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REVIEW

# Epigenetic regulation of neuroblastoma development

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## Abstract

In recent years, technological advances have enabled a detailed landscaping of the epigenome and the mechanisms of epigenetic regulation that drive normal cell function, development and cancer. Rather than merely a structural entity to support genome compaction, we now look at chromatin as a very dynamic and essential constellation that is actively participating in the tight orchestration of transcriptional regulation as well as DNA replication and repair. The unique feature of chromatin flexibility enabling fast switches towards more or less restricted epigenetic cellular states is, not surprisingly, intimately connected to cancer development and treatment resistance, and the central role of epigenetic alterations in cancer is illustrated by the finding that up to 50% of all mutations across cancer entities affect proteins controlling the chromatin status. We summarize recent insights into epigenetic rewiring underlying neuroblastoma (NB) tumor formation ranging from changes in DNA methylation patterns and mutations in epigenetic regulators to global effects on transcriptional regulatory circuits that involve key players in NB oncogenesis. Insights into the disruption of the homeostatic epigenetic balance contributing to developmental arrest of sympathetic progenitor cells and subsequent NB oncogenesis are rapidly growing and will be exploited towards the development of novel therapeutic strategies to increase current survival rates of patients with high-risk NB.

**Keywords** Histone code · Chromatin remodeling · Core regulatory circuits · Non-coding RNA · Epigenetic therapy

## Introduction

The 'Hallmarks of Cancer' concept as proposed by Hanahan and Weinberg has provided a framework to understand the complex principles of cancer biology (Hanahan and Weinberg 2000, 2011). These hallmarks explain how cancer cells breach multiple cellular safeguards driving proliferation and self renewal, unlimited growth, angiogenesis, invasion and metastasis and evasion of cell death, maintenance of genomic stability, sustained energy supply related to the rewired metabolic cancer cell circuitry and tumor promoting inflammation (Hanahan and Weinberg 2011). In the past decade, altered epigenetic states, with a main focus on **promotor hypermethylation** coupled to inactivation of tumor suppressor genes, were already recognized to play a major role in many cancer entities. Recently, a bewildering number of **mutations** were found that affect the expression/function of a wide variety of proteins involved in **chromatin modification** as well as local and higher-order chromatin structure. In addition, there is increasing evidence that malignant transformation in many different tumor types depends on the formation of **super-enhancer-driven core regulatory circuits**. Last, a vast majority of transcriptional states is not only instructed through the action of regulatory proteins but also relies on **protein/non-coding RNA interactions**. This has dramatically fueled ongoing fundamental and translational epigenetic cancer research, together with the notion that these perturbed epigenetic states present a novel target for therapy.

Neuroblastoma (NB) is considered as a developmental disorder resulting from the disruption of normal sympathetic neuronal progenitor maturation. Furthermore, like most other pediatric cancers, the mutational landscape at diagnosis is relatively silent with an average of only 5–10 mutations. Therefore, it can be assumed that deviation from the normal epigenetic homeostasis during this process could play a key role in malignant transformation. While the basic concepts of the 'epigenetic landscape' explaining the underlying processes of normal cell differentiation were proposed more than

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50 years ago by Conrad Waddington, we have now learned that (1) transcription factors are key actors in establishing and guiding cellular identity (reflecting the developmental grooves going down the developmental Waddington hill) while interacting with various DNA regulatory elements embedded in a specific epigenetic constellation, and (2) chromatin ensures the stability of these lineages and cell fates (the depth of the grooves on the hill). In a recent review, Flavahan et al. proposed the concept of abnormal epigenetic resistance and plasticity resulting from altered chromatin regulator activity and remodeling as well as DNA methylation as important processes taking part in tumorigenesis (Flavahan et al. 2017).

*We propose that a full understanding of the epigenetic control of normal sympathetic neuron differentiation and uncovering the key epigenetic perturbations during NB development will be instrumental to fully understand the molecular basis of NB oncogenesis.* Moreover, given the limited number of mutated druggable targets, these insights can also guide us towards the development of novel therapeutic interventions to increase survival rates and reduce long-term effects of current cytotoxic treatment.

Knowledge from different ongoing research efforts may converge towards this goal, e.g., novel neural crest or sympathetic progenitor cell-derived NB models, as nicely illustrated with the recently developed model system by Olsen and colleagues allowing ex vivo generation of MYCN-driven transformed neural crest stem cells to be transplanted into recipient mice, leading to tumor formation consistent with our understanding of human MYCN-driven NB and thus providing an excellent toolkit for the discovery of novel driver genes in this malignancy (Olsen et al. 2017). Among others, transcription factors that are key in normal neuronal development (e.g., *SOX11*, *TWIST1* and *TCF3*) as well as cell cycle regulators (*FOXM1*, *E2F3* and *MYBL2*) were found to be upregulated in this experimental murine NB model in accordance with human NB tumors. Future studies should provide further in-depth profiling of epigenetic landscaping in cellular models and primary tumors including enhancer mapping, screening for gene essentiality or cancer dependency, and further in-depth bioinformatics analyses of developmental and gene regulatory networks in large transcriptome and proteome datasets. Ultimately, this should guide us towards exploiting the epigenetics toolbox to develop more effective and less toxic therapies in combination with other small molecules (e.g., ALK and/or RAS/MAPK inhibitors) and immunotherapy to reach the full extent of precision medicine for these patients.

## Mapping of neuroblastoma-specific epigenetic DNA modifications

DNA methylation is a stable epigenetic modification of which the global pattern can be faithfully inherited through multiple cell divisions. During development and cell differentiation, DNA methylation is dynamic, but some patterns may be retained as a form of epigenetic memory, while cancer initiation and progression can lead to genome-wide and gene-specific DNA methylation changes. Methylation states can thus inform both on oncogenic altered epigenetic states of genes as well as a cell-of-origin epigenetic profile (Feinberg et al. 2016). For a detailed overview of the most prominent DNA methylation markers in neuroblastoma, see the review by Decock et al. (2011).

### Methylation and prognostic classification

Like for many other tumor entities, several studies focusing on methylation have been conducted in NB, using increasingly more powerful and sensitive methods. One of the first prognostic methylation studies was reported by Alaminos and co-workers who investigated promoter hypermethylation in 45 candidate genes in 10 NB cell lines and a subsequent selection of 10 genes in 118 primary NBs by means of methylation-specific PCR (MSP). This CpG island hypermethylation portrait showed distinct patterns for *MYCN*-amplified versus non-amplified tumors, among others for the tumor suppressor caspase-8 (Alaminos et al. 2004). Hoebeeck et al. used the same technique to screen the methylation status of 10 selected tumor suppressor genes in 33 NB cell lines and 42 primary NB cases (Hoebeeck et al. 2009). In follow-up of MSP, novel techniques have been developed allowing genome-wide analysis of aberrant DNA methylation patterns, such as various methylation arrays. This also included methyl-CpG-binding domain (MBD) sequencing of NB cell lines, a sensitive and cost-effective method for genome-wide DNA methylation profiling and biomarker identification. Using this method in combination with MSP on primary NB tumors, eight candidate biomarkers (Decock et al. 2012) were identified of which the methylation status could be related to previously known risk factors such as age and *MYCN* amplification status, underscoring the prognostic value of these methylation markers. Later on, we expanded this work by MBD-sequencing based screening of 87 primary NB tumors in search for methylation biomarkers relating to event-free and overall patient survival rates, followed by validation using MSP assays in independent cohorts of 132 and 177 tumor samples. As such, Decock and co-workers established a prognostic **DNA methylation signature** consisting of 58 markers which allowed accurate outcome prediction in the total NB patient cohort; additionally, we revealed a novel specific characteristic of 4S NB tumors (Decock et al. 2016a, b, c). In brief, it was shown that specific chromosomal regions could be identified to be **uniquely hyper- or hypomethylated in stage 4S tumors** compared to the other stages, comprising genes implicated in neuronal differentiation (e.g., targets of MSX1 and GFI1) among others, as well

as genes located on subtelomeres. Based on these findings, it was hypothesized that subtelomeric DNA methylation represents an additional mechanism by which telomere length and spontaneous regression are controlled in NB. A similar study by Olsson et al. (2016) compared high- versus low-grade NB tumors using the Infinium Human Methylation 450 k (HM450K) BeadChip. Screening of 60 primary neuroblastoma cases revealed, among others, that the *TERT* gene is one of the strongest hypermethylated genes in high-risk versus low-risk tumors and that this change in the epigenetic state of *TERT* could be clearly linked to altered expression correlating to poor patient survival.

More recently, novel methods for genome-wide methylation analysis (e.g., 450 k array) resulted in more comprehensive analyses. The Westermann team applied an integrative approach to analyze the methylomes, transcriptomes, and copy number variations of 105 NB cases, complemented by primary tumor- and cell line-derived global histone modification analyses and epigenetic drug treatment in vitro (Henrich et al. 2016). This investigation further supported the presence of distinct DNA methylation patterns identifying divergent patient subgroups, with respect to survival and clinical/biological variables, including *MYCN* amplification. Similar observations were reported by Gomez et al. based on an Infinium Human Methylation 450 k (HM450K) BeadChip analysis of 35 primary NB cases (Gomez et al. 2015). In addition, non-CpG methylation was also observed and mostly associated with tumors characterized by favorable clinical-biological features.

## CpG island hypermethylation

Henrich et al. (2016) also investigated and confirmed the previously proposed CpG island methylator phenotype (CIMP) association with gene body methylation of protocadherin beta family (PCDHB) members in high-risk associated NB (Abe et al. 2005, 2007). Transcriptome integration and histone modification-based definition of enhancer elements revealed intragenic enhancer methylation as a mechanism for high-risk associated transcriptional deregulation. In this same study, the most prominent high-risk associated phenomenon was hypermethylation in combination with transcriptional downregulation of genes enriched for 1p36 genes, including the well-established dosage-dependent NB suppressor candidates, *CAMTA1*, *CHD5* and *KIF1B* (Henrich et al. 2012) (see Fig. 1). This finding suggested that DNA methylation could further reduce expression levels of tumor suppressors of which one allele is deleted. Finally, evidence was also presented for cooperation between the 'Polycomb Repressive Complex' (PRC2) (see below) activity and DNA methylation in blocking tumor-suppressive differentiation programs.

## Altered methylation profiles underlying differentiation therapy

The stage 4S NB phenotype has spurred investigations towards differentiation therapy and led to the integration of retinoic acid (ATRA) administration in particular therapy schemes. The molecular basis of the effects of ATRA in NB are still poorly understood, but (partial) epigenetic resetting towards the normal chromatin state and subsequent altered expression patterns can be assumed to be the underlying driving effector. In this perspective, the Stallings team applied genome-wide DNA methylation analysis to study epigenetic modifications during ATRA-induced neuroblastoma differentiation (Stallings et al. 2011) using an ATRA-sensitive neuroblastoma cell line. In total, 402 and 88 gene promoters were found to be hypo- and hypermethylated, respectively. Subsequent integration with mRNA expression data revealed that 82 hypomethylated genes were over-expressed (>2-fold) and 13 hypermethylated genes were under-expressed (>2-fold), indicating that many of the DNA methylation alterations had functional consequences. Further investigation towards the underlying mechanism revealed a statistically significant decrease in expression of both DNMT1 and DNMT3A upon ATRA treatment, possibly mediated through altered expression levels of miRNAs targeting mRNA stability or translation of these important DNA methylases. Later on, the same research team performed methylated DNA immunoprecipitation coupled to custom tiling array hybridization for 18 primary neuroblastoma tumors and 3 neuroblastoma cell lines to screen the methylation status of 528 miRNA loci (Das et al. 2013). In total, 67 miRNAs were found to be lower expressed (hypermethylated) in neuroblastomas compared to controls, several of which are involved in regulation of cellular differentiation.

## Global hypomethylation

Besides the phenomenon of localized hypermethylation at CpG sites, Feinberg and Vogelstein discovered that also a **genome-wide loss of DNA methylation** (Feinberg and Vogelstein 1983) (see Fig. 1) at non-CpG sites contributes to the process of malignant transformation, mainly through aberrant oncogene activation and genomic instability, the latter inferred by the fact that these sites often serve as hotspots of chromosomal breaks. Mayol et al. (2012) used the 'Infinium Human Methylation27 BeadChip' platform to generate genome-wide DNA methylation profiles from 22 primary NB cases and bisulfite sequencing for an independent cohort of 13 cases, to show that in primary NBs promoter hypermethylation is a more restricted phenomenon compared to gene-specific DNA hypomethylation.

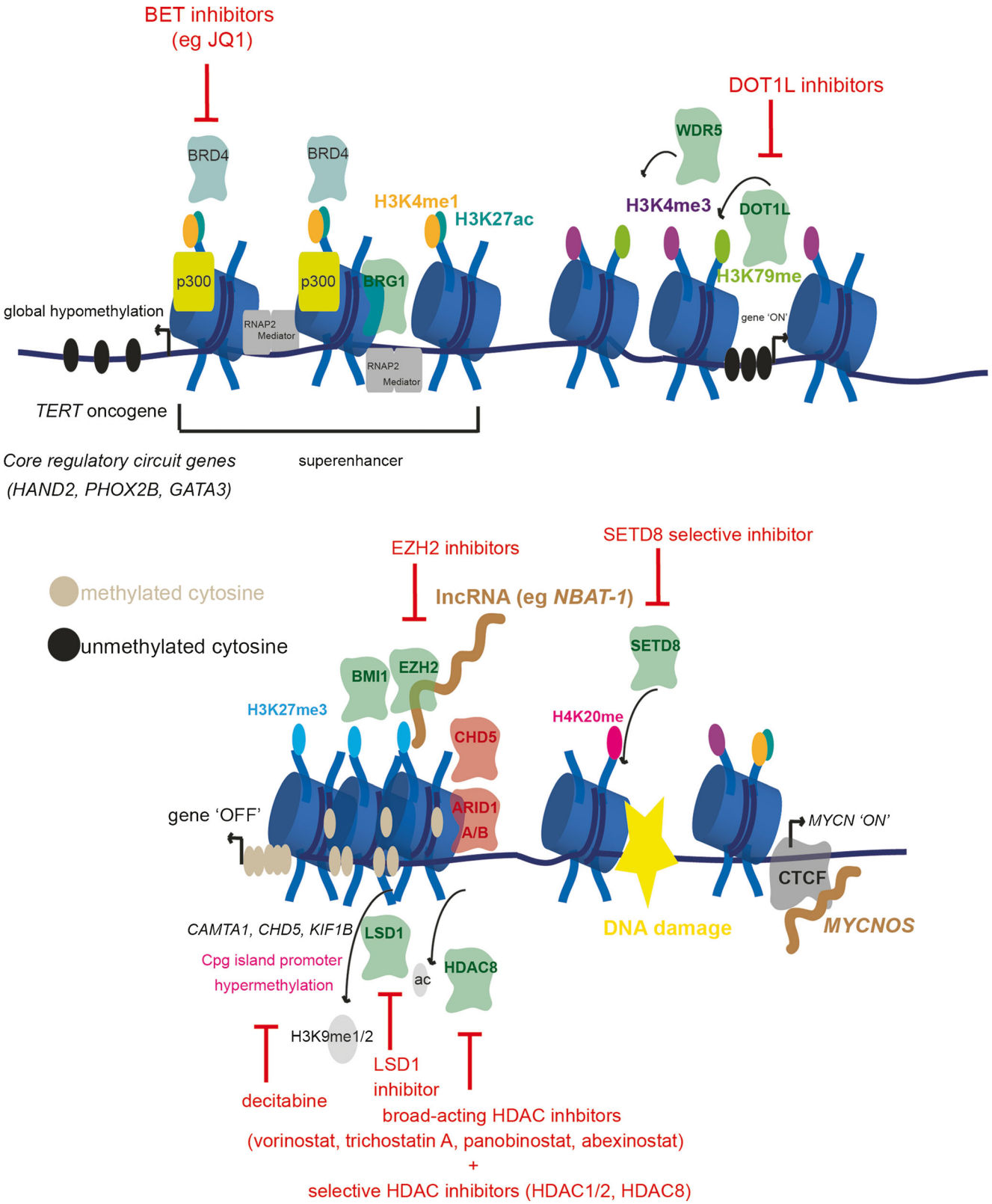


**Fig. 1** Overview of the epigenetic rewiring events that underlie NB tumor formation. Proteins in *green*: epigenetic modifiers that are overexpressed in NB cells; proteins in *pink*: epigenetic modifiers that are downregulated in NB cells through genetic aberrations

The latter was found not only to occur at promoter regions but also at more distal regulatory sites, in agreement with the later finding by the same research team that DNA methylation depletion at distal sites of the *CCND1* NB

oncogene are key to drive its overexpression in NB (Gomez et al. 2015).

### Active DNA demethylation – 5-hydroxymethylation

Initially, cytosine methylation was considered as a stable modification. It is now known that also **DNA demethylation** can take place and also plays an important role in normal development and cancer, underlying genomic instability (Jeschke et al. 2016). Demethylation of the DNA template can occur either actively or passively. Passive DNA demethylation can occur during DNA replication. Instead, active DNA demethylation is a consequence of the catalytic activity of the 'Ten Eleven Translocation' (TET) protein family (TET1, TET2, TET3). These TET enzymes convert 5-methylcytosine (5mC) to the intermediate 5-hydroxymethylcytosine (5hmC), which can be further oxidized to 5-formylcytosine and 5-carboxylcytosine (Delatte et al. 2014). These oxidation products can then be efficiently removed from the DNA template by the activity of the 'thymine DNA glycosylase' enzyme and replaced by an unmethylated cytosine residue during DNA repair (base excision repair). The role of 5hmC is also firmly established in the context of differentiation of various tissues, for example, with strong enrichment of this modification in neuronal tissue (Hahn et al. 2013) and high levels in pluripotent cells (Choi et al. 2014). Mariani et al. (2014) related **global increases in 5hmC** in NB **to the response on hypoxia**. Taking into account that neuronal tissue harbors high levels of 5hmC and that neuroblastoma cells undergo dedifferentiation in hypoxic conditions, **5hmC levels could be involved in regulation of differentiation stage transitions of NB tumor cells.**

## Histone modifier enzymes as novel players in NB tumor development

In addition to DNA methylation, a second major layer of epigenetic information is dictated by a series of post-translational modifications (PTMs) at the N-terminal tails of the core histone proteins (H2A, H2B, H3, H4 and linker histone H1) to form the so-called 'histone code (Strahl and Allis 2000). In addition, a variety of histone variants can replace the canonical ones, such as the H2A variant H2AX, which is incorporated into the nucleosomes at sites where DNA double-strand breaks occur (van Attikum and Gasser 2009) or the histone H3 variant H3.3, that specifically associates with sites of active transcription and affecting transcriptional plasticity as well as playing a key role in the process of tumorigenesis (Yuen and Knoepfler 2013). There are at least eight different types of PTMs possible on histone tails, such as lysine acetylation, lysine and arginine methylation, serine and threonine phosphorylation among others, that can be of different forms (e.g., di- or tri-methylation) (Kouzarides 2007). Particular modifications can mark regions of active or inactive transcription. Importantly, histone modifications are also specific in demarcating various functional elements in the genome such as enhancers or transcribed regions (Zhou et al. 2011). Histone modifications are dynamic in nature, and a plethora of **histone modifier enzymes** has been identified in the last decades that are involved in the deposition ('writers') or removal of the histone PTMs ('erasers'). A third class of proteins can dock to certain histone modifications through a specific binding domain, but have no intrinsic enzymatic activity ('readers') and act to recruit writers and erasers for control of the expression of target genes. For example, the plant homeodomain pocket is one of the most commonly found domains enabling histone methyl-lysine binding.

### Histone acetyltransferases/deacetylases (HATs/HDACs)

One of the most prominent members of the 'writer/eraser' class of histone modifier enzymes are the histone acetyltransferases (HATs)/deacetylases (HDACs), serving as an important switch between repressive and permissive chromatin states. Both HATs and HDACs serve as co-factors, implying that they are recruited by other DNA-binding factors to the site-of-action rather than associating themselves with the DNA template. Three main types of HATs (the GNAT5, CBP/p300 and MYST subfamilies) (Gajer et al. 2015) and 18 different HDAC proteins (Ropero and Esteller 2007) can be distinguished in mammalian cells. One of the most prominent HATs is CBP/p300 (see Fig. 1), expressed in many different tissue types and implicated in the regulation of various normal cellular processes such as cell cycle regulation and differentiation (Sterner and Berger 2000), but also a known player in malignant transformation either as a tumor suppressor or supporting the action of specific oncogenes (Goodman and Smolik 2000). Altered HDAC expression has been reported in NB (West and Johnstone 2014). Oehme et al. (2009a, b) screened the expression of human the HDAC family in a primary NB cohort of 251 patients by micro-array based profiling and an independent cohort of 118 samples by real-time RT-PCR and found that *HDAC8* overexpression significantly correlated to poor patient prognosis. In keeping with this finding, Rettig and colleagues demonstrated in vitro and in vivo potency of selective HDAC8 inhibitors for NB treatment (Rettig et al. 2015) (see Fig. 1). In addition, the key neuroblastoma driver oncogene MYCN has been shown to cooperate with HDAC proteins during malignant transformation to repress its target genes, e.g., with HDAC5 in the silencing of *CD9* (Fabian et al. 2016), with HDAC2 to downregulate the expression of the tumor suppressor *miRNA-183* (Lodrini et al. 2013) and with HDAC3 in silencing of *GRHL1* (Fabian et al. 2014).

## Histone methyltransferases/demethylases (HMTs/HDMs)

The second type of enzymes that can influence the histone code are the **histone methyltransferases (HMTs) and histone demethylases (HDMs)**. Histone methylation can either be placed on lysine or arginine residues and is executed by proteins (total of 48 in mammalian cells) containing a 'suppressor of variegation' (SET) domain (Albert and Helin 2010), with DOT1L being the only member not containing a SET pocket. For the latter, Wong et al. (2017) very recently described a role in NB tumorigenesis together with MYCN in downstream target activation, due to its unique capacity of all HMTs to catalyze methylation of H3K79 (see Fig. 1). In addition, the expression of *DOT1L* itself was also driven by MYCN and its high levels in neuroblastoma tumors were shown to correlate with poor survival. Application of a DOT1L inhibitor proved antitumorigenic potential both in vitro and in neuroblastoma xenografts, indicating the therapeutic potential for treatment of MYCN-driven NB tumors. Besides H3K79 methylation, H3K4me3 is also an established epigenetic mark for permissive gene expression. Among others, WDR5 is a core component of the H3K4me3 writer complex MLL–SET1 (see Fig. 1) and is known to function as a key co-factor of the MYC oncoprotein in cancer (Thomas et al. 2015). In NB, similar to DOT1L, *WDR5* expression levels are upregulated by MYCN and indicate poor survival changes in primary cases. The formation of a transcriptional complex between MYCN and WDR5 leads to downstream target activation, such as the anti-apoptotic factor MDM2 (Sun et al. 2015). Very recently, Veschi and co-workers performed a high-throughput screening strategy combining RNAi (siRNAs) for 400 genes and 21 epigenetic chemical probes (Veschi et al. 2017) to identify epigenetic modulators that suppress NB cell differentiation. From this screen, the H4K20 methyltransferase SETD8 was identified as a top candidate (see Fig. 1), with high SETD8 levels in primary NB tumors correlating to poor patient prognosis. Genetic and pharmacological perturbation of SETD8 resulted in a significant reactivation of p53 signaling, inducing a pro-apoptotic and antiproliferative response both in vitro and in vivo, the latter underscored by a significant reduction of murine xenograft tumor growth.

In contrast to H3K79 and H3K4 methylation which are associated with gene activation, methylation of other lysine residues of histone H3/4 such as K9 or K27 induces transcriptional silencing. The **G9a H3K9 methyltransfersase** was shown by Ke and colleagues to fulfill an oncogenic role in NB development by epigenetic regulation of autophagosome formation and pinpoint G9a as a novel potent NB therapeutic target (Ke et al. 2014). The formation of H3K27me3 is catalyzed by PRC2, a multi-subunit assembly that is essential during normal embryonic development (Hock 2012). The catalytic component of PRC2 is the 'Enhancer of Zeste' (EZH2) protein (see Fig. 1) and its efficiency requires the action of the co-factors EED and SUZ12. In cancer, PRC2 may act either as an oncogene or tumor suppressor, depending on the cellular context and tumor type. In its association with the co-factor MAX and the aforementioned histone methyltransferase WDR5, MYCN is acting as a transcriptional activator. Its role as a transcriptional regulator has now proven to be dual, and MYCN is equally capable of repressing a subset of its downstream target genes, predominantly involved in neuronal differentiation (Gherardi et al. 2013). In this context, MYCN has been shown to physically interact with PRC2 (Corvetta et al. 2013; He et al. 2013), although further research will be required to show what the functional importance of this interaction is in the context of MYCN regulated gene expression programs. In NB, MYCN-amplified tumors show a 'PRC2 hyperactivation' profile, exhibiting very strong expression levels of the PRC2 components, EZH2, EED and RBBP7, versus non-amplified NB tumors (Henrich et al. 2016). This leads to a significant enrichment of H3K27me3 signal for a set of genes of which hypermethylation and concomitant downregulated expression is associated with high-risk NB. In addition, Wang and colleagues have previously shown that EZH2 overexpression in NB is related to poor patient prognosis and that PRC2 suppresses differentiation through epigenetic silencing of tumorsuppressor genes like *CASZ1* (Wang et al. 2012). It was previously shown that MYC is responsible for direct upregulation of EZH2 expression and that MYCN could act likewise in the context of NB (Neri et al. 2012; Tsubota et al. 2017). Recently, Bate-Eya et al. (2017) suggested that the recurrent gain of the *EZH2* locus could contribute to its overexpression. Notably, the survival advantage hereby conferred to NB cells was shown to be independent of its catalytic activity but rather caused by a so far uncharacterized non-canonical EZH2 function. Therefore, in NB, the application of drugs inhibiting EZH2 activity might be required to be directed to the complete protein functionality rather than a moiety targeting only its catalytic pocket, which will require further investigation.

Besides EZH2 as part of the PRC2 complex, the PRC1 component BMI1 also has an established role in NB development. The PRC1 complex can either be recruited to its target genes by K27me3 modifications imposed on the chromatin template by PRC2, or can also bind independently of PRC2 (Hock 2012). The **PRC1 core component BMI1** (see Fig. 1) is overexpressed in as much as 90% of neuroblastoma cases (Huang et al. 2011), and has been shown by Cui et al. (2007) to be a cooperative oncogene in MYCN-driven NB. Its overexpression (together with MYCN) can be driven by E2F1 (Nowak et al. 2006), but in addition MYCN itself can also

induce high *BMI1* expression levels in NB cells (Ochiai et al. 2010), with concomitant inhibition of cell death and differentiation through inhibition of tumor suppressor gene expression (e.g., *KIF1β*).

A key lysine demethylase implicated in NB is LSD1 (KDM1A). The LSD1 protein specifically catalyzes demethylation of H3K9me1/2 and H3K4me1/2 (Hosseini and Minucci 2017) (see Fig. 1) and plays essential functions during normal development (e.g., embryogenesis) as well as malignant transformation, with high LSD1 levels being observed in many different cancer types (Zheng et al. 2015). In NB, elevated LSD1 expression was shown to be correlated with adverse outcome and inversely correlated with differentiation in neuroblastic tumors. LSD1 depletion decreased cellular growth, induced expression of differentiation-associated genes, and increased target gene-specific H3K4 methylation and reduced neuroblastoma xenograft growth in vivo. Subsequently, miRNA-137 was identified as a LSD1 negative upstream regulator and a novel tumor suppressor in NB development, with downregulation of LSD1 mimicking miRNA-137 re-expression in neuroblastoma cells (Althoff et al. 2013). More recently, Amente et al. (2015) revealed a functional cooperation of LSD1 with the central NB oncogene MYCN through a physical interaction, supporting a **transcriptional repressor role of MYCN**, among others, facilitating silencing of *NDRG1*, a tumor suppressor gene normally suppressing NB cell motility and invasion capacity (Ambrosio et al. 2017). Also, the H3K9me2/3 demethylase KDM4B was recently identified as another novel MYCN interaction partner in NB, assisting in sustaining tumorigenesis (Yang et al. 2015). These results thus indicate the therapeutic value of combined MYCN–KDM pharmacological inhibition.

## Chromatin remodeling complexes

Chromatin remodeling complexes are a specific type of regulators catalyzing the eviction or deposition of nucleosomes coupled to ATP hydrolysis, consisting of: the 'Switching defective/Sucrose Non-Fermenting' (SWI/SNF) family, 'Inositol requiring 80' (INO80), 'Imitation Switch' (ISWI) and the 'Chromodomain Helicase DNA binding' (CHD) family (Clapier and Cairns 2009).

From the latter class, the ATP-dependent helicase CHD5 (see Fig. 1) has been proposed as a key tumor suppressor gene (Bagchi et al. 2007) targeted by deletion of 1p36 in high-risk (often MYCN-amplified) neuroblastomas. Low CHD5 levels are associated with poor patient prognosis. Alternatively, high-risk NB patients can also present with *CHD5* distal promoter methylation (Koyama et al. 2012; Fujita et al. 2008), further supporting a tumor suppressor role of CHD5 in NB tumor development. High CHD5 levels can be found in the brain and to a lesser extent in the adrenal glands (Thompson et al. 2003), underscoring its importance in normal neuronal development and neuronal differentiation downstream of TRKA (Higashi et al. 2015) as part of a 'nucleosome remodeling and deacetylation' (NurD) protein complex (Potts et al. 2011). To execute its function, CHD5 associates with the NurD complex, leading to the formation of a transcriptional repressor complex dampening the expression of the G2/M checkpoint kinase WEE1 (Quan et al. 2014). Additionally, several oncogenic miRNAs downstream (e.g., *miRNA-204*) of MYCN have been identified to reduce *CHD5* expression in NB tumors (Naraparaju et al. 2016). Furthermore, the role of CHD5 as a tumor suppressor is also well established in other tumor entities, such as glioma, breast and colon cancer (Kolla et al. 2014).

The SWI–SNF complex fulfills key tasks during normal mammalian development, with evidence for a role in heart and muscle development, neuronal development and hematopoiesis (Cedar and Bergman 2011). Mechanistically, the SWI–SNF remodeler subunits have an important role in nucleosome eviction to allow for transcriptional activation and elongation by POLII (Subtil-Rodriguez and Reyes 2010; Schwabish and Struhl 2007). The complex is structurally conserved and the components that drive its remodeler activity are part of the core, whereas other constituents are rather tissue-/context-specific (Tang et al. 2010). In total, this combinatorial complexity leads to over 100 different possible assemblies (Ho and Crabtree 2010). This diversification in composition also occurs during neuronal development (Lessard et al. 2007), with stage-specific changes, e.g., from embryonic stem cells to neuronal progenitor cells (Son and Crabtree 2014). From the subunits conferring functional specificity, ARID1A is known, together with ARID1B, as a subunit specific to the 'BAF'-type SWI–SNF complex and is one of the most commonly mutated subunits in cancer (Shain and Pollack 2013). Overall, one-fifth of all human tumors exhibit mutations in SWI–SNF complex components, demarcating it as the most frequently mutated epigenetic regulator complex and underscoring a critical tumor suppressor role for its constituents in various human cancer types (Kadoch et al. 2013). In pediatric cancer entities, the crucial role of the complex is underscored by the fact that atypical teratoid/rhabdoid tumors (ATRT) can be divided into three molecular epigenetic subytpes, but with no other recurrent mutations found by exome sequencing besides those found in the *SMARCB1* gene (Johann et al. 2016). A whole genome and exome sequencing effort by Sausen and colleagues revealed ***ARID1A/B* mutations** (see Fig. 1) and deletions in 11% of neuroblastoma cases (Sausen et al. 2013), and targeted sequencing performed by Lee et al. confirmed that cases with *ARID1B* mutations present with high-risk NB (Lee et al. 2017). The importance of ARID1B function in normal neural development is underscored by the causal role of de novo *ARID1B* mutations in the congenital syndrome Coffin–Siris, as these patients display neurodevelopmental deficits (Santen et al. 2012).

From the core constituents of the mammalian SWI–SNF complex, the central ATPase, either represented by BRG1 (SMARCA4) or BRM (SMARCA2), is the critical driving force to alter nucleosome positioning using energy derived from ATP hydrolysis. While, in many tumor entities, BRG1 is known as a tumor suppressor, it may also serve as key driver of an oncogenic expression program, as illustrated by the study of Shi et al. showing that BRG1 in acute myeloid leukemia is required to sustain MYC expression (Shi et al. 2013). In NB, high BRG1 levels are associated with poor patient prognosis, pointing towards its potential as a novel therapeutic target (Jubierre et al. 2016).

## Core regulatory circuits in neuroblastoma development

Recently, super-enhancer-driven core regulatory circuits have been defined that underlie NB development, further illuminating the complex biology of this disease. One of the fundamental driving forces in epigenetic reprogramming has been linked to the activity of so-called super-enhancers (SEs) or clustered enhancers (see Fig. 1). These are the main control elements coupled to genes encoding master transcription factors defining cellular identity, and are typically characterized by binding of a multi-factorial regulatory complex (Pott and Lieb 2015; Heinz et al. 2015) and a very high density of the enhancer-associated histone modifications, H3K27ac and H3K4me1, that mark high chromatin accessibility of these regions. Key players at SE sites include Mediator, RNA Pol II, CBP-p300 and a plethora of chromatin modifiers, with a prominent role for SWI–SNF complex components and the 'bromodomain and extra-terminal motif' (BET) protein family member BRD4 (see Fig. 1) (Hnisz et al. 2013). The recruitment of BRD4 occurs through its capability of recognizing and binding of acetylated histones and its direct interaction with the Mediator complex (Pott and Lieb 2015), ultimately facilitating both transcriptional activation and elongation (Li 2002). Tumor cells typically exploit the power of this specific class of regulatory elements to aberrantly boost the expression of oncogenes driving the major cancer hallmark dependencies (Hnisz et al. 2013; Whyte et al. 2013).

A first example of a SE-driven transcription factor in NB is *LMO1* (Oldridge et al. 2015), with oncogene addiction driven by a **single nucleotide polymorphism** (SNP), previously found as a germline SNP in a neuroblastoma GWAS study, residing in a super-enhancer identified in the first exon of *LMO1* and serving as a critical determinant for GATA3 binding to the SE site. Besides polymorphisms, **chromosomal rearrangements** involving super-enhancer sites form another mechanism for aberrant gene activation. Through whole-genome sequencing of 75 high-risk neuroblastoma cases, Valentijn and co-workers recently identified that rearrangements involving the *TERT* gene (Peifer et al. 2015) comprise about 23% of high-risk NB cases, with half of those cases bearing a translocation leading to SE hijacking (Valentijn et al. 2015).

Although the majority of gene expression programs involves many different transcriptional regulators, it has been shown that only a handful of them, denominated as **core transcription factors**, define the framework of cell-specific networks (Saint-Andre et al. 2016), with their target genes marked by SE sites. These core regulators, of which the expression is also SE-driven, work within a constellation of a 'core regulatory circuitry', in which they also regulate one another expression (formation of an auto-regulatory loop). These circuitries are also key in the process of malignant transformation, involving essential oncogenic factors. This concept was previously illustrated by Sanda and colleagues in the context of acute T-cell leukemia (Sanda et al. 2012), where TAL1 together with GATA3 and RUNX1 forms a positive feed-forward loop converging to drive MYB expression, in its turn activating TAL1 complex target genes. More recently, Van Groningen et al. (2017) identified super-enhancer-associated transcription factor regulatory circuits underlying interconvertible neuroblastoma cell states of the adrenergic versus mesenchymal type, with neuroblastoma tumors consisting of a heterogeneous mixture of both cell types. Whereas MEOX1/2 and SOX9 among others were identified as master regulators driving the mesenchymal cell state, GATA3 and HAND1 were found to support the adrenergic state. Binding of these transcriptional regulators to their own enhancer, as well as with the enhancers driving the expression of the other core regulators driving that cell state, enforces regulatory loops that sustain the required identity program. In addition, switching between these identities was shown to be supported by the plasticity of the super-enhancer profile underlying the actual reprogramming from one state to the other. In a parallel paper, Boeva et al. (2017) defined mainly PHOX2B as well as HAND2 and GATA3 as the main module controlling the sympathetic noradrenergic cell type in NB tumors, and defined a second population of a neural crest cell-like population sustained by the FOS/JUN transcription factor family of which the expression anticorrelates to the noradrenergic transcription factor module, with PHOX2B and AP-1 found to regulate the super-enhancer landscape of the two respective cell types. In MYCN-amplified NB tumors, the neural crest cell module was found to be repressed. Based on an integrated landscaping of NB transcriptome and epigenome landscapes, these studies reveal tumor heterogeneity as a novel aspect to be taken into account in the rationalization of novel therapeutic strategies for NB patients.

## Long non-coding RNAs and chromatin architecture dynamics in gene expression regulation

The study on functional annotation of the non-coding genome has yielded further insights into the complex epigenetic regulation of gene transcription. In addition to miRNAs, implicated in negative regulation of mRNA stability or translation of its target transcripts, long non-coding RNAs (lncRNAs) are a newly emerging important class of non-coding RNAs (Goff and Rinn 2015; Rinn and Chang 2012). While the function of most lncRNAs is still unknown, organism complexity is strongly correlated to the proportion of the genome that is non-coding (Liu et al. 2013a, b). Of further notice, in contrast to miRNAs, lncRNAs have been shown to display a wide variety of mode-of-actions (Bartonicek et al. 2016). The diversity of their folding or structure is crucial to support the interaction with protein complexes and the specific functions that lncRNAs are able to execute (Yan et al. 2016). In addition, recent discoveries have proven their cell-type-specific expression, with important implications both in normal development and during malignant transformation (Haemmerle and Gutschner 2015) and thereby influencing various processes such as genomic stability, cell proliferation and survival (Huarte 2015).

Further, given the tissue-specific expression pattern of many lncRNAs, they may represent important and **powerful biomarkers** in diagnosis and monitoring of disease and are currently being scrutinized to that end. This has been illustrated in prostate cancer where researchers identified lncRNA *PCA3* as being highly prostate-specific (Ronnau et al. 2014). As a further consequence, lncRNAs can act as suitable therapeutic targets as their specific expression will avoid unwanted toxic side effects in normal tissues (Gloss and Dinger 2016), as illustrated by the identification of the melanoma-specific lncRNA *SAMMSON* (Leucci et al. 2016). Taken together, it can be expected that a better understanding of the previously so-called 'dark matter' of the genome will have a profound impact on our understanding of the complex regulation of various cellular processes, including replication, transcription, splicing, DNA repair and chromatin conformation. Together with steadily evolving opportunities for genome editing and RNA therapeutics, a new era of revolutionized targeted therapy is now emerging.

Just like protein-coding genes, lncRNAs can serve as **biomarkers for prognosis** as well as **diagnosis**. Pandey et al. (2014) identified a signature of 24 unannotated lncRNAs that allows the discrimination between low- and high-risk NB cases, with *NBAT-1* as a top-candidate novel biomarker for patient survival. In high-risk NB tumors, the *NBAT-1* locus is silenced by **DNA methylation**. In addition, a single nucleotide polymorphism (SNP) on 6p22 was shown to also significantly affect *NBAT-1* expression. Functionally, *NBAT-1* is acting as a tumor suppressor through its interaction with the **PRC2 component EZH2** (see Fig. 1), silencing the expression of, e.g., *SOX9*. Similar to *NBAT-1*, another risk-associated SNP mapping on 6p was associated with the locus encoding the lncRNA *CASC15-S* (Russell et al. 2015), with its low expression also linked to poor patient prognosis. Similarly, loss of *GAS5* lncRNA expression is also a poor prognostic marker in NB, as recently reported, in line with previously observed marked reduction of *GAS5* levels in aggressive breast and gastric cancer. Loss of this tumor suppressor lncRNA is mainly contributing to malignant transformation through the **induction of p53 signaling** leading to a cell cycle arrest (Mazar et al. 2017). Although no differential *GAS5* expression could be observed between MYCN-amplified versus non-amplified NB cases, *GAS5* splice variants may be different between the two groups.

One of the best characterized lncRNAs so far implicated in cancer is *MALAT1*, involved in many tumor types such as prostate, ovarian and colorectal cancer. Very recently, Bi et al. (2017) described the functional implication of *MALAT1* as a novel oncogene in NB development through its positive regulation of AXL expression, thereby promoting the invasive and migration potential of NB cells. Poor prognosis of NB patients is often linked to *MYCN* amplification, but in this same region Liu et al. (2014) also identified *lncUSMycN* and the MYCN antisense transcript *NCYM* to be co-amplified. Mechanistically, the lncRNA *lncUSMycN* upregulates *NCYM* expression followed by its binding to the RNA-interacting protein NonO, thereby upregulating *MYCN* expression (Liu et al. 2016). Another MYCN upstream regulatory lncRNA is *MYCNOS* (chr2p24). To this end, the *MYCNOS* transcript binds to CTCF (see Fig. 1), facilitating its association to the *MYCN* promotor region, resulting in its transcriptional upregulation. Sahu et al. applied an integrative analysis of lncRNA expression data determined by either micro-array or RNA-sequencing of primary NB cases. From the 51 lncRNA candidates derived from the RNA-seq dataset, only 6 were overlapping with those determined as differentially expressed between the two groups, among others the lncRNA *SNHG1* (Sahu et al. 2016). This study revealed a positive correlation between *SNHG1* and *MYCN* expression levels, with high *SNHG1* levels corresponding with poor disease outcome, and, if co-expressed with the protein-coding gene *TAF1D*, a potential interaction effect may be observed on patient outcome.

## Deregulated 3D nuclear architecture in NB

The chromatin landscape is physically distributed yet functionally connected in the three-dimensional nuclear space. To this end, a modular organization takes place into three-dimensional folds of so-called 'topologically associating

domains' (TADs) that form in a cell-type specific manner (Ciabrelli and Cavalli 2015). The TAD borders form an insulated neighborhood and are evolutionary conserved. This organization involves several architectural proteins, with the 'CCCTC binding factor' (CTCF) as the well-known insulator protein that recruits cohesins to stabilize the TAD borders. The occurrence of CTCF at TAD borders comprises only 15% of genome-wide CTCF binding, and the majority is comprised within TADs where CTCF instructs proper enhancer–promoter interactions (Symmons et al. 2014). Disruption of these insulated neighborhoods can lead to aberrant activation of proto-oncogenes within a certain TAD, through interaction with previously inaccessible enhancer regions outside the TAD region and can be a consequence of several genomic insults. In gliomas, **hypermethylation of the CTCF binding sites** are shown to disrupt the insulation of the *PDGFRA* proto-oncogene from an enhancer region, leading to its aberrant activation (Flavahan et al. 2016). Besides DNA methylation, insulated neighborhoods can also be distorted as a consequence of genetic aberrations. A prototypical example is the identification of microdeletions in acute T-cell leukemia, perturbing the CTCF borders of a TAD and thus presenting a novel mechanism to drive *TAL1* or *LMO2* overexpression (Hnisz et al. 2016). In NB, the studies of Peifer et al. (2015) and Valentijn et al. (2015), as previously discussed, link chromosomal rearrangements to TAD distortions, ultimately resulting in increased *TERT* expression associated with high-risk NB tumor development.

CTCF plays a key role in normal neuronal development (Hirayama et al. 2012) and has been previously described in NB as one of the main targets of the tumor suppressor *miRNA-34a* (itself a known MYCN target gene in NB development) (De Antonellis et al. 2014). Zhao et al. (2016) identified CTCF through an integrative data-mining approach of transcriptome and ChIP-sequencing as a novel driver of *MYCN* expression in NB cells (as stated previously to be facilitated by its interaction with the previously discussed non-coding RNA *MYCNOS*), thereby serving as a driving force for neuroblastoma progression.

## Towards drugging of the NB epigenome

### Targeting DNA methylation

The dynamic and reversible character of the epigenome, as well as its cancer- and cell-type-specific signature, make it a very attractive target for therapy. Agents that modulate DNA methylation, like the DNA methyltransferase inhibitor 5-aza-2-deoxycytidine, have been previously shown to be effective in the context of NB, both in vitro and in vivo, for reactivation of tumor suppressor genes like '*thrombospondin-1* (*THBS1*)' and '*heat-shock protein 47*' (*HSP47*) (Yang et al. 2003, 2004).

### HDAC inhibition - tumor suppressor re-activation

Although it was initially anticipated that pharmacological inhibition of HDAC enzymes for cancer treatment would evoke toxicity towards normal cells (and thus elicit strong side-effects), the rationale for the introduction of HDAC inhibitors in cancer therapy regimens has been proven successful with promising antitumor effects in clinical trials due to their very high levels in tumors compared to normal cells, thereby creating a therapeutic window. In NB, as in several other cancers, HDAC inhibition leads to p21 upregulation, thereby inducing antiproliferative effects (Oehme et al. 2009a, b). Broad-spectrum HDAC inhibitors (see Fig. 1) like vorinostat and trichostatin A (TSA) have been shown to be effective in the context of NB, but evoke unwanted side-effects. However, in combination with other existing therapeutic strategies or small-molecules, their potency can be increased. Mueller and co-workers proposed the use of vorinostat to potentiate the response of metastatic NB tumor cells to radiation therapy, given that vorinostat interferes with the expression of key DNA damage repair enzymes like RAD51 (Mueller et al. 2011). The potency of this combinatorial therapeutic strategy is now being tested in clinical trials for various solid tumors. Following a similar reasoning, Wang et al. showed synergistic effects of combining the pan-HDAC inhibitor panobinostat with DNA damage-inducing agents like cisplatin or etoposide, partially related to repressive effects of panobinostat to CHK1 signaling (Wang et al. 2013). Waldeck and colleagues evaluated the in vivo potential of panobinostat treatment against NB tumors using the well-established TH-MYCN mouse model. Long-term (9 weeks) exposure to panobinostat resulted in strong antitumor regression without regrowth as a consequence of induced tumor cell differentiation (Waldeck et al. 2016). In the course of potent combination strategies with broad-spectrum HDAC inhibitors, the study of Kroesen et al. (2016) provided a basis for clinical testing of combined anti-GD2 antibody and vorinostat NB treatment, given that vorinostat treatment leads to upregulation of surface GD2 expression on NB tumor cells and its modulation of the tumor microenvironment to be more permissive for tumor-targeting antibody therapy. Another example of this strategy was illustrated by Sholler et al. (2013) showing in vitro synergism between the HDAC inhibitor abexinostat and the proteasome inhibitor bortezomib, through upregulation of NOTCH signaling and repression of MYCN expression, but mainly effective through induction of reactive oxygen species induced apoptosis, an effect previously shown to underlie the potency of this drug combination to combat Hodgkin lymphomas (Bhalla et al. 2009). Now, more selective HDAC inhibitors are being developed, such as HDAC8 specific inhibitors (see Fig. 1) that have been shown to induce neurite formation and differentiation in NB cells (Oehme et al. 2009a). In the same line, HDAC5 would be an interesting therapeutic target given its

capability to promote invasion/metastasis and suppress differentiation (Fabian et al. 2016; Sun et al. 2014). Shahbazi and co-workers identified that MYCN cooperates with HDAC2, itself a target of MYCN (Marshall et al. 2010), to silence TP53INP1 expression in NB leading to reduced p53 activation (Shahbazi et al. 2014). Frumm and co-workers discovered, through a chemical probing approach for compounds that can induce NB cell differentiation, the selective HDAC1/2 inhibitor BRD8430 (Frumm et al. 2013). Furthermore, this study provided evidence for synergism of BRD8430 with *cis* retinoic acid as a pro-differentiation agent by non-dependent mechanisms. In addition, 'Sirtuin 2' (SIRT2) inhibitors are also indicated for MYC(N) driven cancer entities like NB, given the role of SIRT2 in MYCN protein stabilization and through repression of NEDD4 ubiquitin ligase expression (Liu et al. 2013a, b). Ling et al. showed that the potency of HDAC inhibitors such as vorinostat can be increased by a combined treatment with transamidation activators, based on the rationale that HDAC inhibitors like SAHA lead to increased expression of specific transglutaminase enzymes, rendering tumor cells less sensitive to their cytotoxic effects (Ling et al. 2012).

### Targeting oncogenes and tumor dependency genes through interference with the epigenetic transcription machinery

As mentioned above, the cancer epigenome landscape dictates a transcriptional regulatory network. This represents a druggable vulnerability as nicely illustrated by Veschi et al. (2017). Shahbazi et al. (2016) recently described that MYCN-amplified tumors are particularly sensitive to 'BET bromodomain inhibitors (see Fig. 1), given that BRD4 inhibition leads to a general downregulation of the MYCN downstream transcriptional network (Puissant et al. 2013). Moreover, the central role of MYCN in neuroblastoma oncogenesis with 25% of all primary cases harboring a MYC(N) amplification, but with limited expression in other mature tissues, makes it a very attractive target in neuroblastoma treatment. Interestingly, it was shown that JQ1, besides a consensus gene expression signature, also evokes a gene signature uniquely regulated in neuroblastoma, including genes for neuronal cell identity, e.g., *PHOX2B*. Recently emerging studies have proved that JQ1 is a very potent tool compound for promising synergistic drug combinations. Shahbazi and co-workers identified JQ1-panobinostat as a drug combination that could serve as a very potent approach for treatment of high-risk NB cases, given their synergistic downregulation of MYCN and LIN28B expression levels (Shahbazi et al. 2016). Besides JQ1, other BET inhibitors have also been shown to be very potent to kill NB cells, such as I-BET726, through suppression of apoptosis and MYCN downstream signaling (Wyce et al. 2013). A more pre-clinically potent BET inhibitor, OTX015, has recently been developed and proven its efficacy both in murine and human MYCN-driven in vivo tumor models (Henssen et al. 2016).

A further approach to target this transcriptional network was published by Chipumuro et al. (2014). This paper started from the rationale that cycles of transcription initiation and elongation are particularly dependent on the action of specific cyclin-dependent kinases (CDKs), with CDK7 as one of the most prominent members implicated in this process. In this respect, they reasoned that hampering of MYC(N)-driven transcriptional amplification could be an interesting feature to exploit for therapeutic purposes. The expression of MYC(N) is in large part driven by an associated SE site and so particularly sensitive to interference to perturbations at the transcriptional level. In this way, they showed that pharmacological inhibition of CDK7 activity using the small molecule THZ1 leads to selective targeting of MYC(N)-amplified NB linked to its transcriptional interference inflicted to SE-linked oncogenes like MYCN.

## Epilogue

As recently proposed, epigenetic plasticity and resistance are essential aspects forging the cancer cell phenotype and they are logically interconnected with the hallmarks of cancer cells (Hanahan and Weinberg 2011).We have summarized the various layers of epigenetic (re)wiring during neuroblastoma tumor formation and how those insights can be exploited for the improvement of patient diagnosis and prognosis, and the development of innovative and targeted treatment protocols. Future efforts should also be focusing on circumvention of potential resistance mechanisms and prevention of relapse, given that epigenetic processes also support tumor cell escape to drugging effects and host immune surveillance (Frumm et al. 2013). Given that, as for chemotherapy, novel therapies using single compounds are destined to lead to treatment resistance, new combinatorial approaches including epigenetic drugs, together with other established treatment protocols like chemotherapy or immunotherapy, are considered as the future path towards more successful treatment. Detailed epigenomic landscaping of NB tumor cells can therefore be expected to become an essential part of the diagnostic genetic work up, given that about 20% of mutations in pediatric cancers are found in genes encoding key epigenetic regulators (Huether et al. 2014). These genomic changes contribute to major changes in the epigenetic lands of tumor cells that also play a significant role during malignant transformation. Integrative epigenome analysis will therefore be key to better characterize tumors in a diagnostic setting and will further open novel opportunities for therapeutic intervention. One could think of the possibility to perform whole-genome bisulfite

sequencing on circulating tumor DNA obtained from liquid biopsies to integrate methylation profiling in a diagnostic setting or ATAC-seq to obtain chromatin accessibility profiles on primary patient-derived cells.

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