norepinephrine or epinephrine gets metabolized by COMT to metanephrines, or by MAO to DOPEGAL, unless it is actively transported back into the DCVs by vesicular monoamine transporters (VAMTs). While there are some differences in the metabolism of catecholamines in adrenal medulla and sympathetic ganglia, both cell types rely upon the storage of catecholamines in DCVs by VAMTs.

VAMTs belong to an evolutionarily conserved family of genes that includes transporter proteins involved in multidrug resistance (MDR) in cancer cells<sup>71</sup>. The driving force for VAMTs is a hydrogen electrochemical gradient produced by an ATP-dependent vesicular proton pump<sup>67</sup>; any change in ATP production or intracellular pH can tilt this delicate balance against the tumor cell and may provide novel therapeutic approaches for NB. For example, ouabain is a Na,K-ATPase inhibitor that lowers intracellular pH and perturbs the electrochemical gradient that sequesters catecholamines in DCVs, thereby reducing the viability of NB cells<sup>72</sup>. These observations highlight how a deeper understanding of NB's unique cellular physiology and metabolism can lead to new therapeutics that are not directly related to any particular genetic lesion.

## Chromosomal instability and tumor heterogeneity

A patient's age and stage of disease at diagnosis and the presence of *MYCN* amplification in NB cells are the three strongest determinants of clinical outcome<sup>3</sup>. These determinants, combined with the loss of chromosome 11q, histologic properties, and ploidy, are now the foundations of risk-group stratification for patients with NB. Not surprisingly, NB is much more heterogeneous when examined at the genetic level. As with other cancers, nongenetic heterogeneities (e.g., epigenetic or differentiation state) also influence risk<sup>74–75</sup>. However, how heterogeneity evolves with treatment and disease progression remains unknown.

In general, low risk, intermediate risk and stage 4s NBs (Table 1) have numerical chromosomal gains while high risk NBs have intrachromosomal rearrangements<sup>1</sup>. The



incidence of these chromosomal aberrations increases with age at diagnosis and is strongly prognostic of outcome<sup>5, 52, 56</sup>. Together with oncogene amplifications, these large-scale genomic alterations may lead to deregulation of messenger RNAs, micro RNAs and other non-coding RNAs that interfere with apoptosis, differentiation, and immune surveillance<sup>76–78</sup>. In short, connecting clinical behavior to these complex molecular/genetic profiles is an ongoing challenge.

Genomic instability drives human genome evolution, both in healthy and disease states, and is common in many forms of cancer<sup>79</sup>. Hyperdiploidy reflects chromosome-segregation failure during mitosis<sup>80</sup>. Telomere dysfunction, DNA-repair defects, and chromothripsis are possible mechanisms of genome instability. Defects in the p53 pathway, loss of genes mapping to 1p and 11q, or gains of genes mapping to 1q and 17q may also play a role<sup>56</sup>.

DNA index in the hyperdiploid[G] range, typical for low risk NB, confers favorable outcome<sup>81</sup>. However, we do not know whether hyperdiploidy is simply a genomic biomarker or whether some mechanism links ploidy, NB-cell differentiation, and outcome. Patients whose high risk tumors harbor either large segmental chromosomal lesions fare much worse<sup>82</sup>. Established markers of poor prognosis include losses of 1p<sup>83</sup> and 11q<sup>84</sup> and gain of 17q<sup>85</sup>; losses of 3p, 4p, 9p, and 14q and gain of 1q, 2p, 7q, and 11p have also been implicated<sup>86</sup>. The loss of 11q is associated with an older age at diagnosis, absence of *MYCN* amplification and more chromosomal breaks. Because breakpoints occur on multiple chromosomes, they probably reflect an underlying defect in DNA maintenance or repair<sup>82</sup> or elevated levels of double-stranded DNA breaks as a result of the unique cellular physiology of NB.

Chromosome instability is a dynamic process that cannot be accurately measured at a single time point. It is important to distinguish between dynamic processes that reflect the continuous accumulation of genetic lesions and more acute genomic events such as multiple chromosome trisomies in hyperdiploid NB or chromothripsis<sup>87</sup>, the acute shattering of genomic regions. Chromothripsis, which occurs in 2% to 3% of human cancers, may result from the uncoupling of DNA replication between individual chromosomes in micronuclei and those in the nucleus. If a chromosome in the micronucleus is still replicating its DNA when the cell enters mitosis, a massive break can occur that is limited to that chromosome. In a WGS study of NB, the investigators identified structural variants consistent with chromothripsis in 18% of high-risk NB<sup>5</sup>. In a separate study of 40 stage 4 NBs, there was only one tumor with evidence of chromothripsis<sup>52</sup>. Thus, additional studies are required to elucidate the frequency and significance of chromothripsis in NB.

## Immunology and Immunotherapy

Because NBs can spontaneously regress, de novo anti-tumor immunity in patients seems logical. However, an active adaptive immunity against NB has been difficult to demonstrate especially in high-risk patients. This is not unexpected, given the exceptionally large tumor bulk (both primary and metastatic) and their rapid proliferation that overwhelm the immature immune system in a child. Beyond the paucity of somatic mutations making NB poorly immunogenic, this tumor has developed a sophisticated immunosuppressive

microenvironment to ensure that no effective T-cell immunity can develop or accomplish its functions.

### **NB escapes the immune system**

NB cells evade T-cells and natural killer (NK) cells by down-regulating human leukocyte antigen (HLA)<sup>88–89</sup> and adhesion molecules<sup>90–91</sup>. They express or release proteins to inhibit<sup>92–94</sup> and to kill, T-cells and NK cells<sup>97</sup>. They even recruit tissue macrophages to disable these lymphocytes<sup>98</sup>. NB cells carry on their cell surface high levels of ganglioside and sialic acid-containing sugars and proteins<sup>65</sup>, which are important for migration, adhesion and metastasis<sup>99</sup>. These highly negatively-charged carbohydrate epitopes are poorly immunogenic<sup>100</sup> and sometimes even immunosuppressive<sup>101</sup>. Natural antibodies against NB are rare, with the possible exception of IgM<sup>102</sup>. Natural anti-ganglioside antibodies are even rarer, thereby allowing NB to survive in circulation despite their lack of complement decay accelerating factor (CD55)[G]<sup>103</sup>. In addition, NB can exploit protectin (CD59)[G] to resist complement-mediated lysis<sup>104</sup>. While NB downregulate HLA to escape T-cells, ironically they can also re-express HLA to resist NK cell mediated antibody-dependent cell mediated cytotoxicity (NK-ADCC)[G] when treated with MAbs<sup>105–106</sup>. This duplicity highlights the plasticity of NB as treatment pressures are applied. Clinically, NB evades the immune system by escaping to sanctuaries such as the CNS, which is not accessible to circulating antibodies. In fact, the increasing frequency of isolated relapses in the CNS, soft tissues (e.g., lymph nodes), and bone (despite having the marrow space in remission) signals a new curative challenge in the era of MAb therapy<sup>106</sup>.

### **NB vaccines to stimulate T-cell mediated immunity**

Given these escape mechanisms and the complexity of the T-cell mediated immunity<sup>107</sup>, constructing an effective T-cell vaccine is daunting. NB antigens recognizable by cytotoxic T lymphocytes (CTLs)<sup>108</sup> exist; they include cancer-testes antigens (MAGE and NY-ESO-1<sup>109</sup>), MYCN<sup>110</sup>, and survivin<sup>89</sup>. However, despite compelling evidence that T-cell vaccines using these antigens can be effective in syngeneic mouse models<sup>111–112</sup>, clinical success has been limited<sup>113</sup>. Dendritic cells as T-cell vaccines are still early in clinical testing<sup>114</sup>. Until the patient's weak T-cell immunity following high-dose chemotherapy can be recharged, strategies to circumvent T-cell blockades (e.g., anti-CTLA4 and anti-PD-1<sup>115</sup>) may be ineffective.

### **Anti-GD2 MAb**

The discovery that high-risk patients with NB can be maintained in continual remission with anti-GD2-specific MAb therapy was unexpected and is rapidly becoming the standard of care after 2 decades of research (Figure 3). GD2 belongs to a unique class of T-cell independent carbohydrate antigens with high density<sup>66</sup>, membrane proximity, homogeneity within and across NBs, and rare occurrence of antigen loss<sup>116</sup>. As an oncofetal differentiation antigen, it is expressed during fetal development and in mature neurons, pain fibers, and skin cells<sup>117</sup>. Two intravenous (iv) anti-GD2 IgG antibodies have been tested in the clinic, chimeric 14.18 (ch14.18) and mouse 3F8 (Table 2). Ch14.18 combined with iv interleukin-2 (IL-2) and iv granulocyte-macrophage colony-stimulating factor (GM-CSF),



and oral 13-*cis*-retinoic acid (CRA), was proven efficacious in a randomized trial<sup>118</sup>. 3F8 plus subcutaneous GM-CSF and oral CRA without IL-2 in a single arm study showed a 5-year PFS of 62% and OS of 81% among patients with high-risk stage 4 NB treated in first remission<sup>106</sup>; although without a randomized placebo control, the efficacy cannot be certain. Because of the pain side effects, the anti-GD2 MAb dose is limited, and efficacy has only been observed to date in patients with minimal residual disease (MRD), while rarely seen in patients with bulky NB. Persistent MRD early during immunotherapy was highly predictive of ultimate treatment failure<sup>106</sup>. Granulocyte-mediated ADCC[G]<sup>119</sup> and NK-ADCC<sup>105</sup> are important effector mechanisms.

A better understanding of the immunology of anti-GD2 MAb therapy should help explain its success and offer future directions. Anti-GD2 MAb does much more than passively attaching tumor cells to NK cells; they rescue NK cells from being suppressed or inhibited by NB. Normally, inhibitory receptors on NK cells (e.g., killer cell immunoglobulin-like receptors [KIRs]) [G] efficiently brake the network of synergizing activating receptors<sup>120</sup> until Fc $\gamma$ RIII (CD16)-mediated signaling releases that brake. Since KIRs and HLA (the cognate KIR ligands) genes are polymorphic and segregate independently; some individuals (up to 60%) have KIRs on their NK cells with no corresponding HLA ligands during maturation<sup>121–122</sup>. These cells with missing inhibitory KIR ligands are “uneducated” and hyporeactive until their CD16 is activated during ADCC. On the other hand, “educated” NK cells are restrained by HLA which can be upregulated by cytokines released during ADCC. This restraint by HLA explains why patients with “missing inhibitory KIR ligands” have better outcome following anti-GD2 MAb therapy<sup>105–106, 121–122</sup>.

Like NK cells, myeloid effectors are also awakened by anti-GD2 MAb. Granulocyte-ADCC of NB is unique among cancers and is clinically important<sup>106, 123</sup>. It does not depend on oxidative intermediates<sup>124</sup>, requires azurophil (primary) granule exocytosis<sup>123</sup>, and is enhanced by GM-CSF<sup>123, 125</sup>. Among Fc $\gamma$ Rs, Fc $\gamma$ RIIA (CD32) is the receptor for ADCC on granulocytes<sup>123, 126</sup> and its affinity for MAb correlates with patient outcome<sup>122, 127</sup>. Besides Fc $\gamma$ R, complement receptors CR3 and CR4 on granulocytes are critical adhesion molecules for this type of ADCC<sup>123, 126, 128</sup>. One of their natural ligands is membrane-bound C3bi, a breakdown product of C3, resulting from complement activation on NB cells<sup>128</sup>. When activated, CR3 acquires the conformational neoepitope CBRM1/5<sup>129</sup>, and this is correlated with better patient survival<sup>119</sup>, especially in the presence of high-dose GM-CSF administered subcutaneously<sup>106</sup>.  $\beta$ -glucan binds and activates CR3, and when administered orally, enhances antibody therapy of neuroblastoma<sup>130</sup>. Tumor-associated macrophages (TAMs) represent another class of myeloid effector gaining prominence in NB research<sup>131</sup>. Macrophage migration inhibitory factor regulates NB growth, angiogenesis, and metastasis<sup>132</sup> and is associated with dedifferentiation in *MYCN* amplified tumors<sup>133</sup>. Depending on the microenvironment, TAMs can become polarized into type 1 antitumor or type 2 pro-tumor phenotypes<sup>134</sup>. In the presence of anti-GD2 MAb, ADCC can turn pro-tumor M-CSF-activated macrophages in vitro into efficient antitumor “killing machines”<sup>135</sup>.

By activating ADCC to kill NB, anti-GD2 MAb is most efficient when effector cell populations and functions are amplified by cytokines. ADCC requires leukocytes, which are typically depleted following induction chemotherapy or autologous stem cell transplants.

Since NK cells and granulocytes are effectors for ADCC, the cytokines IL-2 and GM-CSF are obvious candidates for combination with MAb. IL-2, which activates NK cells, natural killer T (NKT) cells, T-cells, and the undesirable regulatory T-cells (T<sub>reg</sub>)[G]<sup>136</sup>, has a modest anti-NB effect as a single agent<sup>137</sup>. Unlike GM-CSF, IL-2 is associated with substantial toxicity (e.g., 23% of patients experienced capillary leak<sup>118</sup>). Similar to IL-2, IL-15 activates NK, NKT, and CD8+ T-cells<sup>138</sup>. However, it does not cause capillary leak, activation-induced cell death, or increased T<sub>reg</sub> activation in non-human primate studies<sup>139</sup>. Another type of anti-NB lymphocytes called NKT cells<sup>140</sup> can infiltrate NB, kill TAMs<sup>96</sup>, and they are associated with superior patient survival<sup>96, 141</sup>. However, for NKT cells to survive the hypoxic NB environment, IL-15 is essential<sup>98</sup>. Immunocytokines derived from humanized 14.18 (hu14.18-IL-2<sup>142</sup> or hu14.18-GM-CSF<sup>123</sup>) are genetic fusion proteins of anti-GD2 antibody and cytokines targeted to tumor sites as immune stimulants when applied systemically<sup>143</sup> or locally<sup>144</sup>. Despite compelling data in syngeneic mouse models<sup>145</sup>, intravenous hu14.18-IL-2 produced bone marrow remission only if NB was minimal<sup>143</sup>, and no benefit was seen for soft-tissue tumors<sup>143, 146</sup>. The clinical advantage of hu14.18-IL-2 over ch14.18 plus IL-2 awaits confirmation because grades 3/4 capillary leak with abnormal liver functions have been seen in both<sup>118, 143</sup>. These considerations provide further rationale to consider IL-15 as an alternative to IL-2 for combination with MAb in NB.

Even though MAb therapy is often regarded as passive immunotherapy, the induction of a host anti-tumor or anti-idiotypic[G] network following MAb therapy may be important for long term tumor control<sup>147</sup>. Human anti-mouse antibody response (HAMA), an indirect measure of the host anti-idiotypic network, was consistently correlated with long term survival<sup>106, 147</sup>. Because of these observations, anti-idiotypic vaccines such as mouse IgG1 antibody 1A7 specific for ch14.18<sup>148</sup> and rat IgG1 antibody A1G4 specific for 3F8 seem logical<sup>149</sup>. The GD2 peptide mimotope<sup>150</sup> and its DNA vaccine<sup>151</sup> can induce serum antibodies and protective anti-GD2 IgG responses in mice. However, in contrast to whole-antigen vaccines, single-epitope vaccines will probably be limited by their narrow target coverage. The whole GD2 antigen has also been conjugated to keyhole limpet hemocyanin (KLH) to overcome the poor immunogenicity of carbohydrates<sup>100</sup>.

Besides its utility as a target to direct leukocyte-mediated killing, GD2 is also ideal for tumor-selective delivery of radioisotopes, liposomes, or nanoparticles<sup>70</sup>. However, the pain side effect<sup>106, 118</sup>, thought to be a consequence of complement activation<sup>152</sup>, is a major limitation of anti-GD2 MAb therapy. Mutating the Fc region to reduce complement activation<sup>152</sup> and using blocking antibodies<sup>153</sup> have decreased but not eliminated pain. Humanized 3F8 (hu3F8)<sup>154</sup> has low immunogenicity, and deglycosylating hu3F8 can further decrease complement activation and possibly its pain side effects. For immunoconjugates that do not require Fc-effector functions, a pain-free targeting agent is ideal. Other potential NB cell surface targets include GD3, ALK<sup>155</sup>, polysialic acid<sup>108</sup>, L1CAM<sup>156</sup> and B7-H3<sup>157</sup>. B7-H3-specific MAb 8H9 has been used successfully for compartmental radioimmunotherapy of NB metastasis to the CNS<sup>158</sup>.

### Adoptive T-cell therapy

The new paradigm of including T-lymphocytes in MAb therapy of NB is taking shape. Although autologous tumor-reactive T-cells are rare in patients with NB and fail to home to tumor sites<sup>89</sup>, genetically redirected T-cells (e.g., using chimeric antigen receptors [CAR] hold great promise<sup>159–160</sup>. CAR connects a single-chain variable fragment (scFv) (anti-GD2) with a T-cell intracellular-signaling domain. When they were virally transfected into activated T-cells (ATC), clinical benefit was seen in NB<sup>160</sup>. CARs built with anti-L1CAM (MAb CE7) have been tested for NB, although the persistence of these ATCs was short-lived ( $\leq 42$  days)<sup>159</sup>. To enhance their survival, Epstein-Barr virus-specific CTLs (instead of polyclonal ATC), which are life-long from continuing antigenic challenge, have been successfully used<sup>160</sup>. These dual-specific CTLs (anti-Epstein-Barr virus through the natural T-cell receptor and anti-GD2 through the CAR) can persist and control tumors for years<sup>160</sup>. Additional possible genetic modifications include luring T-cells with chemokine receptors<sup>141, 161</sup> and reducing T<sub>reg</sub> interference by turning up the Akt pathway<sup>162</sup>. Similar strategies of adoptive cytotherapy with NK cells and NKT cells are being explored following their expansion ex vivo using engineered IL-15<sup>163</sup> or IL-21<sup>164</sup>.

### The future of NB immunotherapy

The current immunotherapy strategy uses anti-GD2 MAb to direct the traffic of FcR-bearing NK cells and granulocytes, stimulating them through the FcR CD16 and CD32, respectively. By activating through CD3, T-cell engaging bispecific antibody (anti-GD2 x anti-CD3) will exploit T-cells that do not carry FcR<sup>165</sup>, a compartment of underused effectors with potentials for long term anti-tumor immunity. Since the Fc function is not required for these bispecific MAb and therefore dispensable, the pain side effects of anti-GD2 antibodies could potentially be eliminated. A rational integration of anti-tumor MAb, effector cells, and cytokines to induce tumor cell death, while combining with small molecules to prevent death evasion, can be tested in the appropriate mouse models to guide ongoing and future clinical trials.

### Translational Model

Genetic and epigenetic aberrations carried by high-risk NB are continually exploited by these tumors to survive the selective pressures of competing nutrients, hypoxia, immune surveillance and cytotoxic therapy. The latter two prescriptions have successfully been applied to NB, with glimpses of cure but not for all patients. With cytotoxic therapy, increasing dose and dose intensity have been explored<sup>166–167</sup>, and could be further maximized with better supportive care. Small molecules that target specific genetic and epigenetic aberrations are rapidly transitioning into the clinic for NB (see Table 2). *ALK* is an exemplary tumor target for NB therapy<sup>45</sup>, ahead of other promising gene candidates (Figure 2).

Translational and clinical research of pediatric cancers is fundamentally different from that of adult cancers, because there are relatively few patients, and clinical trials are often carried out by cooperative groups. Also, drug metabolism, acute toxicities, and late effects vary greatly between children and adults. Most pediatric preclinical cancer studies use cell lines

or flank xenografts in immunocompromised mice<sup>168</sup>. For immunologic analysis, mouse effectors are poor human surrogates for testing monoclonal antibodies (MAbs)<sup>169</sup>; humanized mice (Table 3), i.e., those grafted with human immune cells, should be more appropriate<sup>170–171</sup>. Many research groups incorporate genetically engineered mouse models<sup>172</sup>, yet few use comprehensive preclinical studies to directly guide clinical trials<sup>173</sup>. For example, most studies are short-term (2–3 weeks), using drug doses and schedules that are not clinically relevant. They often fail to incorporate combination chemotherapy, and do not have an appropriate benchmark (e.g., current standard of care). Most preclinical studies also lack statistical design, appropriate randomization of animals to treatment regimens, or a mechanism to “blind” researchers to treatments received by study cohorts. An ideal translational research “roadmap” (Box 2) should engage a multidisciplinary team comprising clinical researchers, laboratory-based scientists, chemists, pharmacologists, and biostatisticians.

With the exception of *ALK*, genomic characterization of NB has provided few leads for druggable pathways that can be directly moved into clinical trials. We propose that a multipronged approach will be required to improve outcomes for NB patients over the next decade. As presented here, neuroendocrine differentiation of NBs can be exploited in two ways. First, epigenetic processes are important for coordinating proliferation and differentiation during development. In other pediatric cancers with features of neuronal differentiation such as retinoblastoma, epigenetic profiling provided important new insights into tumor initiation and progression and led to the identification of novel therapeutic approaches<sup>174</sup>. Therefore, epigenetic profiling of NBs may lead to the identification of differentiation or cancer pathway that can be targeted in the clinic. Second, it may be possible to exploit the unique features of NB differentiation with respect to catecholamine biosynthesis, transport and metabolism by using therapeutics to force intracellular accumulation of toxic catechoaldehydes or other perturbations. Another approach is to identify novel therapeutics using an unbiased high-throughput drug screening as recently used for pediatric brain tumors<sup>175</sup>. For such approaches, it is essential to have validated cell-based screening assays that faithfully recapitulate the molecular, cellular and genetic heterogeneity of NB found in patients. While small molecules used in single-agent targeted strategies will provide proof of principle, their curative potential in NB will likely require exploiting multiple pathways, and careful integration with active death induction through either chemoradiotherapy or immunotherapy.

## Overall conclusion

We have witnessed tremendous advances in our understanding of the genetics and biology of NB that will greatly improve the precision in the stratification of this complex heterogeneous disease. There have also been remarkable advances in our understanding of how NB tumor cells evade the immune system, and some of the most promising therapeutic approaches for NB now involve immunotherapy. With the ability to further refine the classification of patients accurately into risk groups<sup>3</sup>, the challenge we face in the next decade is to identify the most effective and least toxic therapy for these patient subgroups. The focus must now shift toward multidisciplinary translational research teams using validated preclinical models to select the most promising combinations of molecular

targeted therapies, broad-spectrum chemotherapy and immunotherapy to cure patients who currently have a poor prognosis and reduce therapy for those with more favorable outcomes. Emphasis on targeted therapies and reduction of cytotoxic treatments are the overarching goals – to cure NB while sparing young children adverse treatment-related late effects.

## Acknowledgements

We want to thank Dr. Irene Cheung, Dr. Brian Kushner, and Dr. Shakeel Modak for their critical review of this manuscript. This work was supported in part by NCI-CA161978, DOD-PR111043 and NCI-CA154754 (NKC), as well as NCI-CA21765, NIH-EY014867, NIH-EY018599, NCI-CA168875, the American Lebanese Syrian Associated Charities, and the Howard Hughes Medical Institute Early Career Scientist Award (MAD).

## Glossary Terms

<b>GD2</b>	Disialoganglioside expressed on tumors of neuroectodermal origin, including human neuroblastoma, melanoma, small cell lung cancer and many sarcomas, with highly restricted expression on normal tissues (cerebellum and peripheral nerves). Two monoclonal antibody families specific for the oligosaccharide epitope of GD2 have been tested extensively in patients, mouse IgG3 antibody 3F8 and its humanized version (hu3F8), and mouse IgG2a antibody 14G2a, and its chimeric (ch14.18) or humanized (hu14.18) forms
<b>Myeloablative therapy</b>	Bone marrow ablation owing to the loss of haematopoietic stem cells following high-dose radiation or chemotherapy
<b>Adrenal gland</b>	Endocrine organ responsible for making stress hormones, aldosterone and androgens
<b>Sympathetic ganglia</b>	Masses of nerve cells that are part of a network controlling autonomic/"fight-or-flight" responses
<b>Hirschsprung disease</b>	A congenital disease where large intestines lack innervation
<b>Congenital Central Hypoventilation Syndrome (CCHS)</b>	A congenital brain stem disease where autonomic control of breathing is defective causing sleep apnea
<b>Alternative Lengthening of Telomeres (ALT)</b>	A recombination-based mechanism that allows telomere length maintenance in the absence of telomerase activity
<b>Schwannian stromal content</b>	Glial cells in the surrounding stroma, interspersed among neuroblastoma cells, usually abundant in tumors that are more differentiated
<b>Mitotic-karyorrhexis index</b>	A measure of the frequency of cells in mitosis with karyorrhexis (nuclear fragmentation associated with cell death)

<b>Transcribed ultraconserved regions (T-UCR)</b>	T-UCRs are non-coding RNAs transcribed from 481 ultraconserved regions (UCRs) defined as being at least 200 bp in length and 100% conserved between the human mouse and rat genomes
<b>Hyperdiploid</b>	Having more than the diploid number of chromosomes, where the DNA index is >1.15
<b>Event-free survival</b>	A measure of time being continually alive without a life-threatening adverse event
<b>Complement decay accelerating factor (CD55)</b>	A 70 kDa membrane protein that prevents the assembly of the C3bBb complex (C3-convertase of the alternative pathway of complement activation), thereby blocking formation of the membrane attack complex (MAC)
<b>Protectin (CD59)</b>	A membrane protein that inhibits MAC by binding C5b678 and preventing C9 from binding and polymerizing
<b>NK cell mediated antibody-dependent cytotoxicity (NK-ADCC)</b>	Killing of tumor cells by natural killer cells whose Fc receptor adheres to the antibody attached to the target
<b>Granulocyte mediated antibody-dependent cytotoxicity (granulocyte-ADCC)</b>	Killing of tumor cells by granulocytes whose Fc receptor adheres to the antibody attached to the target
<b>Killer cell immunoglobulin-like receptors (KIRs)</b>	Highly polymorphic NK cell surface proteins that interact with MHC class I molecules, and most KIRs mediate NK inhibition instead of activation
<b>Regulatory T-cells (T<sub>reg</sub>)</b>	A T-cell subtype that releases suppressive cytokines and serves to silence immune responses
<b>Anti-idiotype</b>	An antibody binding specifically to an epitope in the variable region of another antibody
<b>when the epitope is the actual antigen-binding site</b>	an anti-idiotypic antibody can mimic the de novo antigen
<b>QS21 adjuvant</b>	A soluble triterpene glucoside saponin discovered in the extract from the Soap bark tree ( <i>Quillaja saponaria</i> ) that stimulates humoral and cell-mediated immunity
<b>Chromaffin cells</b>	Neuroendocrine cells in the adrenal medulla that receive sympathetic input and release catecholamine neurotransmitters to the systemic circulation



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**[Box 1]****AT A GLANCE**

- Neuroblastoma (NB) is a heterogeneous disease. Fifty percent of NBs arise in young children, carry whole-chromosomal gains with relatively few somatic mutations, and are highly curable with either surgery alone or surgery and low-dose chemotherapy. The other 50% of NBs are usually metastatic, and most are diagnosed after 18 months of age, with half of the tumors carrying *MYCN* amplification and *ALK*, or *ATRX* mutations. Other somatic mutations accumulate with increasing age at diagnosis. Neural crest cells and NB share common pathways and genes, including *PHOX2B*, *MYCN*, *ATRX*, and *ALK*, which offer therapeutic targets.
- A predictive profile of genetic predisposition to NB is emerging via genome-wide association and whole-genome sequencing analyses. However, there is general paucity of somatic mutations in contrast to adult cancers.
- The unique biology of catecholamine transport has been successfully exploited to provide the tumor-specific neurotransmitter analogue MIBG for diagnosis and anti-NB therapy. This advance exemplifies how understanding unique tumor metabolism can lead to new therapeutics that is not directly related to specific genetic lesions.
- Chromosomal aberration is common in NB; numerical whole chromosomal gains are typically found in low-risk tumors, while segmental chromosomal gains or losses and somatic mutations are associated with high-risk disease.
- Epigenetic regulation and miRNA control are other prognostic and therapeutic directions likely to uncover new markers and targets for NB.
- NB can evade T-cells and NK cells while exploiting inflammatory macrophages to enhance its survival. Monoclonal antibodies, cytokines, and multifunctional antibodies could potentially reactivate antitumor activity in these cells.
- Anti-GD2 antibody, when combined with GM-CSF with or without IL-2, is one of the most successful and important strategies for the curative approach to NB. Both myeloid effectors and NK cells and their cell surface activating or inhibitory receptors play crucial roles in the clinical response.

**[Box 2]****Translational research roadmap****Step 1 Identify key clinical challenges**

For NB, this may include patients with high-risk disease and particular emphasis on those with refractory disease. It may also include patients who have very good prognosis, where translational research may reduce treatment intensity and treatment-associated toxicities.

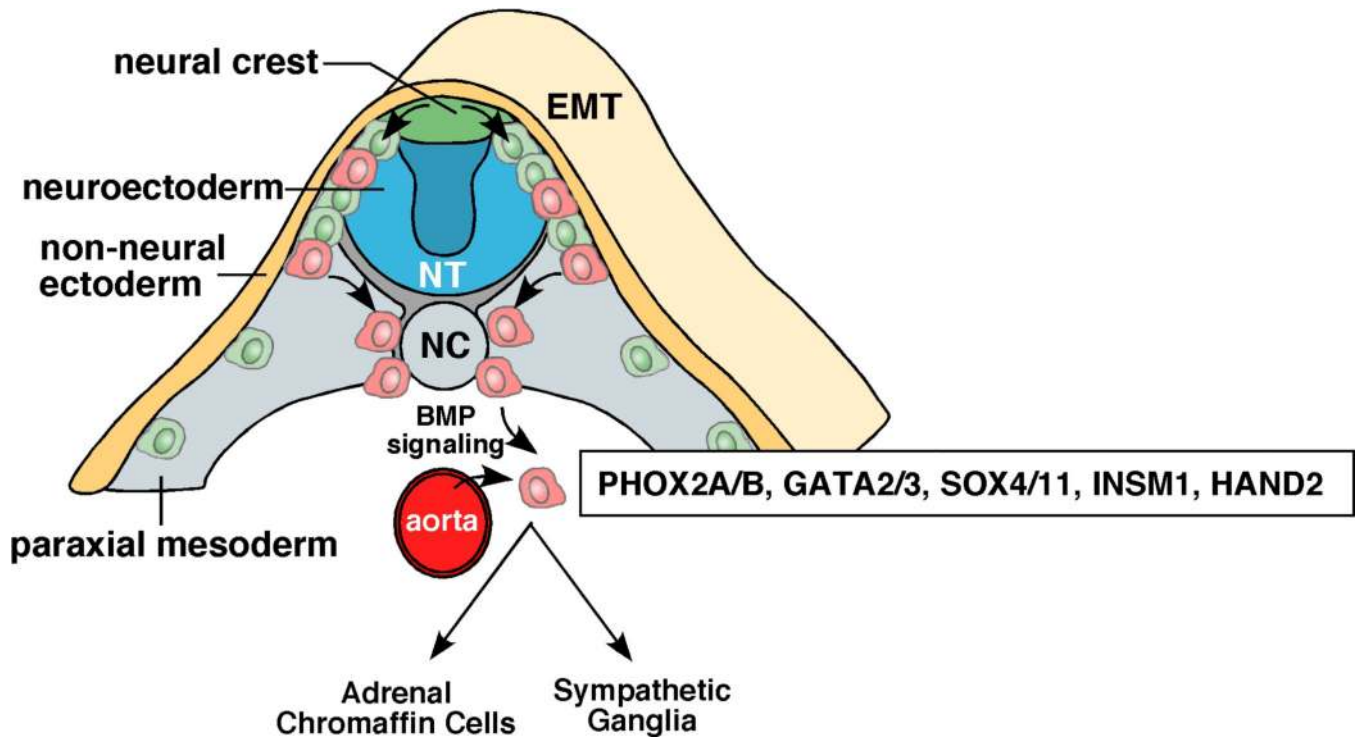
**Step 2 Identify druggable targets and pathways**

Besides specific kinases (e.g., ALK) or enzymes (e.g., drug transporters), entire pathways and cellular programs (e.g., ALT) should be considered. In some cases, biomarkers (e.g., GD2) can be used for molecular-targeted therapeutics, even if the marker is not directly related to perturbations in cellular pathways required for tumorigenesis.

**Step 3 Well-designed preclinical testing**

For orphan diseases, only the most promising new agents should move forward, and every available resource should be used to compare/contrast that agent with the standard of care. A comprehensive preclinical trial should consider genetic mouse models and orthotopic xenografts of human primary tumors to establish the relative efficacy of competing treatment options. The choice of mouse models (Table 3) is crucial, especially when immunologics are being tested, human immune cells are required, and the tumor microenvironment is part of the pathway being tested. Ideally, mice should undergo the same diagnostic testing (magnetic resonance imaging, ultrasound, positron emission tomography) and functional assessments (blood counts and chemistries, urine catecholamine monitoring) that patients receive. Minimal residual disease measurement using blood samples is another potentially informative endpoint.

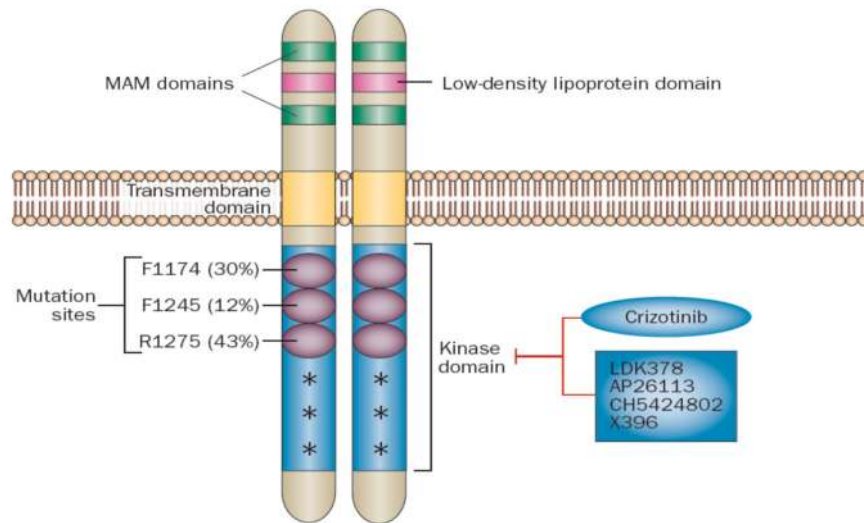




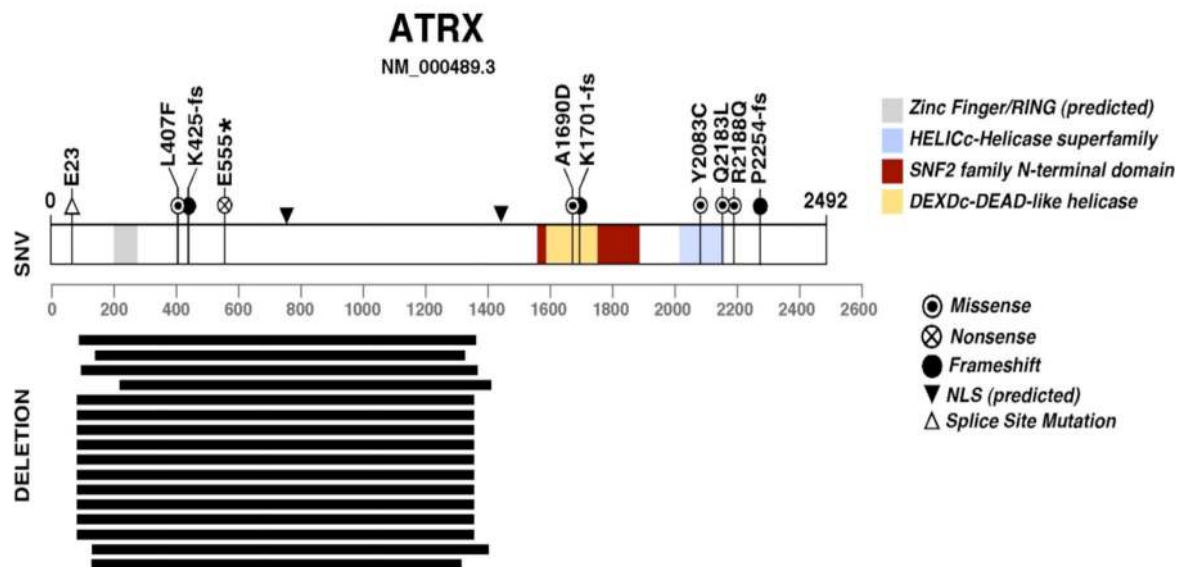
**Figure 1. Development of the sympathoadrenal lineage of the neural crest**

As cells of the neural crest (green/red cells) migrate, they undergo epithelial-mesenchymal transition (EMT). A subset of cells (red) migrates toward the dorsal aorta as they commit to the sympathoadrenal lineage. This migration is directed in part by the expression of the chemokine receptor CXCR4 on the migrating neural crest progenitor cells (red) and the expression of the SDF-1 chemoattractant on the dorsal aorta. At the dorsal aorta, the migrating neural crest progenitor cells committed to the sympathoadrenal lineage initiate their differentiation program in response to BMP signalling emanating from the dorsal aorta<sup>199–200</sup>. A series of transcription factors including PHOX2A/B, ASCL1, GATA2/3, SOX4/11, INSM1 and HAND2 are upregulated. Shortly after these transcription factors are induced, neuronal markers are upregulated along with genes that encode enzymes required for catecholamine biosynthesis such as tyrosine hydroxylase (TH) and dopamine beta hydroxylase (DBH). From that point, the cells commit to the adrenal chromaffin lineage[G] or become sympathetic ganglia. Abbreviations: NT, neural tube; NC, notochord; EMT, epithelial to mesenchymal transition.

A.



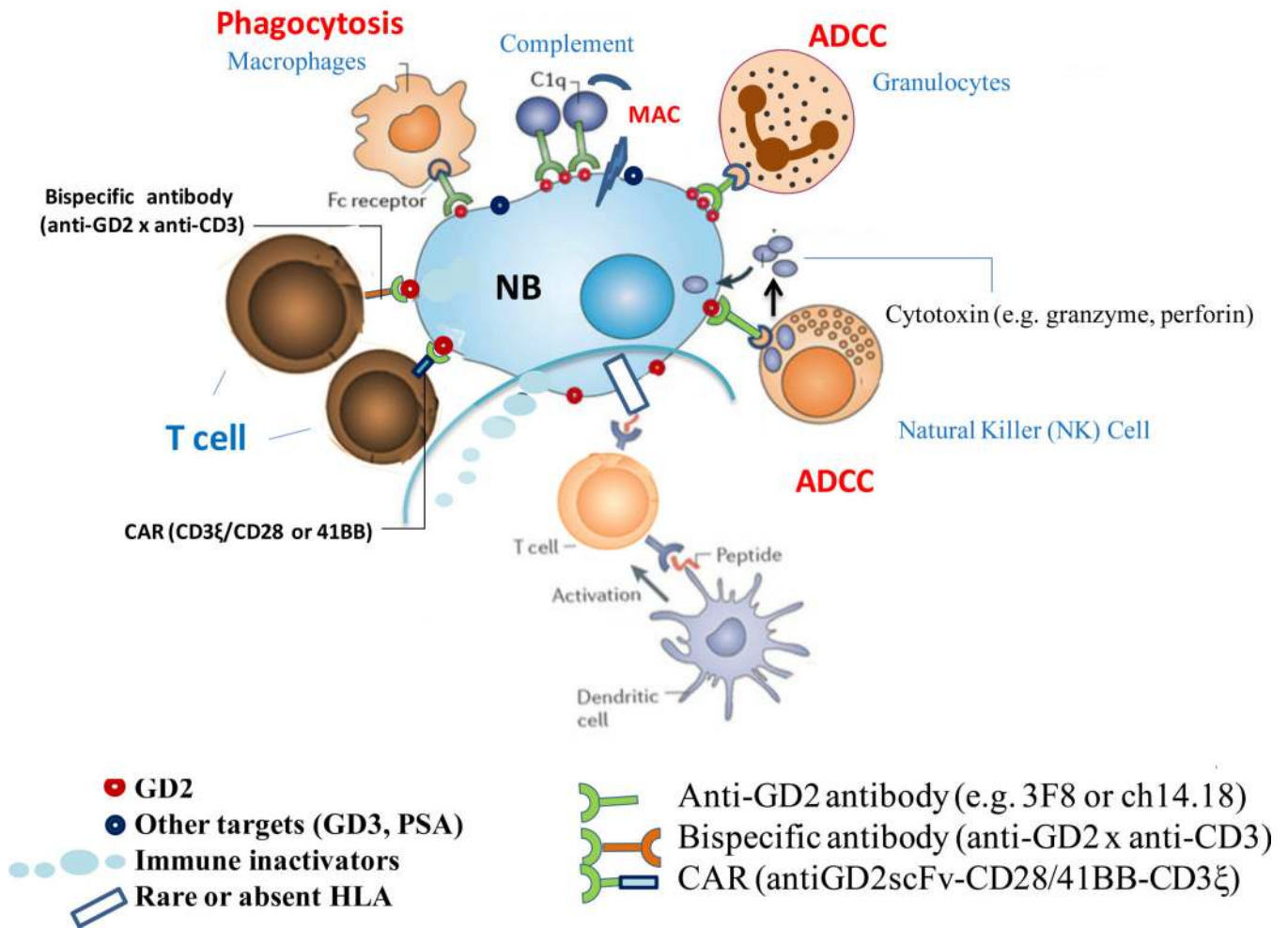
B.



**Figure 2. ALK and ATRX mutations in NB**

A | Schematic representation of ALK protein structure and mutations found in NB. The low-density lipoprotein domain, two MAM domains, and the transmembrane and kinase domains of ALK are shown. R1275, F1174 and F1245 are three most common ALK mutations in neuroblastoma; the frequency of these mutations is provided in parenthesis. Other low frequency mutations are denoted with an asterisk. The tyrosine kinase inhibitor crizotinib is in clinical trials and other second-generation ALK inhibitors are in development. (Reproduced with permission from Carpenter et al<sup>45</sup>).

**B** | In a recent WGS study of stage-4 NBs from different age groups, *ATRX* mutations were identified, including in-frame deletions, missense, nonsense and frame-shift single-nucleotide variations. Mutations in *ATRX* were significantly associated with age at diagnosis. Mutations in *ATRX* were mutually exclusive of *MYCN* amplification and were associated with alternative lengthening of telomeres (ALT). Importantly, previous studies have shown that ALT in NB is mutually exclusive of *MYCN* amplification<sup>201</sup>. More common among older patients (Group 4, 1), ALT is associated with chronic disease and poor survival. The 5-year OS for patients with ALT-positive NB is 0%; that of patients with ALT-negative is 52%<sup>201</sup>. *ATRX* mutations may contribute to ALT in NB cells and are associated with poor OS among older patients. (Modified with permission from Cheung et al<sup>52</sup>).



**Figure 3. Immunotherapy of Neuroblastoma**

NB evades T-cells by downregulating or losing HLA expression, thereby interfering with the afferent arm (priming through dendritic cells), homing of T-cells to NB, and the CTL effector phase of adaptive immunity. Soluble inhibitors of immune response (e.g. FasL, gangliosides) are constantly released into the tumor stroma to impair cellular immunity. In addition, NB recruits pro-tumor macrophages and silences NK cells. Myeloid suppressor cells and T-reg can also suppress immunity. The paucity of mutations in NB compared to adult cancers like melanoma, the immaturity of the immune system in young patients, their massive disease and the intensive chemotherapy all combine to make NB poorly immunogenic for T-cells. Carbohydrate differentiation antigens (e.g. GD2, GD3 and polysialic acid (PSA)), all of which being classically T-independent antigens, offered alternative targets for antibody-based therapies. In the presence of monoclonal antibodies (e.g. 3F8 or ch14.18) specific for GD2, NB loses their defense and becomes highly susceptible to (1) NK (natural killer) cell mediated antibody-dependent cell mediated cytotoxicity (ADCC), (2) granulocyte mediated ADCC, (3) complement mediated cytotoxicity by binding to C1q thereby activating the complement cascade, delivering membrane attack complex (MAC) to tumor cell membrane, and (4) monocyte-macrophage mediated cytotoxicity. Even polyclonal T-cells can be retargeted to kill NB through MABs

in the form of chimeric antigen receptors (CAR) or bispecific antibodies (anti-GD2 x anti-CD3). CARs are anti-tumor single chain Fv fragments (scFv) genetically fused through a transmembrane domain to T-cell activating motifs (CD3  $\xi$  and CD28/41BB) and transfected into killer lymphocytes. (Modified with permission from Scott et al<sup>202</sup>.)

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**Table 1**

Survival from NB depends on MYCN status, age and stage of disease at diagnosis

Prognostic Category	MYCN amplification	Stage* at Dx	Age at Dx	Treatment**	5-year OS*** (%)
<b>Low Risk</b>	No	4s	<12 m	Supportive Care	>90
	No	Locoregional	<12 y	Surgery ± Rx	>90
<b>Intermediate Risk</b>	No	4	<18 m	Surgery + Rx	>90
<b>High Risk</b>	Yes	Locoregional	<12y	Intensive Rx + XRT + Surgery + SCT + CRA	53
	Yes	4	<18 m	Intensive Rx + XRT + Surgery + SCT + CRA	29
	No	4	≥18 m, <12 y	Intensive Rx + XRT + Surgery + SCT + CRA	31
	No	4	≥12 y	Intensive Rx + XRT + Surgery + SCT + CRA	<10##

\* Stage 4 176 = metastatic disease; Stage 4s = special metastatic pattern in infants with resectable primary tumors and distant spread to liver and skin, plus minimal bone marrow involvement. Abbreviations: CRA, 13-cis-retinoic acid; Dx, diagnosis; OS, overall survival; Rx, chemotherapy; SCT, myeloablative therapy with autologous stem cell rescue; XRT, radiation therapy to primary tumor and resistant metastatic sites.

\*\* OS was extracted from a large international retrospective (1990–2002) analysis, before the era of immunotherapy, when treatment was not uniform.

## NB in adolescent and young adults is indolent with OS at 10 years <10%<sup>52,177</sup>.



**Table 2**

## Hallmarks of High Risk Neuroblastoma

Hallmarks <sup>79</sup>	Druggable Targets or Cells <sup>#</sup>	Potential Therapeutic Inhibitors or Activators	Developmental Status in NB
Proliferative signaling	26S proteasome <sup>178</sup>	<b>Bortezomib</b> *	Phase I
	Akt <sup>179</sup>	MK2206, Perifosine	Phase I
	ALK <sup>45</sup>	<b>Crizotinib</b>	Phase II
	Aurora Kinase A <sup>50</sup>	MLN8237 <sup>180</sup>	Phase I
	Cdk4/6 <sup>181</sup>	LEE011	Phase I
	IGF-I/IGF-II <sup>182</sup>	MEDI-573, m708.5	Preclinical
	IGF-1R <sup>183</sup>	Mab R1507, IMC-A12	Phase I
	MYCN (BET) <sup>51</sup>	(+)-JQ1	Preclinical
	PI3K/mTOR <sup>179</sup>	<b>PI103/Rapamycin</b>	Phase I
	TrkA/TrkB <sup>184</sup>	Lestaurtinib (CEP-701)	Phase I
Evading growth suppressors	Bcl-2 <sup>185</sup>	ABT-737	Preclinical
	Bmi-1 <sup>186</sup>	<b>Vorinostat</b>	Phase I
	MDM2-p53 <sup>186</sup>	Nutlin	Preclinical
Resisting cell death	Methylation (CASP8, RASSF1A, DcR1, DcR2, DR4, DR5) <sup>74</sup>	<b>Decitabine</b>	Phase I
	HDAC <sup>187</sup>	<b>Vorinostat</b>	Phase I
Enabling replicative immortality	ATRX (ALT) <sup>58</sup>	CHK1 inhibitor (LY2606368)	Preclinical
	Telomerase <sup>56</sup>	Imetelstat	Phase I
Inducing angiogenesis	VEGF, HIF-1 $\alpha$ , HIF-2 $\alpha$ <sup>188</sup>	<b>MAb (Bevacizumab)</b>	Phase II
Activating invasion and metastasis	c-Met/HGF <sup>189</sup>	<b>Crizotinib</b>	Phase I
	EMT/Twist1 <sup>186</sup>	<b>Vorinostat</b>	Phase I
	MMP2, MMP9 <sup>190</sup>	AZD1236	Preclinical
	Rho/Rac <sup>5</sup>	Y27632	Preclinical
Genome instability	ATRX?, MYCN, Telomerase	--	--
Tumor promoting inflammation	TAM <sup>191</sup>	IL15 (NKT) <sup>98</sup>	Preclinical
Evasion of immune destruction	B7-H3 <sup>192</sup>	MAb (8H9) <sup>157</sup>	Phase I/II
	CD59 <sup>104</sup>	rILYd4 <sup>193</sup>	Preclinical
	Granulocytes/macrophages	<b>GM-CSF</b> <sup>119</sup>	Phase I/II/III
	HLA	<b>IFN-gamma</b> <sup>194</sup>	Phase I
	KIR <sup>105</sup>	MAb (anti-KIR2DL1/2/3) <sup>140</sup>	Preclinical
	NK cells/Granulocytes	MAb (ch14.18, 3F8, hu14.18-K322A, hu3F8) <sup>118,152,154</sup>	Phase I/II/III
	NK/NKT/T-cells	IL-15 <sup>138</sup>	Preclinical

Hallmarks <sup>79</sup>	Druggable Targets or Cells <sup>#</sup>	Potential Therapeutic Inhibitors or Activators	Developmental Status in NB
		<b>IL-2</b> <sup>118</sup>	Phase I/II/III
	T-cells	CAR (14G2a, 5F11, hu3F8, CE7) <sup>159-160</sup>	Preclinical/ Phase I
		MAb (Bispecific 3F8 or bispecific hu3F8) <sup>165</sup>	Preclinical
Energy metabolism	GLUT1	3-Bromopyruvate, 3-BrOP <sup>195</sup>	Preclinical

<sup>#</sup> Though being assigned under a single hallmark, a specific target could be responsible for multiple hallmarks of high-risk NB

\* Bold face in blue cell = FDA approved agents for non-NB indications.

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**Table 3**

## Animal models of neuroblastoma

Model	Type	Gene; Lineage	Reference
<i>TH-MYCN</i>	transgenic	human <i>MYCN</i> ; sympathoadrenal lineage	Weiss et al. (1997) <sup>48</sup>
<i>TH-ALK<sup>F1174L</sup>;TH-MYCN</i>	transgenic	human <i>ALK<sup>F1174L</sup></i> ; sympathoadrenal lineage	Berry et al. (2012) <sup>33</sup>
<i>ALK<sup>F1174L</sup></i>	conditional transgenic	human <i>ALK<sup>F1174L</sup></i> ; sympathoadrenal lineage	Lukas et al. (2012)
<i>Lin28</i>	conditional transgenic	mouse <i>Lin28b</i> ; sympathoadrenal lineage	Molenaar et al. (2012) <sup>43</sup>
<i>TH-MYCN;Caspase 8</i>	conditional knockout	mouse caspase 8; sympathoadrenal lineage	Teitz et al. (in revision)
Xenografts in immune-deficient mice	orthotopic/xenograft	human orthotopic xenografts cell lines and primary tumors	Chanthery et al. (2012) <sup>196</sup> Teitz et al. (2011) <sup>197</sup>
Xenografts in NOD/SCID/IL2 $\gamma$ <sup>null</sup> or BALB/c-rag2KO/ $\gamma$ c <sup>null</sup>	humanized mice	human orthotopic xenografts cell lines and primary tumors	Shultz et al.(2007) <sup>170</sup> Pek et al.(2011) <sup>146</sup>
Syngeneic	A/J inbred mouse	mouse neuroblastoma (NXS2, Neuro-2a)	Huebener et al.(2009) <sup>198</sup> Jing et al.(2011) <sup>112</sup>
<i>MYCN; ALK<sup>F1174L</sup></i>	transgenic zebrafish	human <i>MYCN</i> and <i>ALK<sup>F1174L</sup></i> <i>dβh</i> promoter	Zhu et al. (2012) <sup>47</sup>