

## Review

# Intrinsic transcriptional heterogeneity in neuroblastoma guides mechanistic and therapeutic insights

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## SUMMARY

Cell state is controlled by master transcription factors (mTFs) that determine the cellular gene expression program. Cancer cells acquire dysregulated gene expression programs by mutational and non-mutational processes. Intratumoral heterogeneity can result from cells displaying distinct mTF-regulated cell states, which co-exist within the tumor. One archetypal tumor associated with transcriptionally regulated heterogeneity is high-risk neuroblastoma (NB). Patients with NB have poor overall survival despite intensive therapies, and relapsed patients are commonly refractory to treatment. The cellular populations that comprise NB are marked by different cohorts of mTFs and differential sensitivity to conventional therapies. Recent studies have highlighted mechanisms by which NB cells dynamically shift the cell state with treatment, revealing new opportunities to control the cellular response to treatment by manipulating cell-state-defining transcriptional programs. Here, we review recent advances in understanding transcriptionally defined cancer heterogeneity. We offer challenges to the field to encourage translation of basic science into clinical benefit.

## Introduction

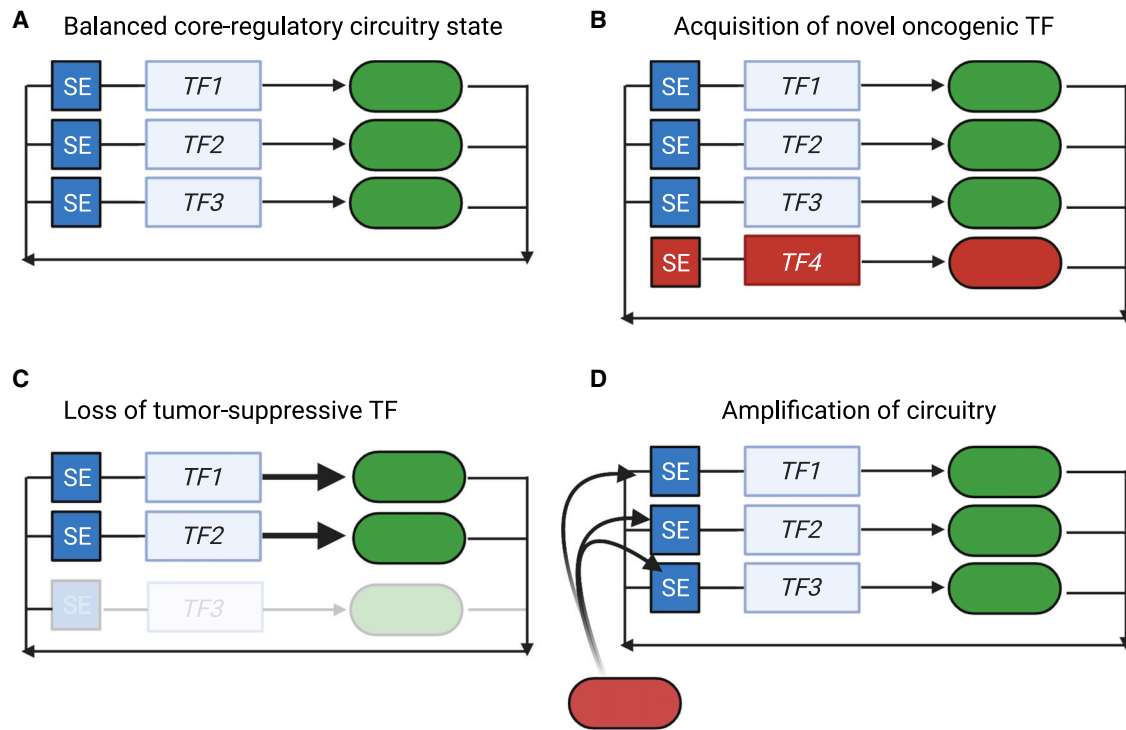
The collective activities of the expressed master transcription factors (mTFs) in a given cell type result in the recruitment of co-factors and the licensing of the RNA polymerase II complex to perform mRNA transcription at gene enhancers and promoters. This process establishes the transcriptome of a cell, which manifests the identity of that cell (reviewed in Bradner et al.<sup>1</sup>). This fundamental process is dysregulated in cancer by a variety of alterations in mTF activity. This may include changes in upstream signaling, chromosomal translocations yielding *de novo* fusion TFs, overexpression/amplification, or epigenetic dysregulation resulting in changes in chromatin accessibility to TFs (reviewed in Bradner et al.<sup>1</sup>). The capacity of mTFs to determine a cell state are shaped in cancer by superimposed mutational processes. The combined effects of the underlying transcriptional cell state and superimposed mutational effects result in dysregulation of transcription, facilitating lineage-specific oncogenesis and heightened tumor-cell dependency on transcriptional processes (so-called “transcriptional addiction”).<sup>1–4</sup> Within individual tumors, distinct subpopulations of tumor cells form a complex and dynamic heterogeneous entity. These different subpopulations may respond differently to exogenous stimuli, resulting in selected tumor evolution in the context of cancer therapy.

Tumor heterogeneity is a fundamental property of several different types of cancer and has been described in acute lymphoblastic leukemia,<sup>5</sup> non-small cell lung carcinoma,<sup>6</sup> colo-

rectal carcinoma,<sup>7</sup> breast carcinoma,<sup>8,9</sup> pancreatic carcinoma,<sup>10</sup> melanoma,<sup>11</sup> diffuse intrinsic pontine glioma,<sup>12</sup> and malignant glioma and medulloblastoma,<sup>13</sup> among others. One tumor displaying hallmark cellular heterogeneity associated with intrinsic transcriptional heterogeneity is pediatric high-risk neuroblastoma (NB).<sup>14–16</sup> NB is the most common extracranial solid tumor of childhood, and despite multimodality chemotherapy, radiation, surgery, immunotherapy, and differentiation therapy, patients still have poor overall survival.<sup>17</sup> Notably, there are no available curative options for patients at relapse,<sup>17</sup> a time when tumors have endured the enormous selective pressures of conventional therapies. NB tumors are commonly characterized by mutational events that drive the high-level expression of members of the MYC family of oncogenic TFs.<sup>18</sup> This occurs by multiple mechanisms, including gene amplification,<sup>19</sup> stabilized protein expression<sup>20</sup> or enhancer hijacking.<sup>21</sup> Among other oncogenic effects, dysregulated MYC proteins cause amplification of transcriptional output, resulting in selective reliance on transcriptional processes.<sup>2–4</sup> Notably, other than these, the high-risk NB tumor genome has very few other recurrent mutations, save for mutations in either *ALK* (~9%), *ATRX* (~7.1% deletion, 2.5% mutation), or *PTPN11* (2.9%) cases.<sup>18</sup> Compared with other malignancies, the presence of relatively few mutations in the NB genome<sup>14–16,22,23</sup> presents it as an ideal model to study transcriptionally driven tumor heterogeneity.

Recent evidence, including that derived from single-cell mRNA sequencing atlases, indicates that NB tumors are





**Figure 1. Conceptual mechanisms by which changes in core regulatory circuitry members result in dysfunctional circuitries and oncogenesis**

(A) Regulatory circuitries are shown as genes (boxes) regulated by super-enhancer elements (SEs). These genes produce transcription factor protein products (ovals) that autoregulate their own loci and that of other TFs within the network. This produces a “balanced” core regulatory circuitry state.

(B) This balanced state may be dysregulated by acquisition of a new oncogenic transcription factor member (TF4), which introduces protein-protein or protein-DNA interactions, interactions with RNA species, or co-activators, to co-opt the regulatory circuitry to an oncogenic outcome.

(C) Loss of a transcription factor (TF3) that suppresses tumorigenesis, resulting in altered transcriptional activity, re-targeting of residual complexes to different gene loci, or both, resulting in oncogenesis.

(D) Invasion of regulatory elements by oncogenic effectors (red box) such as MYC family TFs can induce mRNA amplification across single, collections of, or all genes, resulting in a dysregulated CRC.

heterogeneous entities composed of transcriptionally distinct groups of cells that together comprise a complex and dynamic primary tumor.<sup>14–16,24–27</sup> This heterogeneity poses a therapeutic challenge since a primary tumor may alter its composition of subpopulations in response to the selective pressure of cancer therapy, resulting in the enrichment of a different cell state at relapse, as compared with diagnosis.<sup>16</sup> These distinct cell states are characterized by fundamental differences in expression and activity of mTFs, which drive different transcriptional networks. Dissecting intratumoral heterogeneity by studying these subpopulations in an isolated manner is a reductionist approach to identifying therapeutic strategies for each of these populations independently, termed “subpopulation-directed therapy.” However, understanding the co-existence of these subpopulations also forms the basis for approaches that aim to understand how they cooperatively promote the growth of a primary tumor.

### Core regulatory circuitries establish the transcriptome of the cell

The potency of mTFs in establishing transcriptional networks and cell identity was conclusively demonstrated by Yamanaka and colleagues.<sup>28</sup> They identified that the transcriptome of a

cell, which determines cell state, is flexible and can be rewired in the context of forced expression of small, selected sets of mTFs.<sup>28</sup> The concept of re-programming cells from terminally differentiated to pluripotent states was transformative to the paradigm that postulated terminal differentiation to be an irreversible, “hard-wired” state. Thus, the transcriptional program must be malleable and controlled by the coordinated activities of networks of mTFs that establish it,<sup>28</sup> and individual cohorts of mTFs induce different transcriptional programs that yield distinct cell states and phenotypes.

Further evidence indicates that these mTFs form autoregulatory positive-feedback loops, in which each mTF regulates its own expression and that of the others<sup>29</sup> (model in Figure 1A). The regulation of each mTF gene is accomplished by the physical binding of each mTF protein to enhancer regulatory elements distal to the gene body. This network organization model is termed the “core regulatory circuitry” (CRC).<sup>29–31</sup> The coordinated binding to and regulation of other effector genes by CRC mTFs establishes the transcriptome of the cell, termed the “extended regulatory network” (ERN).<sup>30</sup>

A CRC is a meta-stable arrangement of mTFs and regulatory elements. By this model, the effects of slight and transient

reductions in the protein expression of one mTF can reverberate throughout the other TFs within the network, as they bind to the promoter and/or enhancers that regulate the mTF.<sup>30</sup> This results in feedforward pressure exerted by the other TFs in the network and restored gene expression of the previously reduced mTF.<sup>30</sup> In *E. coli*, positive autoregulation loops slowly respond to changes in TF concentration, yielding cell-to-cell variability in expression that enhances the ability of a population of cells to respond to environmental stimuli.<sup>32,33</sup> Changes in mTF composition also occur during development, implying that flexibility in control of the transcriptome is sufficient to drive morphologic and phenotypic change in cells. For example, pluripotency and self-renewal of embryonic stem cells are maintained by a well-defined CRC, including the mTFs NANOG, OCT4, and SOX2.<sup>29</sup> During development, specification of pluripotent stem cells in the inner cell mass into the primitive endoderm is induced by expression of the fibroblast growth factor FGF4.<sup>34</sup> Pluripotent embryonic stem cells in the epiblast produce and secrete FGF4, which causes downregulation of NANOG and upregulation of GATA6 in other cells within the inner cell mass, leading to the formation of the primitive endoderm.<sup>34</sup> Thus, loss of one CRC member (NANOG) and gain of a new member (GATA6) in response to extracellular stimuli result in changes in cell fate.<sup>34,35</sup> Notably, these networks are primed for collapse in pathological conditions, as loss of a single TF member of the CRC by sporadically occurring endogenous mechanisms (mutation, deletion, translocation) or by exogenous, experimental methods may yield an “unraveling” of the network. This collapse can occur if the expression of a CRC member falls below a threshold such that the above-mentioned and other mechanisms, including collaboration with signaling TFs, are unable to instantly support cooperative gene expression. Thus, a CRC presents a metastable population-based arrangement whereby cells may dynamically respond to external factors that drive slight changes in gene expression but may also be incapable of responding to considerable disruption of these networks by genetic or perhaps pharmacologic manipulation.

The constituent members of the CRC are typically DNA-binding TFs. However, linker proteins, co-factors, and non-coding RNAs are also involved in CRC-anchored regulation, such as in the case of microRNAs that suppress the expression of CRC TFs.<sup>36</sup> Further, mTFs can co-localize with RNAs within transcriptional condensates,<sup>37,38</sup> suggesting possible RNA-based regulatory mechanisms through physical interactions.

### CRCs in cancer

Multiple orthogonal approaches have been used to define CRCs in a range of tissue and disease settings. One of the most common approaches is to associate specific TF-encoding genes with super-enhancers (SEs), usually identified as extensive stretches of acetylation on histone H3, lysine-27 (H3K27ac).<sup>39–42</sup> SEs are a helpful surrogate to identify CRCs since they are often associated with lineage-specific genes and oncogenes, and the technique used to resolve them, H3K27ac chromatin immunoprecipitation sequencing (ChIP-seq) to the H3K27ac mark, is an unbiased and relatively simple experiment to perform.<sup>41,43–46</sup> This association has led to a

model by which SE-regulated mTFs define the component members of the CRC.<sup>30</sup>

The model of SE-controlled mTFs forming CRCs was initially identified in murine embryonic stem cells<sup>29</sup> but subsequently expanded from untransformed to transformed cells by several key findings. First, SE-marked loci were found to be enriched for tumor oncogenes in addition to lineage-specific genes.<sup>41,47</sup> Second, mTFs and their circuitries were demonstrated to be dysregulated by known oncogenic drivers in cancers such as T cell acute lymphoblastic leukemia (T-ALL).<sup>31,46</sup> This example also provided critical insight into the mechanisms by which tumor-specific CRCs differ from those found in untransformed cells. In some T-ALL samples, the activity of the CRC formed by GATA3, TAL1, RUNX1, LMO1/2, E2A, and HEB is co-opted by mutationally derived non-coding insertions that cause ectopic binding of the hematopoietic mediator and the T-ALL oncogene MYB at the *TAL1* locus.<sup>31,46</sup> This finding provides an example of a more general phenomenon: dysregulation of transcriptional networks may occur by mutationally derived addition of an “extra” mTF oncogene.<sup>48</sup> This example informs a generalized hypothesis for ectopic, oncogenic activities of CRCs, wherein oncogenic dysregulation of CRCs may conceptually result from either the addition of an oncogene, loss of a repressive TF, or inclusion of a factor that globally dysregulates transcription (Figures 1B–1D). This hypothesis is rooted in the growing descriptions of CRCs in cancer (reviewed in Chen et al.<sup>49</sup>) and, indeed, in evidence from other species such as *Candida*, where the addition or subtraction of distinct sets of TFs results in the rewiring of transcriptional circuitries and acquisition of new biological activities.<sup>50,51</sup>

Subsequently, by the combined effort of multiple groups, CRCs have been defined for many tumor types, including NB,<sup>14,15,44</sup> chronic lymphocytic leukemia,<sup>52</sup> dedifferentiated liposarcoma,<sup>53</sup> esophageal carcinoma subtypes,<sup>54,55</sup> rhabdomyosarcoma,<sup>56,57</sup> and others (recently discussed in Chen et al.<sup>49</sup>). Further, given the central role of CRCs in establishing cell fate,<sup>29,30,44</sup> and the broad dependence of cancer cells on CRC mTFs for growth,<sup>58</sup> others have attempted to perturb or control these TFs for therapeutic benefit. For further details on the general role and structure of CRCs in cancer, the reader is referred to a recent review.<sup>49</sup> Herein, our discussion of the CRC will focus on advancements as they relate to definition and control of NB transcriptional cell state and tumor heterogeneity.

### The role of CRCs in establishing NB heterogeneity

Several studies have demonstrated that individual NBs display heterogeneity in the cell state. By pairing SE analysis with principal-component analysis on H3K27ac ChIP-seq performed in various NB patient-derived xenograft (PDX) and cell-line samples, NB cell lines could be subdivided into two distinct SE landscapes, termed neural-crest-like or noradrenergic.<sup>14</sup> Separately, transcriptional signatures were used to distinguish different subtypes of NB cell lines and isogenic tumor-cell-line pairs, termed mesenchymal (MES) or adrenergic (ADRN) cell states.<sup>15</sup> In both cases, the transcriptional and SE loci signatures were interlinked. Individual cell lines could be distinguished that dominantly belonged to either group—mesenchymal/neural-crest-like (now commonly referred to as MES) or

noradrenergic/adrenergic (now widely referred to as ADRN)—based on either transcriptional signatures or SE profiles.<sup>14,15</sup> Importantly, cell lines that were mixtures of these subpopulations could be separated into populations enriched for one or the other. For example, while the cell line SK-N-SH was demonstrated to be composed of predominantly ADRN cells, single-cell subclones of this cell line, SH-EP and SH-SY5Y, produced largely MES or ADRN states, respectively.<sup>14,15</sup> These findings are somewhat reminiscent of foundational studies in the transformed human mammary epithelial cell (HMLER) engineered models of breast cancer, which display populations of cells existing predominantly in an epithelial or MES state.<sup>59,60</sup> In contrast to this model where restricted epithelial or MES cells are rare within the larger tumor population,<sup>59</sup> the majority of NB cells and tumors appear to be in either the MES or ADRN state.<sup>16,25</sup>

Further supporting that distinct ADRN and MES subpopulations exist *in vivo*, individual human NB tumor transcriptomes cluster with those of either ADRN- or MES-dominant cell lines.<sup>58,61</sup> These two cell states may be plastic, since ADRN or MES single-cell subclones from NB cell lines appear to be capable of re-populating both transcriptional states.<sup>15,62</sup> Further, the generation of heterogeneous cell pools from single-cell subcloned pools implies that flexibility in the cell state may be independent of mutational status.<sup>15,16,24,62</sup> The prominence of several specific TFs was recurrently highlighted across these analyses, including the presence of SEs at their loci and exceptionally high mRNA expression. These TFs include *PHOX2B*, *GATA3*, and *HAND2* in the ADRN cell state and a large and highly variable number of TFs including *PRRX1*, *RUNX1/2*, *FOSL1/2*, *IRF1/2*, *TCF7L2*, and *JUN* in the MES state.<sup>14,15</sup>

In parallel to these analyses, exome-wide CRISPR-Cas9 dropout screens on >350 cancer cell lines, including 11 NB cell lines, were used to identify genes selectively required for NB cell growth.<sup>44</sup> In this analysis, several TFs, including *HAND2*, *ISL1*, *PHOX2B*, *GATA3*, *TBX2*, *ASCL1*, and *TFAP2 $\beta$*  were each marked by SEs and are also commonly required to promote the growth of high-risk NB cells.<sup>44,63,64</sup> The function of most of these TFs was specifically defined using transient small interfering RNA (siRNA) to disrupt each gene, which demonstrated loss of growth phenotypes and, indeed, loss of gene expression of each other member.<sup>44</sup> These findings established that these mTFs, which co-bound at each other's enhancers, represented a CRC in high-risk NB.<sup>44,63</sup> Importantly, most cell lines in this analysis had more dominant ADRN subpopulations. However, the two MES-dominant cell lines in this analysis, SKNAS and CHP212, were equally dependent on many of these TFs, including *HAND2*, *ISL1*, *PHOX2B*, *GATA3*, and *TBX2*.<sup>44</sup> Thus, while the transcriptomes of MES- and ADRN-dominant subtypes of NB cluster distinctly,<sup>14,58,61</sup> there is a significant overlap in expression of *HAND2*, *ISL1*, *GATA3*, *TBX2*, and *ASCL1*, which may reflect a critical role for these genes as lineage-specifying factors across all subtypes of NB. *PHOX2B*, in contrast, appears to be either lowly expressed or absent in some MES cell lines.<sup>14</sup> Importantly, these TFs, including *PHOX2B*, are generally exceptionally highly expressed in ADRN subtypes relative to MES subtypes.<sup>14,15</sup> A different set of TFs are exceptionally highly expressed in MES subtypes, compared with ADRN, including *PRRX1*, *RUNX1/2*, *FOSL1/2*, *IRF1/2*, *TCF7L2*, and *JUN*. However,

er, the specific mTFs that form the MES CRC have yet to be determined. Comparing transcriptional signatures by RNA-seq across many tumor types has demonstrated that while ADRN NB develops a well-defined cluster, MES NB displays a large variability in expressed genes and clustering.<sup>58,61</sup> This suggests that the mTFs responsible for driving the MES CRC may be more varied. This is echoed in a recent study by Gartlgruber et al., where MES cells displayed many more putative mTFs than ADRN cells.<sup>16</sup> While NBs dominated by either subtype express different levels of *HAND2*, *ISL1*, *PHOX2B*, *GATA3*, *TBX2*, and *ASCL1*, the expression of these mTFs in NBs as a whole, regardless of subtype, is collectively typically high when compared with other tumor types.<sup>44</sup> Coupled with CRISPR-Cas9 dropout data, this suggests these TFs may be required for the growth of both ADRN and MES cells and may reflect a fundamental central NB CRC based in cell lineage.

Further, there are functional implications to these distinct cell states where MES and ADRN cells also have different capacities to form tumors in animal models. ADRN cells display enhanced tumorigenesis in xenograft assays compared with MES cells.<sup>62</sup> Here, Lecca et al. transplanted ADRN- or MES-dominant subclones from the same cell line, subdivided by CD133 status (ADRN demonstrating low CD133 levels and MES cells showing high).<sup>62</sup> This finding is consistent with early work on NB tumor-initiating cells, which were CD133-negative<sup>65,66</sup> and therefore presumably correlate with the ADRN subtype. These results, viewed through the lens of CRCs, are essentially now explained by cell lines and primary tumors existing in heterogeneous populations of both MES and ADRN subtypes.<sup>14–16,24,44,58,62</sup> Intriguingly, sorting NB cells into CD133<sup>LOW</sup> and CD133<sup>HIGH</sup> populations, followed by xenografting, resulted in tumors with a mixed population of cells.<sup>62</sup> These data provide a hypothesis that these subpopulations may interconvert between each other; however, these experiments did not rule the possibility of contaminating cells of either the ADRN or MES subtype within the xenografted populations.<sup>67</sup> Thus, while there is a possibility that ADRN and MES cells may spontaneously interconvert between transcriptional cell states, further examination of this point is needed. In addition, in this study, cell-line xenografts composed of MES-dominant tumor clones grew more slowly than ADRN-enriched ones.<sup>62</sup> This is reminiscent of patients who appear to have no evidence of disease at the end of therapy but experience a late relapse. To this end, analysis of primary and relapsed tumors by single-cell RNA-seq has demonstrated enrichment for MES cells at relapse.<sup>16</sup> If MES cells are enriched at relapse and are relatively resistant to commonly used forms of chemotherapy, then a complete description of the signals and switches that drive MES to ADRN subtypes and vice versa will have significant implications for the clinical management of patients with NB.

#### Expanded complexity of CRCs in NB

Further expanding the complexity of regulatory circuitries in NB, Gartlgruber et al. demonstrated that additional SE profiles exist among NBs. These correspond to an MES subtype, along with other clinically relevant groupings: (1) *MYCN*-amplified, (2) *MYCN*-non-amplified high risk, and (3) *MYCN*-non-amplified low risk. The identities of the *MYCN*-amplified and

*MYCN*-non-amplified high-risk groups appear to overlap with the ADRN group, suggesting that these represent distinct subgroupings of ADRN cells.<sup>16</sup> These subgroups appear to converge on cell-cycle regulation through cyclin D1, a common dependency across NB subtypes.<sup>16,44,58</sup> While the mechanisms by which this convergence occurs are unknown, genetic modeling indicates that this may be mediated by loss of *ARID1A* in *MYCN*-amplified tumors and by loss of *CHD5* in *MYCN*-non-amplified tumors.<sup>68</sup>

The *MYCN*-non-amplified low-risk group is a distinct subgroup of patients with a disease strikingly different than typical high-risk NB. This group is characterized by excellent overall survival.<sup>17</sup> While little is known about the transcriptional cell state of *MYCN*-non-amplified low-risk tumors and the causes of their excellent prognosis and occasional involution, Gartlgruber et al. identified a group of TFs, including *MEIS2*, that appear to be SE regulated in these specimens.<sup>16</sup> These data reflect prior findings where *MEIS2A*, an alternatively spliced isoform of *MEIS2*, is highly expressed and implicated in metastatic-stage-4S tumors,<sup>69</sup> a self-involuting form of low-risk NB. This indicates that a TF isoform may be involved in establishing the transcriptome within a self-involuting tumor. In contrast, other recent findings have implicated *MEIS2* as an ADRN CRC factor,<sup>70</sup> suggesting that isoform-specific regulation of *MEIS2* may be critical to growth. The identification of SE-regulated loci in *MYCN*-non-amplified low-risk groups also includes other TFs, including *RORA*, *STAT3*, *TCF4*, *SOX6*, and *EBF1*, which provides a tantalizing selection of genes for further study of differences in transcriptional regulation between metastatic high- and low-risk tumors, which include the enigmatic self-involuting stage-4S NB.

### Perturbation of cell state alters tumor heterogeneity in NB

#### Genetic perturbations

The roles of few mTFs in the regulation of the NB cell state have been specifically examined. *PHOX2B*, which is mutated in germline predisposition syndromes of high-risk NB,<sup>67,71</sup> is associated with the ADRN cell state, with low expression in MES cells.<sup>14,15,24</sup> Further, *PHOX2B* is a genetic dependency in NB,<sup>44</sup> and downregulation of *PHOX2B* expression is associated with slower, but not ablated, tumor growth in ADRN cell xenografts.<sup>14</sup> In contrast, stable knockout of another ADRN CRC mTF, *GATA3*, in ADRN SH-SY5Y cells yields rare cell clones that are viable and that have a more MES-like gene expression pattern.<sup>72</sup> Importantly, *PHOX2B* and *GATA3* both have homologs that are common NB dependencies and similarly expressed in NB, *PHOX2A* and *GATA2*, suggesting an incomplete “buffering” capacity within these transcriptional networks on the transcriptome that may be sufficient to maintain some component of cell growth in the absence of the first gene. In contrast, expression of the TF *PRRX1* appears to be restricted to MES cells, with overexpression of this protein leading to conversion of ADRN to MES cells.<sup>15</sup> These genetic experiments imply that *PHOX2B*, *GATA3*, and *PRRX1* may be mTF controllers of these two cell states.

These experiments also imply that external stimuli impacting upon *PHOX2B*, *GATA3*, or *PRRX1* expression may be sufficient to induce interconversion between transcriptional cell states. Indeed, this concept is echoed in the HMLER model, where

cellular plasticity is controlled by extracellular canonical and noncanonical WNT signaling.<sup>59</sup> In NB, experimental evidence indicates that overexpression of *NOTCH3* in SH-SY5Y ADRN cells is sufficient to downregulate *PHOX2B* and reprogram to a MES cell state that eventually also results in transcriptional downregulation of the other ADRN CRC members *TFAP2 $\beta$* , *GATA3*, *ISL1*, and *ASCL1*.<sup>24</sup> Associated with this downregulation is re-programming at the *ASCL1* locus, with loss of the SE at this site. Since SH-SY5Y is a strongly ADRN cell line, these data indicate that forced expression of *NOTCH3* may be sufficient to alter the expression of mTF networks, yielding a switch in cell state from ADRN toward MES.<sup>14</sup> Associated with these findings are enhanced resistance to chemotherapies in *NOTCH3*-induced MES-like SH-SY5Y cells, including doxorubicin, etoposide, and cisplatin.<sup>14</sup> These findings echo prior work, demonstrating that MES cells may be more chemoresistant than ADRN cells.<sup>14,15,62</sup> Similarly, in zebrafish models of NB, *ARID1A* loss results in increased penetrance of *MYCN*-driven tumors, associated with loss of the ADRN and acquisition of an MES cell state.<sup>73</sup> Paradoxically, however, the NBs formed in this study display a more rapid onset and are more highly penetrant, despite being of MES state.<sup>73</sup> These data that conflict with the slower growth rate identified in MES, as compared with ADRN, xenografts.<sup>62</sup> Further, genetic deletion of *ARID1A* in the human ADRN NB cell line NGP did not affect cell growth but induced a motility and invasiveness phenotype *in vitro*.<sup>73</sup> These findings indicate key disparities between human cell lines, murine and zebrafish genetic models, and animal xenografts of NB that need to be further explored to fully understand the different growth, invasion, and metastatic properties of ADRN and MES cells.

#### Chemical perturbations

Identifying CRCs in high-risk NB and other tumors has ignited an eagerness to target them with small molecules, intending to modulate the cell state for potential therapeutic benefit. Early studies using the BET bromodomain inhibitor compound JQ1 demonstrated that disruption of BRD4 reader activity was associated with loss of *c-MYC* expression in acute myeloid leukemia.<sup>74</sup> These results led to an explosion in inquiry in various tumors, yielding numerous epigenetic targets for study in distinct tumor states (reviewed in Wimalasena et al.<sup>75</sup>). Save for a few exceptions, the direct physical targeting of TFs by small molecules, however, has been challenging to accomplish *in vitro* or *in vivo* (reviewed in Wimalasena et al.<sup>75</sup>). Thus, most efforts in NB have focused on disrupting regulators of TFs such as epigenetic enzymes and binding factors (Table 1). In NB, disruption of transcriptional processes in single-agent or combination strategies involving inhibition of BRD4 with JQ1,<sup>76</sup> CDK7-, 12-, and 13-mediated transcriptional elongation with THZ1 or THZ531,<sup>77</sup> have striking effects on the collapse of CRCs. Inhibition of CDK9 also causes generalized loss of transcriptional output by suppressing *MYCN*-regulated transcription in *MYCN*-amplified NB. Intriguingly, CDK9 inhibition is also associated with the selective loss of ADRN transcripts such as *HAND2*, *HAND1*, *PHOX2A*, *PHOX2B*, *GATA3*, *ISL1*, and *KLF7* without the induction of MES transcripts, yielding growth suppression of ADRN NB cells.<sup>78</sup>

Other approaches, including the use of targeted inhibitors of histone deacetylases (HDACs) 1 and 2, resulted in enhanced

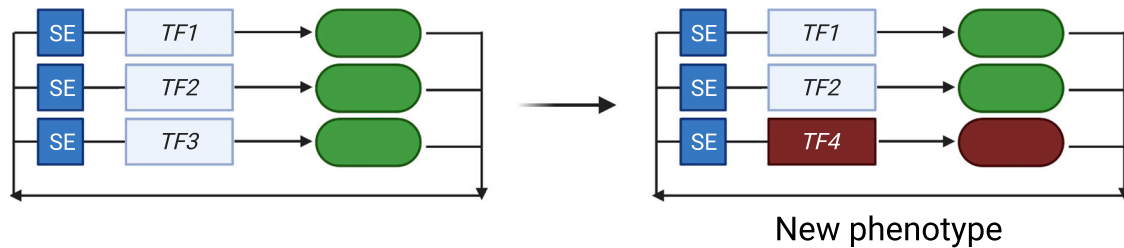
**Table 1. Indirect targeting of CRC members in neuroblastoma**

Molecule and reference	Type of neuroblastoma targeted	Target	Mechanism of action	Effect on CRC or CRC members	Phenotype
5'azacytidine <sup>79</sup>	human <i>MYCN</i> -amplified neuroblastoma cell lines induced to form lung metastases	DNMT1, DNMT3A, DNMT3B	epigenetic control of transcription	suppressed expression of <i>PHOX2B</i>	not reported
GSK126 and JQEZ5 <sup>80</sup>	<i>MYCN</i> amplified and non-amplified	EZH2	inhibition of epigenetic-mediated transcriptional repression	EZH2 inhibition upregulates expression of a 37 gene module including the MES transcription factors <i>PRRX1</i> and <i>TCF7L1</i>	inhibition of cell growth <i>in vitro</i> ; GSK126 inhibits xenograft growth ( <i>MYCN</i> amplified and non-amplified)
BRD8430 <sup>81</sup>	<i>MYCN</i> amplified and non-amplified	HDAC1, HDAC2, HDAC3	epigenetic control of transcription	loss of HDAC1 and HDAC2 by siRNA inhibits expression of <i>GATA3</i> and <i>ASCL1</i>	decreased cell viability and induced differentiation
JQ1 <sup>82</sup>	<i>MYCN</i> amplified and non-amplified	BRD4, BRD2, BRD3, BRDT	epigenetic control of transcription	indirect suppression of <i>MYCN</i> protein levels	induces differentiation, loss of proliferation, reduced xenograft growth
OTX015 <sup>83</sup>	<i>MYCN</i> amplified	BRD4, BRD2, BRD3, BRDT	epigenetic control of transcription	loss of <i>c-MYC</i> or <i>MYCN</i> expression with associated loss of <i>MYC</i> -driven gene expression	enhanced killing of <i>MYCN</i> -amplified cells, xenograft growth.
THZ1 <sup>77</sup>	<i>MYCN</i> amplified and non-amplified	CDK7, CDK12, CDK13	transcriptional initiation/elongation	loss of active transcription causing suppression of <i>MYCN</i> -induced global transcriptional amplification; loss of super-enhancers compared with typical enhancers and loss of H3K27ac notably at the <i>MYCN</i> , <i>PHOX2B</i> , and <i>GATA2</i> loci	enhanced effects on cell-cycle arrest and apoptosis in <i>MYCN</i> -amplified cell lines; tumor regression in <i>MYCN</i> -amplified xenografts
THZ1+ JQ1 <sup>44</sup>	<i>MYCN</i> -amplified	CDK7, CDK12, CDK13 (THZ1); BRD4, BRD3, BRD2, BRDT (JQ1)	inhibition of transcriptional initiation/elongation and epigenetic control of transcription	inhibits expression of ADRN CRC members <i>MYCN</i> , <i>HAND2</i> , <i>PHOX2B</i> , <i>ISL1</i> , <i>GATA3</i> , and <i>TBX2</i>	synergistic inhibition of cell-line and xenograft growth

(Continued on next page)

**Table 1. Continued**

Molecule and reference	Type of neuroblastoma targeted	Target	Mechanism of action	Effect on CRC or CRC members	Phenotype
THZ1+ panobinostat <sup>84</sup>	<i>MYCN</i> amplified and non-amplified	CDK7, CDK12, CDK13 (THZ1); non-selective HDAC inhibition (panobinostat)	inhibition of transcriptional initiation/elongation and epigenetic control of transcription.	inhibits c-MYC and MYCN protein levels	synergistic inhibition of cell-line growth in cell culture and xenografts
JQAD1 <sup>64</sup>	<i>MYCN</i> amplified and non-amplified	EP300 degradation	epigenetic control of transcription	inhibition of <i>MYCN</i> mRNA expression; loss of genome-wide H3K27ac with enhanced loss at CRC super-enhancers	apoptosis in cell lines, reduced xenograft growth
CYC065 <sup>78</sup>	<i>MYCN</i> -amplified	CDK9 and CDK2	inhibition of transcriptional initiation/elongation; G1 cell-cycle arrest.	loss of <i>MYCN</i> -regulated transcripts including <i>HAND2</i> , <i>PHOX2A</i> , <i>PHOX2B</i> , <i>GATA3</i> , <i>ISL1</i> , <i>TBX2</i> , <i>HAND1</i> , and <i>KLF7</i> ; minimal change in <i>MES</i> -associated transcription factors	reduced tumor growth in xenografts of <i>MYCN</i> -amplified Kelly cells; reduced murine <i>TH-MYCN</i> ; <i>TH-ALK</i> <sup>F1174</sup> growth, enhanced effects with co-treatment with temozolamide
Retinoic acid <sup>85</sup>	<i>MYC</i> amplified	agonism of retinoic acid receptor dimers	epigenetic and transcriptional re-programming	loss of <i>PHOX2B</i> gene expression; proteasomal degradation of <i>PHOX2A</i> with prolonged treatment	not reported
Retinoic acid <sup>86</sup>	adrenergic <i>MYCN</i> amplified	agonism of retinoic acid receptor dimers	epigenetic and transcriptional re-programming	decreased <i>MYCN</i> expression with loss of <i>PHOX2B</i> , <i>GATA3</i> , and <i>ASCL1</i> ; <i>ADRN</i> CRC is replaced by a new retino-sympathetic CRC containing <i>SOX4</i> , <i>MEIS1</i> , and <i>RARA</i>	differentiation



**Figure 2. Alterations in transcriptional circuitries results in acquisition of new phenotypes**

Regulatory circuitries are demonstrated genes (boxes) regulated by SEs. These genes produce transcription factor protein products (ovals) that autoregulate their own loci and that of other TFs within the network. Perturbation of this circuitry, by loss of TF3 and acquisition of TF4, results in a newly generated regulatory circuitry, with new genomic-binding loci. This causes alterations in the transcriptome, with a new cellular phenotype.

NB differentiation,<sup>81</sup> and non-selective HDAC inhibition synergizes with THZ1 to suppress xenograft growth.<sup>84</sup> Mechanistically, siRNA-mediated targeting of HDAC1 and HDAC2 together resulted in selective suppression of the ADRN CRC members *GATA3* and *ASCL1*, indicating that these mTFs may play a role in repressing normal neuroblastic differentiation.<sup>81</sup> Independently, using the clinically active agent retinoic acid to drive NB differentiation, Zimmerman et al. demonstrated that retinoic acid causes loss of *GATA3*, *ASCL1*, and *PHOX2B* expression, concurrent with acquisition of SE elements at the loci of *SOX4*, *MEIS1*, and *RARA*, associated with upregulation of these transcripts.<sup>86</sup> These data indicate that loss of these specific members of the ADRN CRC and gain of expression of other, distinct TFs is associated with neuroblastic differentiation. In total, these data indicate that global dysregulation of transcription by targeting transcriptional regulators can cause a range of phenotypic changes, ranging from collapse to subtle rewiring of transcriptional circuitries. These alterations in transcriptional circuitries are associated with the acquisition of new cell states and phenotypes (Figure 2). However, since most of these studies have focused on ADRN-dominant cell lines and tumors, there remains pivotal questions to be answered about the relevance of these approaches in phenotypically distinct MES cells.

#### Cell-fate switching caused by CRC plasticity engenders drug resistance

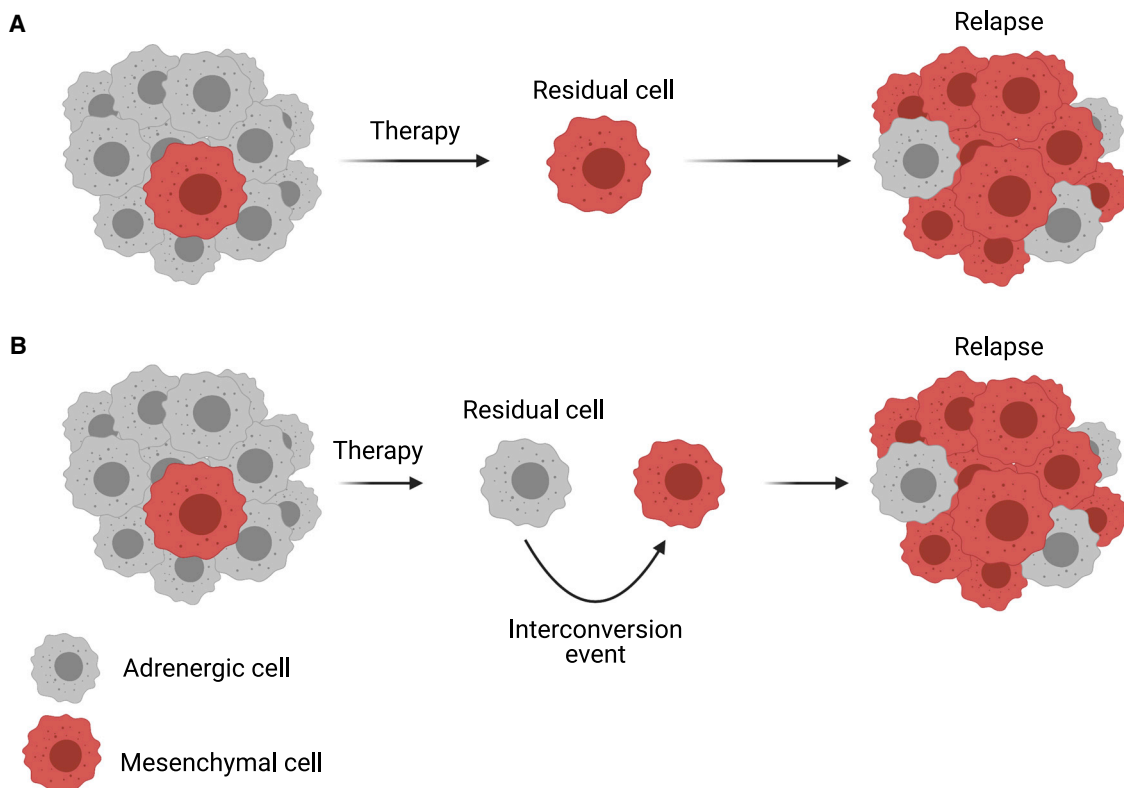
Loss of individual members of CRCs without substitution of other new TF members is typically lethal to the NB cell.<sup>44</sup> However, recent studies have demonstrated that changes in cellular phenotype may be driven by subtle alterations in CRCs, where some members may be lost and new members gained. These findings provide insight into the mechanisms by which the cell state is governed, demonstrating that cellular phenotypes, such as drug resistance, proliferation, invasion, or differentiation, may be derived by changes in regulatory circuitries (Figure 2). Further, two recent studies have shown that changes in the constituent members of the CRC promote NB differentiation. In both studies, *cis*-retinoic acid, a clinically used differentiation agent in high-risk NB, led to fundamental rewiring of the CRC, with loss of members of the ADRN CRC and acquisition of new members previously unrelated to either the ADRN or MES cell states, such as *MEIS1* and *SOX4*.<sup>86,87</sup> When *SOX4* knockout NB cells were treated with *cis*-retinoic acid, they failed to differentiate and instead were committed to apoptotic cell death.<sup>86</sup> These

findings indicate a conserved mechanism by which *cis*-retinoic acid drives changes in cellular phenotype through modulation of CRC members and implies the necessity of these transcriptional changes for achieving the phenotypic outcome of differentiation.

The concept of changes in NB CRC members resulting in altered cellular phenotype has also been demonstrated in the context of drug resistance. For example, resistance to ALK inhibition in *MYCN*-driven NB is mediated in part by upregulation of the CTCF-like protein BORIS.<sup>88</sup> BORIS causes changes in three-dimensional chromatin architecture, resulting in genomic reorganization and newly formed SEs at, and upregulation of, TF genes that are typically upregulated during neuronal differentiation, such as *SIX1*, *NEUROD1*, *NEUROD4*, *NEUROG1*, *SOX2*, and *SOX9*.<sup>88</sup> While the status of conventional ADRN or MES TF loci and gene expression was not commented on in this work, the BORIS-induced cell state is resistant to both ALK and BRD4 inhibitors.<sup>88</sup> These data indicate that BORIS activity reprograms cells to drive a new cell state marked by altered mTF expression and resistance to multiple agents that target transcription.<sup>88</sup>

Beyond targeted therapies, broad-spectra chemotherapies used in the management of NB also differentially affect the heterogeneous components of individual NB tumors. In these studies, MES cells such as SH-EP display enhanced endogenous resistance to the conventional agents etoposide, cisplatin, and doxorubicin, as compared with ADRN cells such as SH-SY5Y. Further, treatment of the “intermediate” cell line SK-N-SH with cisplatin or doxorubicin resulted in changes in gene expression such that the resultant pool clustered more with MES cell lines.<sup>14</sup> Separately, studies of both ADRN and MES cell lines treated with escalating doses of cisplatin to isolate relatively resistant cell lines demonstrated that resistant clones are marked by an enhanced MES gene expression pattern.<sup>89</sup> These data indicate that MES and ADRN cells may show fundamentally distinct responses to conventional chemotherapeutic agents and that these states may exist along a continuum. Further, these data provide potential insights into the mechanisms by which chemotherapies shape tumor evolution, resulting in the reported finding of enhanced MES cell phenotypes at patient relapse.<sup>16</sup> These data suggest two attractive potential hypotheses: (1) MES cells are enriched at relapse due to enhanced intrinsic chemoresistance programmed by MES gene expression patterns or (2) NB tumor cells are a dynamic entity capable





**Figure 3. Principles of selection or interconversion of heterogeneous cancer-cell clones**

Two distinct models of adrenergic and mesenchymal clonal selection due to therapy in high-risk neuroblastoma are shown.

(A) Tumors at diagnosis are heterogeneous populations. Adrenergic cells are depleted by conventional therapies, resulting in residual mesenchymal cells that repopulate the tumor, yielding a mesenchymal-dominant relapsed tumor. Here, adrenergic cells are found in the relapsed tumor due to subsequent stochastic interconversion of mesenchymal to adrenergic cells.

(B) Tumor populations are globally reduced by therapy, affecting both adrenergic and mesenchymal cells. Residual cells undergo cell state switching, and treatment with conventional chemotherapies favor switching to a mesenchymal (MES) gene expression pattern. This results in re-population of tumors yielding mesenchymal-dominant relapsed tumors.

of “switching” between ADRN and MES cell states, and conventional chemotherapies favor switching to an MES gene expression pattern (Figure 3). Evidence exists to support both hypotheses; however, a deeper understanding of how these mechanisms interact to engender NB relapse will be critical to developing strategies to target the disease at relapse.

Intriguingly, relapsed NB demonstrates enrichment for RAS-pathway member mutations<sup>90–92</sup> and MES gene signatures.<sup>16</sup> This leads to a tempting hypothesis that Ras-pathway mutations are, in some way, linked to MES phenotypes and chemoresistance. This hypothesis remains unproven in NB, though activated Ras pathways have long been implicated in driving chemoresistance in other diseases, such as melanoma.<sup>93</sup> It is notable that MES-enriched cell lines commonly contain activating RAS-pathway mutations, such as mutations in *K-RAS* (KPNS19S), *N-RAS* (CHP212, SK-N-SH, SKNAS), and *B-RAF* (NB1, ACN). Other mutations of genes in the RAS pathway implicated in relapsed NB<sup>90,91</sup> include *NF1*, *PTPN11*, and *ALK*, which are rarely demonstrated in MES cell lines. Whether these findings reflect small numbers of sequenced MES-enriched cell lines, adaptation to laboratory study conditions, or the fundamental biology of these cells *in vivo* remains to be identified.

By comparing three pairs of primary and relapsed samples, Gartlgruber et al. also made the critical observation that relapsed tumors may be enriched for cells of the MES state.<sup>16</sup> Further, these samples appeared to be associated with a RAS-driven gene expression signature.<sup>16</sup> These data suggest one of two potential explanations: (1) therapy selects for rare clones containing RAS-pathway mutations that are present at diagnosis or (2) MES lineage clones may preferentially acquire mutations that yield a selective growth advantage by the pro-mutational activity of treatment (Figure 3). Again, these distinct possibilities warrant further exploration and accelerated drug-discovery efforts to identify mechanisms to selectively target rare MES clones present at diagnosis in the first case or, alternatively, to replace mutagenic agents that induce these clones during therapy in the second.

### Conclusions and future directions

Recent studies of transcriptional heterogeneity in high-risk NB and other tumors have yielded important insights into the mechanisms by which transcriptional heterogeneity forms and is controlled. These studies have highlighted heretofore unappreciated complexities in the interplay between enhancer

remodeling, transcriptional changes, and the acquisition of new cellular phenotypes in complex tumor microenvironments and have led to hypotheses of how exogenous control of one of these aspects may impact the others. In the setting of single-cell RNA-seq approaches identifying higher-resolution transcriptional networks based on the highly expressed genes in clinical samples of NB and, indeed, other tumors, the insights of such study will be profound.<sup>25–27</sup> Mapping these regulatory circuitries to fetal and developmental stages will also yield impactful insights into the origins of the disease, the mechanisms by which normal cells become malignant and, ultimately, produce new therapeutic strategies that harness the heterogeneity of NB to identify strategies to ablate distinct tumor-cell populations.

To do so, however, several critical questions must be answered to develop practical approaches that target these distinct subsets of tumor cells for subpopulation-directed therapy. First, single-cell-sequencing technologies have been used extensively to try to pinpoint the cell of origin for NB.<sup>25–27</sup> Knowledge of the CRC of the cell of origin would grant insights into the mechanisms of NB tumorigenesis. Equally important is to determine whether cells existing at relapse represent the clonal outgrowth of a rare mutationally derived cell state enriched by current therapies or interconversion events of existing subpopulations within the primary tumor (Figure 3). Tools such as recent chimeric antigen receptor (CAR)-T cell therapies directed against antigens derived from PHOX2B will be helpful in this regard, as they are likely to solely and persistently ablate ADRN cells.<sup>94</sup> Similarly important are recent findings suggesting that MES cells are more sensitive to immunotherapeutic approaches than are ADRN cells.<sup>95</sup> How strategies such as these that apply selective pressure to one subpopulation within the larger tumor affect tumor growth, cellular heterogeneity, chemoresistance, and relapse are crucial.

Further, it is essential to identify whether all CRC TFs are equivalently and specifically required for NB growth or if some are only critical for specific phenotypic conversion events. Evidence indicates that the ADRN CRC members HAND2, ISL1, GATA3, and TBX2 may be required for survival of both ADRN- and MES-dominant NBs<sup>44</sup>—does this indicate that loss of these factors unravels a “master” CRC and commits all NB to death? Does this imply that other TFs, such as PRRX1, are uniquely required to mediate and maintain a cell-fate switch without effects on growth? If so, then perhaps a nomenclature identifying a master CRC and “accessory” CRCs responsible for the acquisition of phenotypes other than growth is necessary (Figure 2). How deeply must the regulatory circuitry be perturbed to cause a “collapse”? Are some factors within the CRC more dominantly required for survival or alternative behaviors, and if so, is there a potential therapeutic window, given the generation of first-generation tools for degradation of TFs?<sup>96</sup> Is the strategy of targeting CRCs by indirect treatment with epigenetically targeted agents (Table 1) selective and effective enough to cause tumor death while limiting toxicity to untransformed cells, especially progenitor and stem cells?

Before addressing these more advanced questions, however, fundamental ones exist. Efforts to date have demonstrated the mechanisms by which ADRN subpopulations of NB can be induced to switch to an MES fate. Given that MES cell states

are relatively chemotherapy resistant, the opposite—switching from MES to ADRN states—may be a separate approach. To clarify this, analytic prediction of regulatory circuitries may predict drivers of such an interconversion, which may be experimentally validated. These cell-fate switches are clearly of critical importance to the disease, as evidenced by enrichment of the MES state at relapse,<sup>16</sup> and must be understood to be harnessed. After determining genetic drivers of interconversion, identifying minimally toxic compounds that can force interconversion between MES and ADRN cell states and thereby engender sensitivity to chemotherapies, targeted therapies, or immunotherapies would be a critical step forward to help to realize more effective treatments in the relapsed setting. Given the difficulty in drugging the types of sequence-specific TFs known to drive differential CRCs, an alternative strategy might be to reprogram SE landscapes, thereby affecting the transcriptional state, to convert high-risk NB to an involuting, type-4S NB. Much is left to be done, but in the setting of exciting advances in tools, insights, and new mechanisms to describe tumor development, we anticipate significant advances in NB biology and medicine in the coming years.

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