engineered to target GPC2 or a bispecific antibody approach, because the present study is limited to an antibody-drug conjugate approach. Bosse and colleagues lay the exciting groundwork here for future exploration into the role of cell surface proteins in neuroblastoma and other pediatric malignancies as immunotherapy targets.

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When *LMO1* Meets *MYCN*, Neuroblastoma Is Metastatic

Zhihui Liu[1] and Carol J. Thiele[1,*]
[1]Pediatric Oncology Branch, National Cancer Institute, Center for Cancer Research, 9000 Rockville Pike, Bethesda, MD 20892, USA
*Correspondence: ct47a@nih.gov
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***LMO1* is a high-risk neuroblastoma susceptibility gene, but how *LMO1* cooperates with *MYCN* in neuroblastoma tumorigenesis is unclear. In this issue of *Cancer Cell*, Zhu et al. develop a novel zebrafish model that elucidates a mechanism by which *LMO1* and *MYCN* synergistically initiate neuroblastoma and contribute to metastatic disease progression.**

Neuroblastoma (NB) is the most common extracranial solid pediatric tumor arising in presumptive neural crest sympatho-adrenal progenitors. It comprises more than 5% of malignancies in children and accounts for 10.2% of cancer-related deaths in childhood (Bosse and Maris, 2016; Brodeur, 2003). Approximately 70%–80% of patients over 18 months of age present with metastatic disease, usually in the bone marrow, bone, lymph nodes, liver, intracranial, and orbital boney sites. The long-term survival rates of high-risk patients remain at approximately 50% (Maris, 2010). Sporadic neuroblastoma is genetically heterogeneous. Recent genome-wide-association studies (GWAS) and next-generation sequencing (NGS) studies have identified novel germline and somatically acquired genetic mutations, as well as single-nucleotide polymorphisms (SNPs), associated with low- or high-risk disease (Bosse and Maris, 2016). Remaining to be elucidated are the mechanisms by which SNPs contribute to neuroblastoma initiation and impact tumor progression.

Among the NB high-risk GWAS loci identified in NB, the LIM domain only 1 (*LMO1*) locus (Wang et al., 2011) is intriguing because LMO1 is a member of a family of cysteine-rich transcription factors, LMO1-4, that are required for many developmental processes and have been implicated in the onset or the progression of different cancers (Matthews et al., 2013). Interest centered on *LMO1* because DNA copy number alterations within the *LMO1* locus were also identified in 12.4% of NB cases (Wang et al., 2011). A highly associated SNP (rs2168101) within the first intron of *LMO1* was found to occur at a GATA transcription factor-binding motif, which created a novel super-enhancer that led to high-level *LMO1* transcription (Oldridge et al., 2015). In neuroblastomas with *LMO1*-risk SNP or somatic copy number gains, *LMO1* expression is high and is associated with disease progression. However, *LMO1*'s role in NB tumor initiation and progression has not been rigorously demonstrated.

The observation that risk-associated SNPs in the *LMO1* locus are especially enriched in high-risk neuroblastoma patients with metastatic disease (Wang et al., 2011) led to the current study in this issue of *Cancer Cell*, in which Zhu et al. generated transgenic zebrafish lines that express human *LMO1* (Zhu et al., 2017). *LMO1*, under control of the zebrafish dopamine-β-hydroxylase gene ($d\beta h$) promoter, is expressed in the peripheral

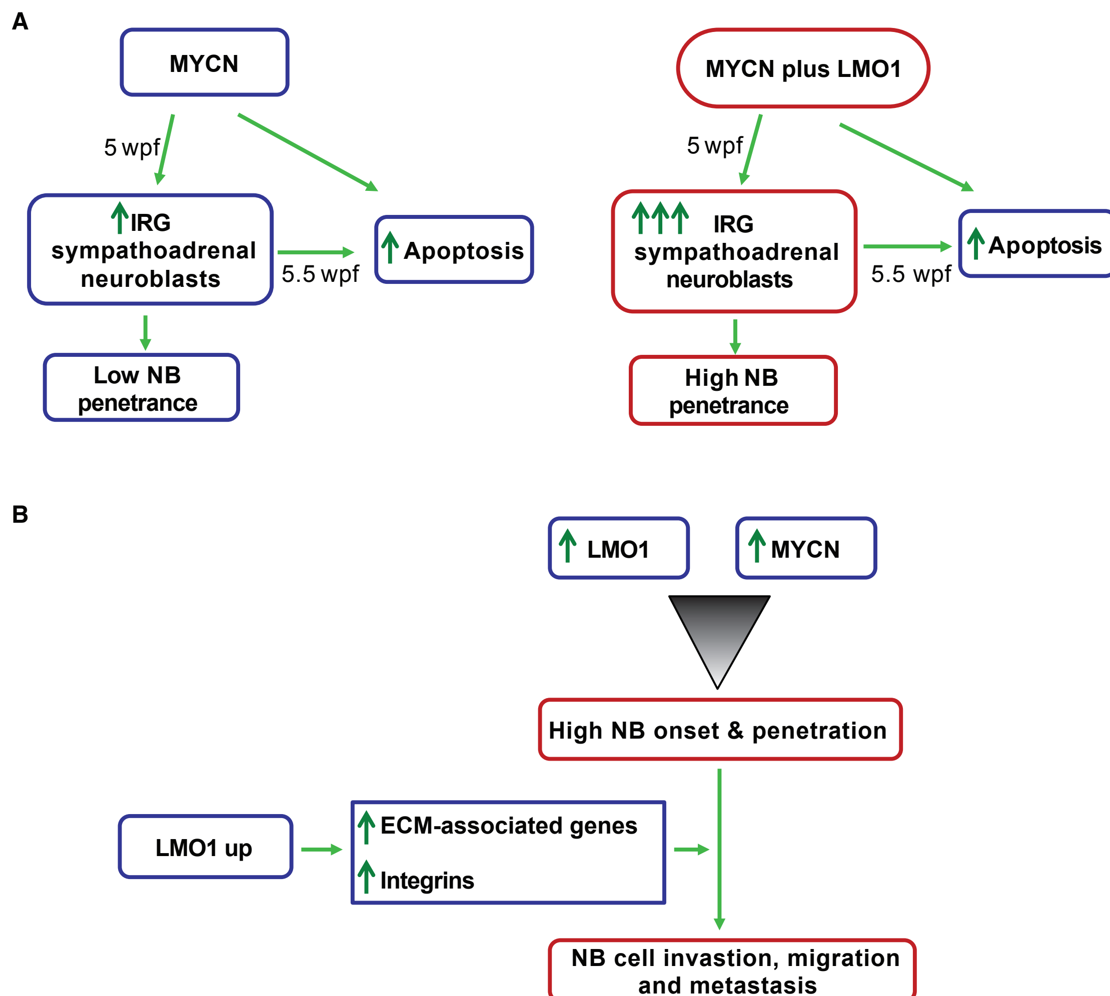

Figure 1. *MYCN* and *LMO1* Synergistically Impact Neuroblastoma Initiation and Metastasis
(A) In zebrafish, the *MYCN* overexpression causes expansion and apoptosis of the sympathoadrenal neuroblasts at 5–5.5 weeks postfertilization (wpf), and a small group of zebrafish with *MYCN* overexpression developed neuroblastoma (left); the overexpression of *LMO1* synergizes with *MYCN* to induce neuroblastoma in a large group of zebrafish by increasing the proliferation of sympathoadrenal neuroblast cells, which overcomes the *MYCN*-induced developmentally timed apoptotic response (right). (B) ECM-associated genes and integrins are downstream targets of *LMO1* that mediate *LMO1* function in neuroblastoma metastasis.

sympathetic nervous system. By co-injecting *dβh:LMO1* and the *dβh:mCherry* constructs into zebrafish embryos at the one-cell stage, the authors are able to visualize tumor development *in vivo*. No tumors were observed in any fish lines with transgenic expression of *LMO1* alone. Because there is a strong association between the expression of *LMO1* and *MYCN* in the subset of high-risk neuroblastoma lacking *MYCN* amplification (Zhu et al., 2017), the authors asked whether the co-expression of *LMO1* and *MYCN* could affect the onset and penetrance of neuroblastoma. By utilizing their *dβh* promoter-driven EGFP-MYCN transgenic fish, which develop neuroblastoma tumors (Zhu et al., 2012), they found that when *LMO1* and *MYCN* transgenic fish interbreed, tumors develop in 80% of the *MYCN;LMO1* progeny by 24 weeks of age, whereas only 20%–30% of the *dβh-MYCN* progeny have tumors. This indicates that high levels of *LMO1* expression enhance initiation of neuroblastoma *in vivo*.

So how does *LMO1* accelerate neuroblastoma onset and increase penetrance in *MYCN* transgenic fish? Using immunohistochemistry, Zhu et al. analyzed the interrenal gland region (the presumptive source of sympathoadrenal progenitors) of 5-week-old transgenic fish. The number of $mCherry^+$ sympathoadrenal cells was similar between *LMO1* transgenic and control fish, indicating that expression of *LMO1* alone does not affect the number of sympathoadrenal progenitors at this stage. In contrast, *MYCN;LMO1* transgenic fish had ~3-fold more GFP^+ sympathoadrenal cells compared to *MYCN*-only fish. To determine whether the increase in sympathoadrenal cell number is due to a faster rate of neuroblast growth, the authors performed EdU pulse-labeling experiments in the control *dβh:EGFP*, *MYCN*, *LMO1*, and *MYCN;LMO1* transgenic fish. They found that the fraction of EdU-incorporating GFP^+ sympathoadrenal cells was significantly increased in the transgenic fish co-expressing *MYCN* and

LMO1, compared to those expressing either *MYCN* or *LMO1* alone. To determine whether transgenic expression of *LMO1* rescued the *MYCN*-induced developmentally timed apoptotic response in the interrenal gland at 5.5 weeks of age, Zhu et al. used activated Caspase-3 to mark apoptotic cell death. A similar number of fish (*MYCN* alone or *MYCN;LMO1*) with apoptotic cells in the interrenal gland expressed activated Caspase-3. This suggested that transgenic expression of *LMO1* was unable to rescue the *MYCN*-induced developmentally timed apoptosis at 5.5 weeks of age. However, because there was a significantly higher number of GFP^+ or $mCherry^+$ sympathoadrenal cells in the fish coexpressing *MYCN* and *LMO1* than in fish expressing *MYCN* alone, at this time, they reasoned that expression of *LMO1* synergizes with *MYCN* to induce neuroblastoma by increasing the proliferation of hyperplastic sympathoadrenal cells, which overcomes the MYCN-induced developmentally timed apoptotic response at 5.5 weeks of age in the transgenic fish expressing both *MYCN* and *LMO1* (Figure 1A).

Clinically, *MYCN* amplification is associated with high-risk, metastatic disease in neuroblastoma patients. However, murine or fish NB models driven by *MYCN* overexpression alone are not associated with metastatic disease. Importantly, Zhu et al. discovered that *MYCN;LMO1* transgenic fish have metastatic disease. In the *MYCN*, *MYCN;LMO1*, and *MYCN;ALK* transgenic fish at 5 to 7 months of age, the primary tumors arising in the interrenal glands are histologically comparable to human neuroblastomas. The authors observed widespread tumor masses in multiple regions distant from the interrenal gland and kidney in the fish co-expressing *MYCN* and *LMO1*, but not in *MYCN*-only or *MYCN* and *ALK* transgenic fish. The metastatic sites include orbit and spleen (analogous to lymph node), common sites of NB metastatic spread in patients, as well as gill (analogous to lung), a less-frequent yet high-risk site in humans. Next, they asked whether *LMO1* affects migration and invasiveness in human NB cells. The BE2C NB cell line has a T/- rs2168101 genotype within the *LMO1*, in which the T nucleotide disrupts the GATA binding motif, resulting in the absence of a super-enhancer and low-level *LMO1* expression. Overexpression of *LMO1* increased the *in vitro* migratory and invasive behavior of BE2C cells.

Clues as to how *LMO1* affected neuroblastoma metastatic behavior were provided by RNA sequencing of BE2C cells expressing *LMO1* or the control vector. Gene set enrichment assays showed significant enrichment of a gene signature encoding "matrisome-associated proteins," consisting of structural extracellular matrix (ECM) proteins and ECM-associated enzymes, as well as gene signatures for "ECM regulators" and "integrins" in LMO1-expressing BE2C cells. Among the upregulated ECM-associated genes, the lysyl oxidase (*LOX*) family encodes enzymes known to promote metastasis (Kagan and Li, 2003; Erler et al., 2006). The authors treated the *LMO1*-overexpressing BE2C cells with a LOX inhibitor, β-aminopropionitrile (BAPN), and found that BAPN reduced the *in vitro* migratory and invasiveness of *LMO1*-expressing cells while having little effect on the vector-control cells. These results suggest that members of the *LOX* family are critical downstream targets of *LMO1* that mediate *LMO1* function in neuroblastoma metastasis (Figure 1B).

In this study, Zhu et al. provide a compelling link as to how a risk allele contributes to NB development and progression. Importantly, they discover that *LMO1* induces metastatic neuroblastoma to sites in *LMO1;MYCN* transgenic fish that are in part analogous to sites in neuroblastoma patients. This *MYCN;LMO1* zebrafish neuroblastoma model not only enhances our understanding of how developmental alterations lead to neuroblastoma initiation and metastasis but will also be an important model for determining critical genes involved in metastatic disease and for using unbiased screens to identify drugs that target metastatic neuroblastoma.

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