

# Recent insights into the biology of neuroblastoma

Guadrin Schleiermacher<sup>1,2,3</sup>, Isabelle Janoueix-Lerosey<sup>3</sup> and Olivier Delattre<sup>3</sup>

<sup>1</sup>Equipe SIRIC Recherche Translationnelle en Oncologie Pédiatrique, Département de Recherche Translationnelle et Inserm U830, Centre de Recherche, Paris Cedex, 05, France

<sup>2</sup>Département de pédiatrie, Institut Curie, Paris Cedex, 05, France

<sup>3</sup>Unité Génétique et Biologie des Cancers, Inserm U830, Centre de Recherche, Institut Curie, Paris Cedex, 05, France

Neuroblastoma (NB) is an embryonal tumor of the sympathetic nervous system which accounts for 8–10% of pediatric cancers. It is characterized by a broad spectrum of clinical behaviors from spontaneous regression to fatal outcome despite aggressive therapies. Considerable progress has been made recently in the germline and somatic genetic characterization of patients and tumors. Indeed, predisposition genes that account for a significant proportion of familial and syndromic cases have been identified and genome-wide association studies have retrieved a number of susceptibility loci. In addition, genome-wide sequencing, copy-number and expression studies have been conducted on tumors and have detected important gene modifications, profiles and signatures that have strong implications for the therapeutic stratification of patients. The identification of major players in NB oncogenesis, including *MYCN*, *ALK*, *PHOX2B* and *LIN28B*, has enabled the development of new animal models. Our review focuses on these recent advances, on the insights they provide on the mechanisms involved in NB development and their applications for the clinical management of patients.

Neuroblastoma (NB) is the most common extracranial cancer of early childhood, with an estimated incidence of 1/8,000–10,000 births.<sup>1</sup> In France, this cancer is diagnosed in 130–150 new patients every year, and accounts for approximately 15% of cancer-related deaths.<sup>2</sup> Median age at diagnosis is 18 months, 40% of cases being diagnosed before 1 year of age. NB arises from embryonic cells that form the primitive neural crest, originating either in the adrenal medulla or in the paraspinal ganglia of the sympathetic nervous system. In case of metastases, these are most frequently observed in bone marrow, bone, lymph nodes, liver or subcutaneous tissue, other metastatic localizations occurring much rarer.

The hallmark of NB is its wide range of clinical courses, with, on the one hand, a possibility of cellular maturation or spontaneous regression even in case of metastatic disease, or on the other hand, life-threatening tumor progression despite

all treatment. Thus, prognostic factors have been sought for to identify risk groups and to stratify treatment approaches. The clinical parameters age and stage have been used, together with pathological features, to define different risk groups at the time of diagnosis.<sup>3</sup> Children diagnosed with a NB before the age of 18 months have a better outcome than those diagnosed at a later age.<sup>4</sup> Patients with localized disease (INRG staging system L1 and L2; localized disease without or with image-defined risk factors precluding surgical resection) have a better prognosis than those with metastatic disease (INRG stage M), whose outcome remains dismal especially in older children. To date, different recurrent genetic markers have also been included in risk stratification schemes. It is likely that with the increase of knowledge about the underlying genetic features of NB, additional genetic information will be included for the definition of prognostic subgroups and treatment stratification in the near future.

**Key words:** neuroblastoma, susceptibility, genetic alterations, expression signatures, progression, animal models

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**Correspondence to:** Olivier Delattre, Inserm U 830, Institut Curie, 26 rue d'Ulm, 75248 Paris Cedex 05, France, Tel.: 33-1-56-24-66-79, E-mail: delattre@curie.fr

## Hereditary Aspects of NB

The observation of rare familial cases and a possible association with congenital malformations has long suggested the possibility of NB genetic predisposition. Recent studies of such underlying predisposition have now contributed important knowledge to the understanding of NB oncogenesis. On the one hand, rare constitutional genetic alterations associated with an important risk to develop NB have been described; on the other hand, genetic variants occurring frequently in the general population might confer an increase of the relative risk to develop NB.

NB has been described in the context of the familial tumor syndrome neurofibromatosis type 1 (NF1).<sup>5</sup> The association between NB and NF1 is rare, but interestingly a few

Table 1. Susceptibility loci identified in NB by genome-wide association studies

Chromosome	Locus/gene	Allelic odds ratio	NB risk group	Reference
1q21.1	NBPF23		High-risk NB	[14]
1q23.3	DUSP12		Low-risk NB	[15]
2q35	BARD1	1.68	High-risk NB	[16]
5q11.2	DDX4		Low-risk NB	[15]
	IL31RA			
6p22	FLJ22536	1.39–1.40	High-risk NB	[17]
	FLJ44180			
6q16	HACE1	1.26	High-risk NB	[18]
	LIN28B	1.38		
11p15.4	LMO1	1.34	High-risk NB	[19]
11p11.2	HSD17B12		Low-risk NB	[15]

cases of NB occurring in association with other RAS–MAPK syndromes such as Noonan and Costello syndromes have also been described, underlining the potential importance of these pathways in NB development.

Familial NB *per se* is rare and concerns only approximately 1% of all NB cases.<sup>6</sup> Missense mutations of *PHOX2B* on chromosome 4p, frequently associated with other neurocristopathies (Ondine's and Hirschsprung's disease), were the first germline mutations to be identified in NB predisposition.<sup>7</sup> Interestingly, expansions of the second polyaniline stretch of *PHOX2B* are mainly observed in patients presenting with Ondine's curse (also called CCHS, for Congenital Central Hypoventilation Syndrome), whereas nonpolyaniline repeat expansion mutations (NPARMs) occur preferentially in patients with CCHS associated with Hirschsprung's disease and NB.<sup>8</sup> More importantly, constitutional activating *ALK* mutations, on chromosome 2p23, have been identified in more than half of all familial cases.<sup>9,10</sup> In a recent study of NB patients with early onset or multifocal disease, two criteria suggestive of a predisposition context, activating *ALK* mutations were detected in only 3/42 NB patients. In two pedigrees, several mutation carriers did not develop any sign of NB.<sup>11</sup> Thus, it is estimated that only about 50% of carriers of constitutional *ALK* mutations are affected by NB.<sup>11,12</sup> Based on the results of genome-wide linkage analyses, it has been suggested that variants in the nonmutated *ALK* gene, or other genes, may affect penetrance of constitutional *ALK* mutations and thus influence the development of NB.<sup>12</sup> Taken together, the presence of constitutional *PHOX2B* and *ALK* mutations constitute key events in NB oncogenesis in affected individuals, but with variable penetrance. A follow-up scheme for nonsymptomatic carriers of these constitutional mutations has been suggested, but the actual individual benefit of such a follow-up remains to be documented.<sup>11</sup> Recently, a syndromic presentation associating congenital NB with severe encephalopathy and an abnormal shape of the brainstem has been reported in two sporadic cases harboring

*de novo* germline F1174V and F1245V *ALK* mutations.<sup>13</sup> This observation together with the description of *ALK* expression in the central nervous system (CNS) of mammals highly suggests that some of the *ALK*-activating mutations may disrupt CNS development and prompt mutation screening in patients with extreme phenotypes.

Recent powerful genome-wide association studies (GWAS) have demonstrated a link between NB and genomic variations at some loci (Table 1), with a correlation with either high-risk or low-risk disease, indicating that favorable and unfavorable forms of NB may represent distinct entities in terms of the genetic events that initiate tumorigenesis.

At chromosome 1q21.1, a common copy-number variation (CNV) has been shown to be associated with NB.<sup>14</sup> Within the CNV, a new transcript shows high sequence similarity with several NB breakpoint family (NBPF) genes, the first of which was identified at a constitutional (1;17) translocation in a NB patient,<sup>20</sup> and hence constitutes a new member of this gene family (*NBPF23*). This transcript shows preferential expression in fetal brain and fetal sympathetic nervous tissues, and its expression level is correlated with the CNV status in NB cells.

A significant association of Stage 4 NB and six single-nucleotide polymorphisms (SNPs) at chromosome 2q35, within the locus of the *BARD1* gene, has been identified, indicating that common variations in *BARD1* contribute to the etiology of the most clinically relevant, aggressive form of NB.<sup>16</sup> The report of a role of *BARD1* variations in NB susceptibility is the first definitive evidence that variations in this gene might be associated with cancer susceptibility. *BARD1* heterodimerizes with the familial breast cancer gene product *BRCA1* and is considered to be essential for the latter known tumor-suppressive function. The oncogenic role of *BARD1* variations has been documented further by the observation that disease-associated variants correlate with increased expression of the oncogenic, activated isoform, *BARD1β*.<sup>21</sup> Silencing of *BARD1β* in NB cells leads to genotype-specific cytotoxic effects,

including decreased substrate-adherence, anchorage-independence and inhibition of cell proliferation. Interestingly, overexpression of *BARD1 $\beta$*  is sufficient for neoplastic transformation in established mouse fibroblasts. *BARD1 $\beta$*  stabilizes the Aurora family of kinases in NB cells, suggesting a rationale for a new therapeutic strategy.

On chromosome 6p22, a significant association between NB and the common minor alleles of three consecutive SNPs at the loci of the predicted genes *FLJ22536* and *FLJ44180* has been identified.<sup>17</sup> NB patients homozygous for the risk alleles at 6p22 are more likely to have stage 4 NB and somatic amplification of *MYCN*. No significant interaction between the loci identified by GWAS at 1q21.1, 2q35 and 6p22 has been detected.

In a subsequent study, two new associations at 6q16, the first within *HACE1*, and the second within *LIN28B*, have been detected.<sup>18</sup> Expression of *LIN28B* correlates with the at-risk genotype in NB cell lines. After the depletion of *LIN28B*, a significant growth inhibition specifically in NB cells that are homozygous for the risk allele. At a somatic level, *LIN28B* has been shown to harbor genomic aberrations in the form of focal amplifications, and an important overexpression in high-risk NB has been observed.<sup>22</sup> *LIN28B* represses microRNAs of the *let-7* family, resulting in elevated *MYCN* protein expression in NB cells, underlining the role of *LIN28B* and *let-7* miRNA in unfavorable NB and in disease progression.

At chromosome 11p15.4, a significant association between NB and alleles within the *LMO1* (LIM domain only 1) gene has been detected, with an odds ratio highest in the subset of patients with the most aggressive form of the disease.<sup>19</sup> *LMO1* encodes a cysteine-rich transcriptional regulator, and its paralogues (*LMO2*, *LMO3* and *LMO4*) have each been previously implicated in cancer. At a somatic level, the *LMO1* locus is aberrant in 12% of NB through a duplication event, and this is associated with advanced disease and poor survival. The germline risk alleles and somatic copy-number gains are associated with increased *LMO1* expression in NB cell lines and primary tumors, consistent with a gain-of-function role in tumorigenesis.

Focusing on low-risk NB, SNPs within the genes *DUSP12* at 1q23.3, *DDX4* and *IL31RA* both at 5q11.2, and *HSD17B12* at 11p11.2 have been identified as being associated with the less aggressive form of NB.<sup>15</sup>

Altogether, although the presence of at-risk variations can transmit susceptibility to NB, other somatically acquired genetic events must occur for cancer cell transformation.

### Somatic Genetic Alterations in NB Determined by Genomic DNA Profiling

To date, a large number of recurrent somatic genetic alterations have been described in NB, most of which concern quantitative alterations with loss or gain of chromosome material. In addition to providing insight into NB oncogenesis, the study of these somatically acquired genetic alterations

has enabled to identify reliable and robust prognostic biomarkers which can be used for treatment stratification in a clinical setting.<sup>23</sup>

Amplification of the oncogene *MYCN*, at chromosome 2p24, is observed in 20–25% of all NB.<sup>24</sup> *MYCN*-amplified NB harbor a particularly poor prognosis, and the *MYCN* genomic status has been integrated into risk stratification and therapeutic decisions in NB treatment for more than 20 years. *MYCN* amplification can occur in a context of coamplification with genes in its vicinity such as *MEIS1*, *DDX1* or *NAG*. Focal, high-level amplifications at other, distant, loci occur more rarely either with or without *MYCN* amplification. Indeed in a recent analysis of genomic profiles of more than 1,000 NB studied by array-CGH, approximately 1% of all cases had focal, high-level amplifications other than *MYCN*, without associated *MYCN* amplification.<sup>25</sup> Recurrent amplifications described to date concern the *ALK* gene on chromosome 2p23, as well as amplicons of chromosome 12q13–14 encompassing, among others, the *MDM2* and *CDK4* genes.<sup>26,27</sup> Preliminary data indicate that NB with focal amplifications other than *MYCN* might present with atypical clinical features, underlining the need for further characterization of these cases, and the precise prognostic role of focal amplifications other than *MYCN* remains to be determined.<sup>25</sup>

Structural chromosome alterations leading to gains or losses of chromosome material in NB occur most frequently owing to unbalanced chromosome translocations<sup>28,29</sup> and are individually associated with a poor prognosis in most cases.<sup>30,31</sup> The most frequently observed segmental chromosome alterations (SCAs) in NB are losses of chromosomes 1p, 3p, 4p and 11q and gains of chromosomes 1q, 2p and/or 17q, the latter being the most frequent genetic alterations in NB observed in approximately 50% of cases. The number of chromosome breakpoints increases with age in NB patients<sup>32,33</sup> and has been shown to be significantly lower in *MYCN* amplified versus *MYCN* nonamplified tumors with SCA, the highest number of breakpoints being observed in tumors with 11q deletion.<sup>34,35</sup>

Numerous studies have investigated the potential role of chromosome breakpoints in tumor development, as well as their consequences in terms of copy-number changes. The chromosome breakpoints are scattered over extensive regions of several megabases.<sup>32</sup> In agreement with this observation, cloning and/or sequencing of breakpoints have shown that region-specific breakpoints do not lead to monomorphic and recurrent genetic consequences as gene disruptions or gene fusions. Indeed, although disruption or fusion events have been characterized for some breakpoints they did not present significant recurrences across tumors. A strong interest has focused on copy-number changes. As these breakpoints are usually hallmarks of unbalanced translocations, they are associated with concomitant gain and loss of defined chromosome regions. Many studies have searched for smallest regions of overlap for the identification of oncogenes or tumor-suppressor genes directly involved in NB. However, in

most cases, these smallest regions of overlap remain quite large and do not point to single-candidate genes with tumor-suppressive or oncogenic functions.

Medium- to high-resolution aCGH and SNP arrays have been used to search for smaller, interstitial, genomic alterations in NB, with an aim to detect genes directly involved in NB oncogenesis, but few recurrent events have been identified. Interestingly, one study has suggested that focal DNA copy-number gains and losses may be enriched for MYCN target genes.<sup>36</sup> Another study using SNP arrays has identified in 10% of the analyzed cases alterations on chromosome 9p, with homo- or hemizygous deletions encompassing the *CDKN2A* gene.<sup>27</sup> Other sporadic copy-number alterations include focal TERT gains,<sup>37</sup> and microdeletions encompassing the *PTPRD* gene.<sup>38</sup> Various studies, based on the integration of genomic with expression data, have shown that SCA-related copy-number changes lead to gene dosage effects. Indeed, detailed comparison of chromosome 1p status with expression levels showed that around 15% of the genes targeted by the deletion had a decreased expression, indicating that chromosome 1p deletion results in a gene dosage effect on a subset of genes that may be crucial for NB development.<sup>39</sup> Further detailed analyses have shown that SCAs may lead to an alteration of gene expression affecting loss of cell-cycle control and deregulation of Rho guanosine triphosphates functioning in neuritogenesis.<sup>33,40</sup> Although copy-number alterations are a hallmark of NB, copy neutral loss of heterozygosity appears to be uncommon in NB tumors, but more frequent in NB cell lines.<sup>41</sup>

Thus, despite the extensive studies focusing on the analysis of copy-number alterations in NB, few recurrently altered genes directly implicated in NB oncogenesis have been identified. However, in addition to the search for recurrently altered genomic regions, these whole-genome approaches have also aimed to search for associations between altered chromosome regions and to develop classifications based on the overall genomic profile, rather than analyzing single copy-number alteration events.<sup>35</sup> Indeed, chromosome 17q gain is frequently observed in association with chromosome 1p loss, whereas chromosome 11q loss, also observed in association with chromosome 17q gain but only rarely with chromosome 1p loss, is frequently associated with more numerous copy-number alterations. These observations led to the proposition of genomic classifications, distinguishing NB with whole chromosome 17 gain *versus* NB with 17q gain with or without 1p loss, with 11q loss, and MYCN-amplified cases.<sup>42,43</sup> More recently, an analysis of nearly 500 NBs using aCGH revealed a 100% overall survival (OS) and an excellent event-free survival (EFS) of NBs with numerical chromosome alterations (NCAs) only. Taking into account all observed copy-number changes, both those observed recurrently and those observed more rarely, it could be shown that in case of any SCA, without or with NCA, EFS was significantly worse ( $92\% \pm 1.8$  vs.  $49\% \pm 4.2$ ,  $p < 0.0001$ ).<sup>35</sup> Other studies have confirmed the prognostic impact of the overall genomic pro-

file in NB, with SCA associated with a poorer progression-free survival.<sup>44,45</sup> In a genomic study of 236 cases, tumors with partial chromosome gains or losses were associated with a 5-year survival rate of 53 *versus* 85% in tumors with whole chromosome gains or losses, according to a classification based primarily on chromosome 17 status.<sup>45</sup> In a study of 165 NBs using high-resolution SNP arrays, NBs with 11q deletion were shown to have a poor survival approaching that of MYCN-amplified tumors.<sup>34</sup> NB with SCAs other than 1p, 11q deletion or 17q gain did not have a worse outcome in this study. Thus, the prognostic impact of the overall genomic pattern in NB is now widely acknowledged, a genomic profile harboring NCA only being associated with an excellent outcome, whereas the presence of SCA of recurrently altered regions in NB is clearly associated with a poorer outcome. More extensive studies are required to further establish the prognostic impact of SCA of regions sporadically altered in NB. Furthermore, with the increased resolution of aCGH and SNP arrays, smaller interstitial alterations might be detected, and additional studies are warranted to analyze the impact of small interstitial copy-number alterations (<3 Mb) observed on higher resolution arrays.

To study the prognostic impact of genomic profiling in low-risk NB, a study by aCGH has been performed on tumors of infants enrolled in the European INES99.1, 99.2 and 99.3 trials.<sup>44</sup> SCAs were observed in 11, 20 and 59% of infants enrolled in trial INES99.1 (infants < 12 months with localized unresectable NB), INES99.2 (infants < 12 months with INSS Stage 4s disease) and INES99.3 (infants < 12 months with INSS Stage 4 disease) ( $p < 0.0001$ ). Although OS in this study was excellent, progression-free survival was poorer in case of a SCA genomic profile, both in infants with localized unresectable disease and with Stage 4s disease, including the subgroup of patients without clinical symptoms at diagnosis (log-rank  $p = 0.04$  and  $p = 0.0003$ , respectively). Thus, in infants with Stage 4s MYCN nonamplified NB, a SCA genomic profile identifies patients with a higher risk of relapse who will require upfront treatment even in the absence of other clinical indication for therapy. These findings are now implemented in a prospective international SIOPEN trial (see below).

### Somatic Genetic Alterations in NB Determined by Next-Generation Sequencing Approaches

In addition to the germline mutations observed in familial NB, activating mutations of the ALK tyrosine kinase receptor have been described to occur in 8–10% of all sporadic NB, with mutations clustering within exons 23 and 25 of the tyrosine kinase domain. The most frequent mutations are observed at positions F1174 and R1275 and a preferential association of the F1174L mutation with MYCN amplification has been reported in a large meta-analysis<sup>9,10,46–48</sup>. Recent genomic sequencing efforts have yielded additional information about genetic alterations in NB (Table 2). Despite the

Table 2. Somatic genetic alterations in NB determined by NGS approaches

Reference	No. of tumors (tumor stage)	Technique	Genomic coverage of each base in targeted regions/ sequence	No. of mutations (per tumor)	No. of structural rearrangements (per tumor)	Recurrent mutations/ structural rearrangements
[49]	87 (All stages)	WGS	50X	Mean: 12 (range: 0–55)	Mean: 15.9 (range: 0–85)	ALK (6% m, 2% sf) ATRX (5.7%) ODZ3 (5.7%) PTPRD (5.7%) TIAM1 (3.4%) FANCM/FAN1 Rac/Rho regulators
[50]	26 6 16	WGS WGS WES	Low coverage: 10X High coverage: 31X 94X	Mean: 13 (range: 1–52)	Mean : 4 within protein coding genes (range : 0–18)	ALK (9%) ARID1B } 11% ARID1A } VANGL ZHX2 LIN28B
[51]	40 (Stage 4)	WGS	35X			ATRX (enrichment in older children)
[52]	222 19 (Stage 4, >18m)	WES WGS	124X 29X (Illumina) 59X (complete genomics)	Mean (total): 18 (range: 0–218) Mean (nonsilent): 14	Mean : 41 breakpoints	ALK (9.2% m) ATRX (2.5% m/7.1% sf) PTPNI1 (2.9%) OR5T1 (1.25%) PDE6G (0.8%) MYCN (1.7%) NRAS (0.8%)
[53]	Two NB cell lines Two NB samples	WGS				FHIT ODZ4 NBAS ALK PTPRD

Abbreviations: WGS: whole-genome sequencing; WES: whole-exome sequencing; m: mutation; sr: structural rearrangement.

analysis of series with different clinical characteristics, the use of different sequencing techniques and different resolutions, these studies clearly indicate that most NB harbor only few mutations, with an average of 10–20 predicted nonsynonymous variations in coding regions *per* genome, indicating an exonic mutation frequency of 0.2–0.4 *per* Mb. The frequency of somatic events strongly correlated with tumor stage, lower stage tumors harboring a lower number of mutations.<sup>49</sup> Two tumors with an important increase of the mutation frequency (7.27 and 4.29 mutations *per* Mb) harbored alterations of the DNA repair genes *MLH1* and *DBI*, respectively.<sup>52</sup> Altogether, these sequencing studies confirmed the occurrence of somatic, activating *ALK* mutations in 6–9.2 % of cases.<sup>49,50,52</sup> Only few additional recurrent mutations were identified, targeting distinct cellular pathways. These included heterozygous missense *PTPN11* mutations (observed in 2.9% of cases),<sup>52</sup> as well as truncating mutations of the *TIAM1* gene,<sup>49</sup> encoding a regulator of the cytoskeleton involved in neuritogenesis, and other regulators of the RAC/Rho pathway. Recurrent mutations of *MYCN* in *MYCN*-nonamplified tumors and missense mutations of *NRAS* were also observed.<sup>52</sup> Interestingly, genes involved in chromatin remodeling were found to be targeted either by mutations or by structural variations in a significant number of cases.<sup>49–52</sup> Somatic alterations of *ATRX*, including missense, nonsense, frameshift mutations and structural variations resulting in in-frame deletions, were found in 5–25% of cases studied by NGS (Next-Generation Sequencing), with a minimum overlapping region mapping to exons 5–10 of the *ATRX* gene, containing a nuclear localization signal. These alterations were associated with an increase in telomere length, and with an absence of the *ATRX* protein in the nucleus. *ATRX* alterations were shown to be enriched in older children.<sup>51,52</sup> Furthermore, hemizygous deletions and splice-site and missense mutations of the *ARID1B* gene, as well as nonsense, missense or truncating mutations of the *ARID1A* gene were also observed, with some cases presenting biallelic inactivation owing to the deletion of the wild-type allele.<sup>50</sup> Other genes involved in chromatin remodeling have also been shown to be affected, such as the histone acetyltransferase genes *EP300* and *CREBBP*, mutations in the SWI2/SN2 member *TTF2*, as well as in the histone demethylase gene *KDM5A* and the chromatin remodeling zinc finger *IKZF1*.

Different pathways have also been shown to be targeted by structural rearrangements. Indeed, recurrent structural alterations of the *ODZ3*, *PTPRD* and *CSMD1* genes were observed, which play a role in neuronal growth cone stabilization. Structural rearrangements were also shown to occur within *NBAS* in the vicinity of *MYCN*.<sup>49,52,53</sup>

Chromothripsis, defined as a local shattering with subsequent random reassembly of fragment in a single event,<sup>54</sup> has been described in up to 18% of high-risk tumors.<sup>49</sup> In two tumors with chromothripsis, inactivating deletions and missense mutations were detected in the *FANCM* and *FAN1* genes, respectively, suggesting a possible involvement of the

Fanconi anemia pathway in the origin of chromothripsis. Although in a subsequent study some cases also presented a high number of chromosome breakpoints,<sup>52</sup> the many complex copy-number states and retention of heterozygosity in lower copy-number regions were not believed to suggest a chromothripsis-linked origin of these alterations. Further evidence of the genomic structure of rearrangements, including those observed in association with chromothripsis, has been provided by whole-genome sequencing of two cell lines and two NBs, the latter with a genomic chromothripsis profile.<sup>53</sup> Analysis of 59 breakpoint junctions of both chromothripsis-associated and -nonassociated rearrangements at a single base resolution revealed frequent microhomologies (40/59) at the junction, as well as complex rearrangements with templated insertions of fragments of nearby sequences in 4/59 cases. The results indicate that both nonhomologous end joining-mediated repair and replicative processes may play a role in the generation of genomic rearrangements in NB. Altogether, given the relative paucity of recurrent gene mutations as compared to the frequency of recurrent copy-number changes, NB could be considered as a copy-number-driven cancer,<sup>55</sup> with gene dosage effects possibly contributing to oncogenesis.

#### Expression Signatures in NB

As SCA are observed in nearly all high-risk NBs, the study of the DNA copy-number profile may not provide additional prognostic information in this patient group. Thus, a large number of studies have focused on the analysis of differential expression patterns in NB, seeking to identify the expression patterns that might enable to distinguish patients with different clinical courses and thus define different prognostic groups in high-risk disease, and to potentially identify new therapeutic targets.

In a recent study, a 144-gene expression signature reliably distinguished patients with distinct clinical courses and was shown to be an independent prognostic factor in a multivariate analysis, with the strongest difference observed in non-high-risk disease.<sup>56</sup> A different study used a 59-gene signature with a sensitivity and specificity of 84.4 and 86.5%, respectively, to predict patient outcome.<sup>57</sup> Multivariate analysis indicated that the signature was an independent predictor of overall and progression-free survival after controlling for currently used risk factors. In another study, using real-time PCR expression data, an expression signature based on three genes (*CHD5*, *PFAH1B1* and *NME1*) discriminated patients with different outcomes.<sup>58</sup> In a study focusing on high-risk patients, an expression profile based on 55 genes defined patient populations with divergent outcome (PFS, 16 vs. 79%).<sup>59</sup> A good *versus* a poor prognosis group could also be identified using a 62-hypoxia gene signature.<sup>60</sup> More recently, based on the hypothesis that tumor-associated inflammatory cells might contribute to the differences in age-dependent outcome of patients with metastatic NB, expression of genes representing tumor-associated macrophages, such as *CD33/CD16/IL6R/IL10/FCGR3* contributed to 25% of the accuracy of a novel 14-gene tumor

classification score. In patients >18 months with metastatic *MYCN*-nonamplified NB, progression free survival was 47 *versus* 12% for patients with a low- *versus* a high-risk score, indicating that interactions between tumor and inflammatory cells may contribute to the aggressive metastatic NB phenotype.<sup>61</sup> Another study has sought to analyze differential expression signatures depending on *MYCN*, and this 157-gene signature identified NB with poor prognosis independent of the genomic *MYCN* status, those without *MYCN* amplification presenting stabilization of *MYCN* at the protein level.<sup>62</sup>

During the last years, it has become evident that the expression levels of noncoding RNAs are also highly variable. Micro-RNAs are the most widely studied noncoding RNA molecules in NB. They function as regulators of gene expression at the post-transcriptional level in diverse cellular processes. *MYCN* modulates the expression of several classes of noncoding RNAs, especially some micro-RNAs, and it can also regulate the expression of long noncoding RNAs such as T-UCRs (Transcribed UltraConserved Regions) and noncoding RNA, whereas other long noncoding RNAs remain to be characterized in NB. The landscape of T-UCRs in NB has been studied recently and has revealed a correlation with the *MYCN* status, and preliminary studies have suggested that T-UCR-based expression signatures might identify short- from long-term survivors in high-risk NB.<sup>63</sup>

The miRNA expression pattern can also be used to classify NB patients according to survival.<sup>64,65</sup> An advantage of the study of miRNA rather than mRNA expression signatures is based on the greater stability of miRNAs, and thus the feasibility even with FFPE samples as opposed to frozen samples.<sup>65</sup>

Gene expression levels also depend on epigenetic modifiers. Some recent studies have sought to identify promoter methylation patterns which might identify patient subgroups. Genome-wide promoter methylation analysis identified prognostic methylation biomarkers, and eight genes with a specific methylation status depending on clinical risk factors could be identified.<sup>66</sup> Further studies are now necessary to determine genome-wide methylation patterns and their correlation to outcome in NB patients.

Taken together, to date, many studies have demonstrated the feasibility of expression profiling of mRNA, miRNA, other noncoding RNAs or epigenetic modifiers to determine different prognostic subgroups among NB patients. However, there is little, if any, overlap between the genes of the different signatures rendering cross-study comparisons unfeasible. Furthermore, although most expression signatures clearly distinguish prognostic groups in the overall population, differences in survival among high-risk patients are frequently not very marked. The routine setup of real-time determination of expression profiles in a prospective clinical trial setting, and their interpretation, remains a clear challenge.

### Therapeutic Implications of Genetic Alterations

The observation that a NCA profile is associated with an excellent outcome, whereas a SCA profile is associated with a

higher risk of relapse is now implemented in an international trial: SIOPEX (<https://www.siopep-r-net.org/>) has recently launched the LINES trial (Low- and Intermediate-risk NB—European Study), the aim of which is to improve outcome with minimal treatment burden in patients with low- and intermediate-risk NB patients by stratifying treatment according to the clinical and radiological findings as well as the genomic profile. Genomic profiling is performed in a clinical, real-time setting, including central review of results. Within this trial, the absence of SCA is used to identify patients with a low risk, INRG Stage L2 NB for whom the possibility of further treatment reduction to observation only is studied in a randomized trial, whereas the presence of SCA leads to more intense treatment as compared to historical controls.

In high-risk NB, current treatment strategies most frequently include a multidrug induction chemotherapy, high-dose chemotherapy with autologous stem cell rescue, local treatment with surgical resection and radiotherapy and maintenance treatment with 13-cisretinoic acid and immunotherapy. Despite significant efforts, OS remains poor in this patient group, and new treatment approaches are warranted. As most high-risk NB patients harbor a SCA profile or *MYCN* amplification,<sup>35</sup> treatment stratification in this group might potentially benefit from expression profiling. However, to date, none of the different expression signatures reported to be of prognostic impact in NB have been tested in a prospective setting, nor have they been compared among each other on the same patient populations. Thus, the place for prospective implementation of expression profiling for risk grouping and determining treatment strategies in high-risk NB remains to be established.

Many efforts are currently placed on the development of new, targeted therapies within important pediatric clinical trial programs, such as the Phase I/II trials run by ITCC (Innovative Therapies for Children with Cancer; <http://www.itcc-consortium.org>). As closer links between pharmacological and biological studies are created, the benefit of biomarker-based approaches becomes more evident. In NB, much effort is now placed on the development of biomarker-driven clinical Phase I/II trials, underlining the necessity of precise knowledge of genetic profiles to be able to take into account predictive biomarkers, such as the *MYCN* status in the development of strategies targeting *MYCN* directly or indirectly.<sup>67</sup> The discovery of activating *ALK* mutations in NB has rapidly led to a phase I/II trial of crizotinib, with preliminary encouraging results especially in the presence of somatic *ALK* mutations known to be sensitive to crizotinib, and new generation *ALK* inhibitors are now being developed.<sup>68</sup>

To improve survival in high-risk patients, it could now be hypothesized that upfront integration of targeted therapy could, in some instances, be tested within prospective clinical trials. It could be suggested that new biomarker-based approaches could potentially include upfront risk grouping according to expression profiling, and search for targetable genetic alterations by whole-genome/whole-exome sequencing.

Also, for an ultra high-risk group of patients, or in the presence of a drugable target, upfront targeted therapy could be included in the overall treatment strategy within a clinical trial setting. Nevertheless, the relative scarcity of somatic mutations in NB at diagnosis indicates that systematic whole-exome sequencing efforts of NB at diagnosis are unlikely to unravel targetable mutations in a significant percentage of children affected with high-risk disease. Disease evaluation at the time of tumor progression might then give additional information with regards to tumor evolution events.

#### Progression of NBs

As described above, genetic analyses of NB have unravelled clinical and genetic subtypes associated with very different outcomes. The determination of whether these distinct profiles distinguish unrelated entities whose mechanisms of development are distinct or whether they form a continuum with possible evolutions from one type to another remains a matter of investigation.

Genetically, two main groups of NBs are triploid cases with only NCA and diploid/tetraploid cases with only SCA, the most frequent being 17q, 2p or 1q gains, 11q, 1p, 3p or 4p deletions. Given the very different ploidy and genomic profiles of these two groups, it is very unlikely that the latter may derive from the progression of the former. In this regard, it is noteworthy that the existence of these very different groups of tumors accounted for the relative failure of screening strategies based on the detection of urinary catecholamines that were implemented in different countries at the end of the past century. Indeed, although most detected tumors were good prognosis, triploid/numerical tumors, this detection did not lead to any decreased incidence later in life of aggressive NB.<sup>69</sup> This also strongly suggested that most aggressive tumors do not evolve from less aggressive tumors.

Nevertheless, a number of arguments suggest that the different histological and genetic subtypes of neuroblastic tumors may share some common mechanisms of development.

The first argument stems from the analysis of hereditary NBs. Indeed, tumors observed in the context of germline mutations of *NF1*, *PHOX2B* or *ALK*, the three main NB susceptibility genes, can be of very variable aggressiveness, from benign ganglioneuromas to highly aggressive undifferentiated NB.<sup>5,7,9,70</sup> In addition, preliminary results strongly suggest that the full panel of different genetic subtypes can be observed in these hereditary tumors. Together with the variable penetrance of these mutations, the diversity of tumors observed in hereditary contexts raises complex issues to propose appropriate strategies for the follow-up of at-risk individuals. Biologically, this, nevertheless, indicates that similar mechanisms of tumor development are involved in these different subtypes.

A second series of arguments is obtained from interesting observations made by investigating heterogeneous tumors. Nodular ganglioneuroblastoma is very interesting in this respect. It constitutes a relatively rare subtype of neuroblastic tumor where neuroblastic nodules and Schwannian areas can

be clearly distinguished histologically. Although in most cases the same histology is observed in different nodules of the same tumor, in a subset of cases nodules of favorable and unfavorable histologies can be seen.<sup>71</sup> This strongly suggests that both types of nodules share common ancestor cancer cells that ultimately diverged and led to nodules of favorable and unfavorable histologies. Interestingly, investigating the clonality of the nodular and Schwannian components of nodular NB through the analysis of the X-methylation pattern, we could show that in most cases the Schwannian and neuroblastic components were monoclonal with identical X-inactivation patterns, suggesting that both components were derived from a common progenitor.<sup>72</sup> One of the hypotheses is that neuroblastic areas emerged after the monoclonal expansion of a glial-neuroblastic precursor which was secondarily transformed into malignant neuroblasts by additional genetic alterations.

Finally, important data arrive from longitudinal analyses of tumor specimen during the course of the disease. Our data suggest that a subset of cases with both NCA and SCA (mixed cases), which are known to share the unfavorable prognosis of pure segmental cases, arose upon progression of pure numerical cases. Indeed, the pattern of numerical abnormalities is extremely similar between mixed cases and numerical cases with identical chromosomes being over- and under-represented. In addition, segmental abnormalities were observed at relapse of cases which were purely numerical at diagnosis.<sup>32</sup> Even more strikingly, we investigated very recently the presence of *ALK* mutations at diagnosis and at relapse and noted that the frequency of *ALK* mutations was higher at relapse. When *ALK* mutations were observed at relapse, the subclonal presence of the mutation could frequently be detected at diagnosis using NGS-based approaches strongly suggesting that the mutated subclone expanded between diagnosis and relapse. In contrast, we did not observe any tumors with an *ALK* mutation at diagnosis that was not detected at relapse. We also observed a very interesting heterogeneous nodular case where the *ALK* mutation could be detected in the poorly differentiated neuroblastic nodules, whereas it was not detected in ganglioneuroblastoma intermixed-like nodules.<sup>73</sup>

Altogether, these different elements suggest that, most frequently, aggressive NBs occur *de novo* without any detectable pre- or poorly malignant step. Yet, some clear evolutions from good to bad prognosis NB can be observed. In addition, the observation that all grades of aggressiveness can be observed in the context of germline mutations of *ALK*, *PHOX2B* or *NF1* indicates that the mechanisms of development of these different NBs are, at least partly, common.

#### Murine and Zebrafish NB Models: From Tools to Understand NB Pathogenesis to Preclinical Models

In 1997, Weiss *et al.*<sup>74</sup> created the first transgenic mice over-expressing human *MYCN* under the rat tyrosine hydroxylase (Th) promoter in neuroectodermal cells and showed that



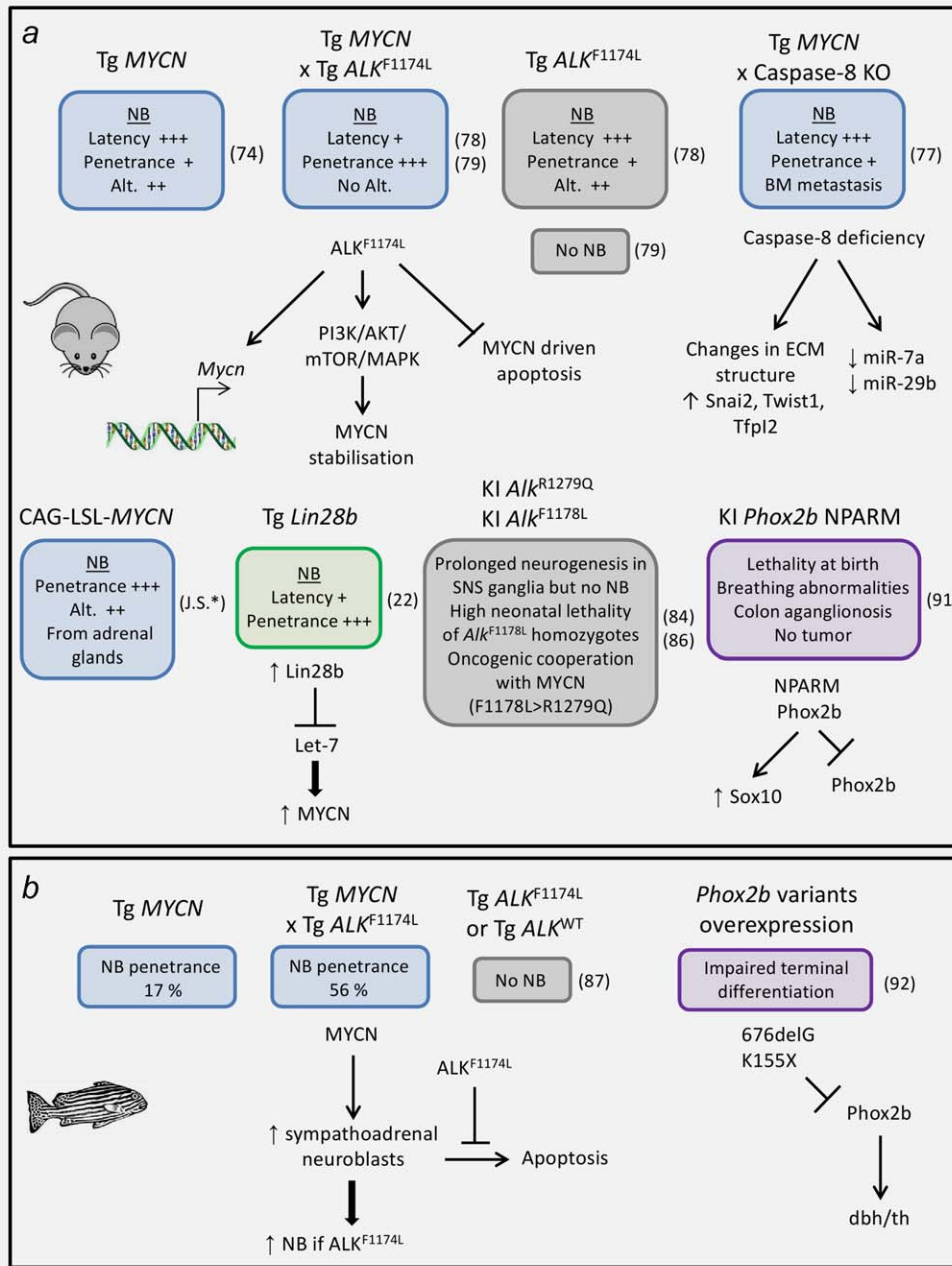
these mice developed tumors with typical features of human NB (Fig. 1a). This approach, therefore, provided the first demonstration that *MYCN* can contribute to the transformation of neuroblasts *in vivo*. Furthermore, these mice were shown to be a relevant preclinical model of NB that has been and still it is widely used to assay therapeutic strategies. This model is characterized by a quite long latency and low penetrance of NB formation. Tumors predominantly originate from abdominal ganglion structures. Genomic profiling documented a number of other genetic alterations occurring in the obtained tumors that are syntenic to the ones commonly found in human NB.<sup>75</sup> Importantly, tumorigenesis in this model is strongly dependent on the genetic background of the strain: whereas tumor penetrance is 70% in the 129x1/SvJ strain background, it is only 5% in the C57Bl6/N background. In addition, mainly locally invasive tumors are obtained in this context and therefore do not reproduce the frequent metastatic disease occurring in patients presenting with *MYCN* amplification. Loss of expression of caspase-8, which exhibits proapoptotic function and is involved in the control of many processes including migration and adhesion, is frequent in metastatic NB.<sup>76</sup> Interestingly, it has been shown recently that breeding Th-*MYCN* mouse with a caspase-8-deficient mouse had no impact on survival upon tumor formation but that bone marrow metastasis was much more frequent<sup>77</sup> (Fig. 1a). No major changes in the proportion of cells undergoing apoptosis could be observed in tumors bearing both alterations. However, transcriptional analysis of the primary tumors revealed extracellular matrix structural changes, suggesting an increased motility and migration of the caspase-8-deficient tumor cells.

A new *MYCN*-driven NB mouse model using Cre-driven conditional expression of *MYCN* has recently been generated (Schulte, personal communication, Fig. 1a). In this model, a *MYCN*-IRES-Luciferase cassette was cloned downstream of the chicken actin gene (CAG) promoter followed by *loxP*-flanked strong transcriptional termination site (LSL) into the *ROSA26* locus. After breeding, mice bearing this construction with *Dbh* (dopamine- $\beta$ -hydroxylase)-iCre mice, *MYCN* expression is induced in *Dbh*-expressing cells. Interestingly, this strategy overcomes several limitations of the original transgenic Th-*MYCN* mice; indeed, high NB penetrance is observed both in the 129x1/SvJ and in the C57Bl6/N backgrounds, tumors arise from the adrenals as well as from the superior cervical or celiac ganglia and they can be detected by bioluminescent imaging. As in the original model, the obtained tumors also exhibit other chromosome aberrations syntenic to those observed in human NBs.

The identification of ALK as a promising therapeutic target in a subset of familial and sporadic NBs bearing activating *ALK* mutations<sup>9,10,46,47</sup> motivated the development of murine models with activated ALK. Two main hotspots of mutations have been identified in sporadic cases at position R1275 and F1174. To date, two transgenic (Tg) mouse lines

have been generated, allowing the expression of human *ALK*<sup>F1174L</sup> in neural crest cells, using the *Dbh* or *Th* promoters<sup>78,79</sup> (Fig. 1a). Although no tumors developed in the model generated by Berry and colleagues, the team of Schulte obtained tumors resembling human NB in a subset of Tg-*ALK*<sup>F1174L</sup> animals. However, the long latency and low penetrance in tumor formation suggested that other genetic alterations might have occurred, which was indeed confirmed by the analysis of the genomic profiles of the tumors. Strikingly, in one case, *MYCN* amplification could be documented. The difference between the two lines may be related to differences in the expression level of the *ALK* transgene, which is difficult to appreciate from two distinct publications. Both groups then bred the Tg-*ALK*<sup>F1174L</sup> mice with Th-*MYCN* mice which revealed a strong cooperation between the two oncogenes. Latency and penetrance were significantly shortened or increased, respectively, and tumors exhibited much less chromosome imbalances compared to tumors from simple Tg-*ALK*<sup>F1174L</sup> animals.<sup>78</sup> Berry *et al.* demonstrated that mutated ALK: (i) induced an increased transcription of the murine *Mycn* gene, in agreement with the previous *in vitro* studies, documenting this upregulation<sup>80,81</sup>; (ii) triggered the PI3K/AKT/mTOR/MAPK pathways, leading to *MYCN* protein stabilization and (iii) inhibited *MYCN*-induced apoptosis.<sup>79</sup> In terms of preclinical models, tumors from double transgenic mice were shown to be resistant to the ALK inhibitor crizotinib<sup>78,79</sup> in agreement with the previous *in vitro* data that documented a lower sensitivity of the F1174L mutation compared to the R1275Q mutation.<sup>82</sup> The identification of the PI3K/AKT/mTOR/MAPK pathway being activated by *ALK*<sup>F1174L</sup> encouraged the study of a combination of crizotinib with torin2, an mTOR inhibitor. This combination was indeed much more efficient than each compound separately to induce tumor regression and increase mice survival.<sup>79</sup> To date, no transgenic mice overexpressing *ALK*<sup>WT</sup> have been described in the literature. With respect to the observation that human tumors with high *ALK* expression display clinical and molecular phenotypes similar to the ones that characterize *ALK*-mutated tumors,<sup>83</sup> it will be of interest to determine whether *ALK*<sup>WT</sup> overexpression may contribute to NB initiation and/or progression in transgenic murine models.

Although only the *ALK*<sup>F1174L</sup> mutation has been investigated in a context of overexpression in transgenic mice, we recently generated and characterized two Knock-in (KI) mouse lines bearing *Alk*<sup>R1279Q</sup> (R1275Q in human) and *Alk*<sup>F1178L</sup> (F1174L in human) mutations (Fig. 1a). Although no tumors developed in these animals, we documented that activated *Alk* triggers a prolonged neurogenesis of the sympathetic ganglia, providing clues to the understanding of NB predisposition linked to germline *ALK* mutations.<sup>84</sup> We demonstrated an increased oncogenic potential of the F1178 mutation compared to the R1279Q mutation in a *MYCN* context and documented that *Alk*<sup>R1279Q</sup> tumors responded to



**Figure 1.** Mouse and zebrafish as model organisms for NB studies. (a) Mouse models. Transgenic mice overexpressing the human *MYCN* gene under the control of the Th promoter were shown in 1997 to develop tumors resembling human NBs. Two lines of transgenic mice overexpressing *ALK<sup>F1174L</sup>* have been described. The occurrence of NB was low in one of them with a long latency, whereas in the second line, no tumors developed in the animals. Breedings of both *ALK<sup>F1174L</sup>* lines with Th-*MYCN* mice demonstrated an oncogenic cooperation between both genes. Activated ALK was shown to potentiate MYCN activity by at least three mechanisms: (i) increased expression of murine *Mycn*, (ii) activation of the PI3K/AKT/mTOR/MAPK pathway leading to stabilization of the MYCN protein (iii) and apoptosis blockage. Although in these models, only locally invasive tumors have been observed, breeding of Th-*MYCN* mice with a caspase-8-deficient (KO) line allowed the development of NB with bone marrow (BM) metastasis. Overexpression of Lin28b has been shown to lead to NB formation in transgenic mice, by inducing repression of Let-7, leading to an increased expression of MYCN. Analysis of Knock-in (KI) mice bearing the *Alk<sup>R1279Q</sup>* (R1275Q in human) and *Alk<sup>F1178L</sup>* (F1174L in human) mutations recently demonstrated that Alk activation in a physiological context is not by itself sufficient to induce neuroblastic tumors. However, a prolonged neurogenesis in sympathetic ganglia was documented in mutant mice, leading to an enlargement of this tissue. Both mutations cooperated with *MYCN* to induce NB. When homozygous, the *Alk<sup>F1178L</sup>* mutation induced a high neonatal lethality, whereas the *Alk<sup>R1279Q</sup>* mutation had no impact on mice survival. KI mice lines bearing some of the *Phox2b* mutations associated with NB (NPARM) have been described. These mice displayed profound breathing difficulties, resulting in neonatal lethality. The analyzed mutations were shown to induce Sox10 expression and impair normal differentiation. (b) Zebrafish models. Zebrafish lines overexpressing human *MYCN*, *ALK<sup>WT</sup>* and *ALK<sup>F1174L</sup>* have been generated. *ALK<sup>WT</sup>* overexpression alone was not sufficient to drive NB formation. As observed in transgenic mice, NB penetrance was quite low in simple transgenic *MYCN* zebrafish but increased upon cooperation with *ALK<sup>F1174L</sup>*. Activated ALK was shown to favor NB formation through apoptosis inhibition. Zebrafish models have been developed to determine the functional consequences of aberrant PHOX2B expression. Overexpression of *Phox2b* variants (corresponding to the NB-associated frameshift mutations, 676delG and K155X) impaired terminal sympathetic neuron differentiation in the presence of endogenous *Phox2b*, demonstrating their dominant-negative effects. Latency + and latency +++ refer to short and long latency, respectively. Penetrance + and penetrance +++ indicate low and high penetrance, respectively. Alt. ++ indicates that tumors exhibit genomic alterations detected by array-CGH. Numbers in parenthesis indicate the corresponding references. J.S.\*: Johannes Schulte, personal communication.

the crizotinib inhibitor. Analysis of human tumors and cell lines as well as murine tumors and sympathetic ganglia at birth uncovered RET/Ret as a target of activated ALK/Alk.<sup>84</sup> Interestingly, Alk activation in sympathetic nervous system ganglia induced opposite effects compared to the ones reported in Ret<sup>-/-</sup> mice.<sup>85</sup> Characterization of heterozygous KI *Alk*<sup>F1178L</sup> mice indicated that these mice did not phenocopy the severe neurological disorders, including major feeding and breathing difficulties, observed in the syndromic patients with *de novo* germline F1245V and F1174V activating *ALK* mutations.<sup>86</sup> However, we noticed a high lethality of homozygotes between 24 and 48 hr after birth. Evaluation of basic physiological functions 12 hr after birth uncovered a dramatic reduced milk intake for KI *Alk*<sup>F1178L</sup> homozygotes,<sup>86</sup> which appears closely related to the feeding difficulties that characterized the patients with encephalopathy. In contrast to the *Alk*<sup>F1178L</sup> mutation, the *Alk*<sup>R1279Q</sup> mutation did not induce neonatal lethality in mice when homozygous. The higher impact of the *Alk*<sup>F1178L</sup> mutation on mice survival is consistent with its higher oncogenic potential. Altogether, these data demonstrate that Alk activation above a critical threshold impairs survival in mice.

The role of *ALK* in NB oncogenesis has also been addressed using the *Danio rerio* model organism. Several transgenic zebrafish lines expressing *ALK*<sup>WT</sup> or *ALK*<sup>F1174L</sup> alone or in combination with *MYCN* under the control of the *Dbh* promoter were characterized<sup>87</sup> (Fig. 1b). Overexpression of *ALK*<sup>WT</sup> alone was not sufficient to induce NB in this model and no oncogenic cooperation with *MYCN* was reported. The consequences of activated *ALK*<sup>F1174L</sup> overexpression on NB formation are highly reminiscent of the mouse situation. Indeed, no NB was observed in simple Tg-*ALK*<sup>F1174L</sup> fish, NB penetrance was low in the simple Tg-*MYCN* line and cooperation between both alterations was reported in double Tg animals. This cooperation resulted from an inhibition of *MYCN*-induced apoptosis in the sympathoadrenal neuroblasts upon expression of activated *ALK*. Such zebrafish models may not only allow addressing basic questions but may also be extremely powerful regarding the possibility of high-throughput screenings of small molecules. In this line, it has been shown recently that expression of the NPM-ALK fusion protein in the zebrafish is able to drive overproduction of iridophores, resulting in a highly visible phenotype.<sup>88</sup> Strikingly, treatment with the ALK inhibitor TAE-684 fully inhibited production of ALK-dependent iridophores. This assay seems very promising to perform high-throughput screening of ALK inhibitors that could be subsequently evaluated in NB models with activated ALK.

Several lines of evidence recently suggested that *LIN28B* may be a critical player in NB oncogenesis (Hereditary Aspects of NB section). Strikingly, a mouse model allowed to demonstrate that *Lin28b* is able to drive NB tumorigenesis<sup>22</sup> (Fig. 1a). Indeed, tumors recapitulating typical NB features and developing from the adrenals were obtained with a short

latency in mice upon CAG-driven expression of mouse *Lin28b* in cells of the sympathoadrenergic lineage that express *Dbh*. Interestingly, it was shown that *Lin28b* signals to repress *let-7* miRNAs which consequently results in *MYCN* protein overexpression.

Although the implication of the PHOX2B homeodomain transcription factor in sporadic NBs remains to be defined, heterozygous germline mutations of this gene have been reported in neurocristopathies of the autonomic nervous system and familial forms of NB.<sup>7,89,90</sup> Recently, two KI lines bearing heterozygous NPARM into the mouse *Phox2b* locus have been characterized. These mice failed to breath spontaneously and exhibited oligoganglionosis or aganglionosis of the colon, therefore recapitulating the clinical features of CCHS and Hirschsprung's disease<sup>91</sup> (Fig. 1a). However, no overt tumor formation was detected in these mice, at least at birth. Sympathetic ganglion progenitors of these mice were shown to be less proliferative but were characterized by sustained Sox10 expression. One may speculate that such a Sox10 aberrant expression may keep these progenitors in an undifferentiated state that may be the target of additional genetic events. The exact mechanism that accounts for NB susceptibility linked to these *Phox2b* NPARM remains, however, to be determined. Interestingly, the functional consequences of two NB-associated frame-shift *PHOX2B* mutations have also been explored in the zebrafish model<sup>92</sup> (Fig. 1b). Impaired terminal differentiation of sympathetic neuron progenitors, revealed by a decreased expression of the *th* and *dbh* markers, was observed after overexpression of the mutant forms in the presence of endogenous *Phox2b*. These observations are at least in part reminiscent of the altered differentiation described in *Phox2b* NPARM mice although different mutations have been explored in both animal models. The zebrafish observations are also compatible with the hypothesis that a differentiation blockage induced by the *Phox2b* mutant forms may provide a source of cells vulnerable to additional transforming lesions.

## Conclusions

Recent technological advances have enabled considerable progress in the determination of both germline alterations associated with a higher NB risk, and somatic genetic alterations involved in NB oncogenesis. Various animal models based on these findings are now providing further knowledge for unravelling events, leading to tumor initiation and progression. Next steps should now focus on the integration of this knowledge into treatment approaches, both by refining current risk groups based on the biological characterization of patients and tumors, and by studying the role and the place of targeted treatment strategies based on the predictive biological markers.

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