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Zebrafish Model of Neuroblastoma Metastasis

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TITLE:

Zebrafish Model of Neuroblastoma Metastasis

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SUMMARY:

This paper introduces the method of developing, characterizing, and tracking in real-time the tumor metastasis in zebrafish model of neuroblastoma, specifically in the transgenic zebrafish line with overexpression of *MYCN* and *LMO1*, which develops metastasis spontaneously.

ABSTRACT:

Zebrafish has emerged as an important animal model to study human diseases, especially cancer. Along with the robust transgenic and genome editing technologies applied in zebrafish modeling, the ease of maintenance, high-yield productivity, and powerful live imaging altogether make the zebrafish a valuable model system to study metastasis and cellular and molecular bases underlying this process *in vivo*. The first zebrafish neuroblastoma (NB) model of metastasis was developed by overexpressing two oncogenes, *MYCN* and *LMO1*, under control of the dopamine-beta-hydroxylase (*dβh*) promoter. Co-overexpressed *MYCN* and *LMO1* led to the reduced latency and increased penetrance of neuroblastomagenesis, as well as accelerated distant metastasis of tumor cells. This new model reliably reiterates many key features of human metastatic NB, including involvement of clinically relevant and metastasis-associated genetic alterations; natural and spontaneous development of metastasis *in vivo*; and conserved sites of metastases. Therefore, the zebrafish model possesses unique advantages to dissect the complex process of tumor metastasis *in vivo*.

INTRODUCTION:

Zebrafish has been widely used and applied to several areas of research, especially in cancer. This model provides many advantages—such as its robust reproduction, cost-effective maintenance, and versatile visualization of tumor growth and metastasis—all of which make zebrafish a powerful tool to study and investigate the cellular and molecular bases of tumorigenesis and metastasis. New techniques for large-scale genome mapping, transgenesis, genes overexpression

or knockout, cell transplantation, and chemical screens have immensely augmented the power of the zebrafish model[1]. During the past few years, many zebrafish lines have been developed to study tumorigenesis and metastasis of a variety of human cancers, including but not limited to leukemia, melanoma, rhabdomyosarcoma, and hepatocellular carcinoma[2-5]. Additionally, the first zebrafish model of neuroblastoma (NB) was generated by overexpressing *MYCN,* an oncogene, in the peripheral sympathetic nervous system (PSNS) under control of the dopamine-beta-hydroxylase (*dβh*) promoter. With this model, it was further demonstrated that activated *ALK* can synergize with *MYCN* to accelerate tumor onset and increase tumor penetrance *in* vivo[6].

NB is derived from the sympathoadrenal lineage of the neural crest cells, and is a highly metastatic cancer in children[7]. It is responsible for 10% of pediatric cancer-related deaths[8]. Widely metastasized at diagnosis, NB can be clinically presented as tumors primarily originating along the chain of the sympathetic ganglia and the adrenal medulla of PSNS[9,10]. *MYCN* amplification is commonly associated with poor outcomes in NB patients[11,12]. Moreover, *LMO1* has been identified as a critical NB susceptibility gene in high-risk cases[13,14]. Studies found that the transgenic coexpression of *MYCN* and *LMO1* in the PSNS of the zebrafish model not only promotes earlier onset of NB, but also induces widespread metastasis to the tissues and organs that are similar to sites commonly seen in patients with high-risk NB[13]. Very recently, another metastatic phenotype of NB has also been observed in a newer zebrafish model of NB, in which both *MYCN* and *Lin28B,* encoding an RNA binding protein, are overexpressed under control of the *dβh* promoter[16].

The stable transgenic approach in zebrafish is often used to study whether overexpression of a gene of interest could contribute to the normal development and disease pathogenesis[14,15]. This technique has been successfully used to demonstrate the importance of multiple genes and pathways to NB tumorigenesis[6,16-20]. This paper will introduce how the transgenic fish line that overexpresses both *MYCN* and *LMO1* in the PSNS was created and how it was demonstrated that the cooperation of these two oncogenes accelerate the onset of NB tumorigenesis and metastasis[13]. First, the transgenic line that overexpresses *EGFP-MYCN* under control of the *dβh* promoter (designated *MYCN* line) was developed by injecting the *dβh-EGFP-MYCN* construct into one-cell stage of wild-type (WT) AB embryos, as previously described[6,17]. A separate transgenic line that overexpresses *LMO1* in the PSNS (designated *LMO1* line) was developed by coinjecting two DNA constructs, *dβh-LMO1* and *dβh-mCherry*, into WT embryos at the one-cell stage[13]. It has been previously demonstrated that coinjected double DNA constructs can be cointegrated into the fish genome; therefore, *LMO1* and *mCherry* are coexpressed in the PSNS cells of the transgenic animals. Once the injected F0 embryos reached sexual maturity, they were then out-crossed with WT fish for the identification of positive fish with transgene(s) integration. Briefly, the F1 offspring were first screened by fluorescent microscopy for *mCherry* expression in the PSNS cells. The germline integration of *LMO1* in mCherry-positive fish was further confirmed by genomic PCR and sequencing. After successful identification of each transgenic line, the progeny of heterozygous *MYCN* and *LMO1* transgenic fish were interbred to generate a compound fish line expressing both *MYCN* and *LMO1* (designated *MYCN;LMO1* line). Tumor-bearing *MYCN;LMO1* fish were monitored by fluorescent microscopy biweekly for the evidence of metastatic tumors in the regions distant to the primary site, interrenal gland region (IRG,

zebrafish equivalent of human adrenal gland)[13]. To confirm the metastasis of tumors in *MYCN;LMO1* fish, histological and immunohistochemical analyses were applied.

PROTOCOL:

All research methods using zebrafish and animal care/maintenance were performed in compliance with the institutional guidelines at Mayo Clinic.

1. Preparation and microinjection of transgene constructs for the development of *LMO1* transgenic zebrafish line with overexpression in PSNS

1.1. To develop the *LMO1-pDONR221* entry clone, amplify the coding region of human *LMO1* from cDNA obtained from human cell line using PCR.

1.1.1. Make a 25 µL reaction as detailed here: 2.5 µL of 10x standard *Taq* Reaction Buffer, 0.125 µL of *Taq* DNA Polymerase, 0.5 µL of 10 mM dNTPs, 2 µL of cDNA template, 0.5 µL of 10 µM forward LMO1 ATTB1 primer, 0.5 µL of 10 µM reverse LMO1 ATTB2 primer, and 18.875 µL of water.

NOTE: Use forward LMO1 ATTB1 primer: 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTACACCATGATGGTGCTGGACAAGGAGGA-3' and reverse LMO1 ATTB2 primer: 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTTTACTGAACTTGGGATTCAAAGGT-3'

1.1.2. Amplify using the following program: 1 cycle of 94 °C, 2 min; followed by 30 cycles of (94 °C, 30 s, 55 °C, 30 s, 72 °C, 1 min) and 72 °C, 7 min.

1.2. Clone *LMO1* PCR product into the *pDONR221* gateway donor vector by BP recombinase reaction[6,13] (PCR fragment + Donor vector = Entry Clone).

1.2.1. For a 10 µL reaction, mix 1 µL of purified *LMO1* PCR product (150 ng/µL), 1 µL (150 ng/µL) *pDONR221* donor vector, and 6 µL of TE buffer (pH 8.0) with 2 µL of BP enzyme mix, incubate for 1 h at 25 °C, and transform to TOP10 competent *E. coli* using the manufacturer's protocol.

1.2.2. Next, spread 50-200 µL from the transformation vial on a Luria Broth (LB) agar plate containing 50 µg/mL kanamycin and incubate at 37 °C overnight.

1.2.3. Select clones by inoculating a single colony into 2-5 mL of LB with 50 µg/mL kanamycin and culturing overnight (16-18 h) at 37 °C.

1.2.4. Use 2 mL of overnight bacterial culture for plasmid isolation according to the manufacturer's protocol. To verify the LMO1 plasmid, send plasmid sample out for sequencing with M13-F primer (5- GTAAAACGACGGCCAG-3').

1.3. To generate a *dβh-pDONRP4-P1R* entry clone, obtain *dβh* PCR product[6,13] by amplifying the

5.2-kb promotor region using the CH211-270H11 BAC clone as template to prepare a 20 µL reaction as previously described in step 1.1.1. Use the following PCR program parameters: 94 °C, 2 min; 10 cycles of 94 °C, 15 s, 50 °C, 30 s, 68 °C, 8 min; 30 cycles of 94 °C, 15 s, 53 °C, 30 s, 68 °C, 8 min; 68 °C, 4 min (with forward primer 5'GGGGACAACTTTGTATAGAAAAGTTGGCGTACTCCCCCTTTTTAGG-3' and reverse primer 5'-GGGGACTGCTTTTTTGTACAAACTTGTGTTGCTTTGTCGTCTTTTGA-3').

NOTE: Due to the long DNA templates in this step, ensure usage of an appropriate PCR system for accurate PCR amplification.

1.4. Clone *dβh* PCR product into the *pDONRP4-P1R* gateway donor vector by BP recombinase reaction[6,13] (PCR fragment + Donor vector = Entry Clone).

1.4.1. Mix 1 µL of purified *dβh* PCR product (172 ng/uL), 1 µL (150 ng/µL) of pDONRP4-P1R donor vector, and 6 µL of TE buffer (pH 8.0) with 2 µL of BP enzyme mix in a total of 10 µL reaction, incubate for 1 h at 25 °C.

1.4.2. Transform to TOP10 competent *E. coli* according to the manufacturer's protocol. Isolate the plasmid as described in steps 1.2.2-1.2.4 and verify plasmid by sequencing with M13-F (5'-GTAAAACGACGGCCAG-3') and M13-R (5'-CAGGAAACAGCTATGAC-3') primers.

1.5. Generate the *dβh:LMO1* expression construct.

1.5.1. Combine 1 µL of each entry clone (*dβh -pDONRP4-P1R, LMO1-pDONR221* and *p3E-polyA*[11]*)* (20 fmole each), 1 µL (60 ng/µL) of modified destination vector with I-SceI recognition sites, and 4 µL of TE buffer (pH 8.0) containing 2 µL of the LR recombinase enzyme mix. Incubate this mixture for 1 h at 25 °C.

1.5.2. While following the manufacturer's protocol, transform bacteria to chemically competent TOP10 *E. coli*. Isolate plasmid as described in steps 1.2.2-1.2.4 and verify plasmid by sequencing with DBH Forward (5'-GAAGCTGTCACAGGGTTGTG-3') and LMO1 (5'-GGCATTGGACAAGTACTGGCA-3') primers.

1.6. Generate *dβh:mCherry* DNA construct with a gateway system by combining entry clones of a 5.2-kb *dβh* promoter, *pME-mCherry,* and *p3E-polyA* into the modified destination vector containing *I-SceI* recognition sites as mentioned previously in step 1.5.1[6]. Isolate plasmid as described in steps 1.2.2-1.2.4 and verify plasmid by sequencing with DBH Forward primer (5'-GAAGCTGTCACAGGGTTGTG-3').

1.7. Linearize *dβh:LMO1* DNA construct and *dβh:mCherry* DNA construct (at a 3:1 ratio, respectively) in a total of a 15 µL reaction volume with 1 µL of I-SceI enzyme (5 U/µL) and 0.75 µL of buffer (10x), and incubate at room temperature for a minimum of 4 h or overnight. Ensure that DNA concentration does not exceed 750 ng in reaction.

1.8. Next day and after linearization of constructs, add 0.5 µL of fresh I-SceI enzyme (5 U/µL) and 0.5 µL of 0.5% phenol red into 5 µL of total DNA mixture from previous step of 1.7. Microinject the total solution (of 50-80 pg of linearized DNA) into one-cell-stage wild-type AB embryos (and as many as 500 embryos) using 1.0 mm diameter glass micropipette needle as previously described[17]. Store the remaining DNA mixture at -20 °C for future injections.

2. Screen and verify *LMO1* transgenic fish line for germline transmission of *LMO1* and *mCherry*

2.1. At 3-4 days-post-fertilization (dpf), anesthetize injected embryos with 0.02% tricaine and screen them for fluorescent mCherry expression, which presents anywhere throughout the fish body due to the mosaic transgenesis. Transfer mCherry-positive embryos into a Petri dish with fresh egg water and raise embryos to sexual maturity in accordance with the zebrafish book[21].

NOTE: Expect the ratio of positive fish to vary depending on the expertise and experience of research personnel. For example, beginning amateurs may have a low integration rate of 10%, compared to an expert's of ≥50%.

2.2. To determine the founder fish with *dβh*:*LMO1* and *dβh*:*mCherry* transgenes integrated into germ cells, outcross single pairs of injected mCherry-positive F0 sexually matured fish from previous step (2.1) with WT AB fish. Screen the F1 generation for mCherry-positive embryos at 3-4 dpf.

2.3. To confirm that the mCherry-positive fish carry *LMO1* transgene, isolate the gDNA from F1 mCherry-positive single embryo using the gDNA extraction buffer, which contains: 12.5 µL of 4x lysis buffer (500 µL of 1 M Tris with pH of 8.4, 2.5 mL of 1 M potassium chloride, and 47 mL of purified water), 35 µL of purified water, and 2.5 µL of proteinase K at 10 mg/mL. Incubate the sample for 16 h at 55 °C, followed by 10 min at 98 °C.

2.4. Use the extracted gDNA (2 µL) as a template for the genotyping PCR with primers: *LMO1* FW: 5'-GGCATTGGACAAGTACTGGCA -3' and *LMO1* RV: 5'-CGAAGGCTGGGATCAGCTTG -3', and the following PCR program: 1 cycle of 94 °C, 10 min; 35 cycles of (94 °C for 30 s, 60 °C for 30 s; 68 °C, 30 s); and 68 °C for 7 min. Confirm the amplified 182 bp fragment by sequencing.

2.5. After confirmation of the genotype, raise the remaining mCherry-positive F1 embryos to maturity in accordance with the standard guidelines of the zebrafish book[21]. Fin clip and genotype them at 2-3 months of age using steps 2.3-2.4 from the protocol above to further confirm the integration of the *LMO1* transgene in the fish.

NOTE: The *LMO1*-positive F1 fish will be the stable transgenic fish [*Tg(dβh:LMO1),Tg(dβh:mCherry*)] (designated as *LMO1* line).

2.6. Breed F1 *LMO1* stable transgenic fish with WT fish and repeat as needed to propagate this line.

3. Outcross of *LMO1* and *MYCN* transgenic lines to create metastatic model

3.1. Once the *LMO1* transgenic zebrafish line is generated, interbreed with *MYCN* line[6,17] to develop heterozygous transgenic fish line overexpressing both *MYCN* and *LMO1*.

3.2. At 1 dpf, sort the progeny of outcross for EGFP expression with stereoscopic fluorescence microscope, which presents as EGFP-positive points in the hindbrain region.

NOTE: Alternatively, if not all embryos are sorted for *MYCN* at 1 dpf, raise the embryos to adulthood and genotype by fin clipping and using formerly stated guidelines from steps 2.3-2.4 for gDNA isolation and PCR genotyping, with primers: *MYCN*-F (5'-ATT CAC CAT CAC TGT GCG TCC-3'); *MYCN*-R (5'-TGC ATC CTC ACT CTC CAC GTA-3', and the following program with standard *Taq* polymerase: 1 cycle of 94 °C for 3 min, 35 cycles of (94 °C for 30 s, 60 °C for 30 s, and 68 °C for 3 min), and 68 °C for 7 min with expected amplicon size of 145 bp.

3.3. After sorting for EGFP at 1 dpf, isolate screened embryos into separate Petri dishes and label dishes as: *MYCN*+ (EGFP-positive) or *MYCN*- (EGFP-negative).

3.4. At 3-4 dpf, visualize and sort embryos of both groups (step 3.3) for *LMO1* expression with a stereoscopic fluorescence microscope. Look for red fluorescent protein expression as spots in both the superior cervical ganglion and non-PSNS dopaminergic neuronal cells in the head region, especially in oblongata medulla of the hindbrain[6,13].

3.4.1. Screen for mCherry/*LMO1* before embryos reach 5 dpf, because air-filled swim bladders fully develop by then and will cause the embryos to float, resulting in difficult microscopic focusing of the PSNS cells during the sorting process.

3.5. Once sorting is completed, isolate sorted fish into four groups of different genotypes and label as below. Raise the sorted fish in identical conditions according to the standard protocols from the zebrafish book[21].

3.5.1. *MYCN*-only (EGFP-positive),

3.5.2. *LMO1*-only (mCherry-positive),

3.5.3. *MYCN*; *LMO1* (EGFP and mCherry double positive),

3.5.4. WT (EGFP and mCherry double negative).

4. **Visualizing tumor burden in transgenic zebrafish lines**

4.1. At 4 weeks-post-fertilization (wpf), anesthetize sorted fish from step 3.5 with 0.02% tricaine in a petri dish.

4.2. Visualize tumors with a stereoscopic fluorescence microscope by gently flipping fish with a metal spatula onto both lateral sides to view tumor. Expect tumors to present as single EGFP-, single mCherry-, or double EGFP-and-mCherry-positive masses that arise from the interrenal gland region (near head and kidney).

NOTE: It is possible that the initial tumor onset is typically presented with a brighter and larger fluorescence positive mass on one side of interrenal gland, which is why it is crucial to visualize both sides of fish to avoid missing early tumor onset.

4.3. After identification of the possible tumor-bearing fish, isolate fish into a separate tank with appropriate labels, which includes date of birth, date when tumor screened, and genotype.

4.4. At 6 wpf, repeat previous steps 4.1-4.3 to screen tumor-bearing fish and non-tumor bearing fish again to confirm the presence of tumors for previously screened tumor-bearing fish or identify new possible tumor-bearing fish, respectively. Look for sustained or increased size of fluorescence-positive mass in confirmed tumor-bearing fish.

4.5. After identifying tumor-bearing fish, monitor them biweekly for evidence of tumor cell migration, which presents as tiny EGFP- and/or mCherry-positive tumor masses far from the primary site of tumorgenesis (interrenal gland region). Isolate these fish into separate tanks as needed and label appropriately to indicate possible metastasis.

4.6. To further confirm the metastasis, continue tracking these fish regularly (every two weeks) until the distant fluorescence-positive tumor masses show clearly increased size, indicating growth of tumors in the metastatic sites.

5. Tissue processing and paraffin sectioning of tumor-bearing fish

NOTE: Perform this step to characterize the spontaneously developed primary and/or metastatic tumors in *MYCN and MYCN;LMO1* transgenic fish.

5.1. After identification and verification of tumor-bearing fish, sacrifice fish from step 4.6 in a Petri dish by submerging fish in 0.02% tricaine to anesthetize first and then increasing tricaine dosage until fish is no longer respiring. Cut the fish into two pieces—with one piece containing the head and a portion of the body's middle section (including tumor), and another piece with the rest of the fish's middle section and tail.

5.2. Fix both fish sections with 4% paraformaldehyde (PFA) in 1x PBS for 1 h at room temperature on a rocker. To enhance fixation efficiency, replace buffer with fresh 4% PFA to increase penetrance to the internal organs effectively and quickly. Leave fish with fresh fixing buffer on a rocker at 4 °C overnight or for 48 h.

CAUTION: PFA is toxic. Since precautionary procedures and proper disposal of reagent depends on the regulations of your institution, seek proper guidelines before using and disposal of

reagent.

5.3. Prior to the processing, place sample(s) in 100% rapid decalcifier solution for 15-20 min at room temperature. Make sure to use a nonmetal container and to check sample(s) throughout incubation to prevent over decalcification. If sample tissue looks heavily degraded, place sample in water or at 4 °C to slow down the decalcification process.

5.4. Once fixed and decalcified, wash fish sample(s) with running tap water for 1 h and place it into processor cassette(s). If there is more than one sample, separate each into its own cassette.

5.5. Dehydrate and prepare the tissue for paraffin embedding by placing cassette(s) with fish into the tissue processor where the sample(s) are submerged in various solutions, respectively, as follows: 70% ethanol (60 min, 40 °C), 85% ethanol (50 min, 40 °C), 95% ethanol (40 min, 40 °C) twice, 100% ethanol (30 min, 40 °C), another fresh solution of 100% ethanol (50 min, 40 °C) twice, xylene (30 min, 40 °C), another fresh solution of xylene (50 min, 40 °C), another fresh xylene (50 min, 45 °C), paraffin (30 min, 45 °C), paraffin (20 min, 58 °C) three times.

5.6. After the tissue is processed, unload the cassette(s) from the processor machine while maintaining organization of each cassette and making sure to prevent mixing up the fish samples.

5.7. Choose the appropriate sized mold based on the size of the fish, making sure the mold will fit the entire cassette with the fish sample. Cover the bottom of the mold with liquid paraffin at 60 °C.

5.8. Immediately place cassette with the fish on top of mold (making sure the fish is laid on its flat lateral side for sagittal sections) with liquid paraffin, and finish filling with paraffin to the top of the mold to completely embed the fish.

5.9. Place the completed mold on the cold plate of the sectioning microtome until the paraffin is hardened.

5.10. Once the paraffin block is hard, which can be judged by visually observing higher opacity and a firm touch to the block, gently remove the block from the mold. Carefully scrape off any excess paraffin for the sides of the cassette.

5.11. Section the paraffin-embedded fish block sagittally at 4 microns on the microtome and float the sections on a warm water bath at 40 °C containing distilled water.

5.12. Carefully transfer the sections onto positively charged slides and bake in oven at 60 °C for 30 min prior to staining before proceeding to either step 6, 7, or 8.

6. Hematoxylin and eosin (H&E) staining of paraffin sections for pathology review

6.1. Place slides containing paraffin sections from step 5.12 into a slide holder. Inside a chemical

fume hood, deparaffinize with xylene 3 times, 5 min each. Discard solution after each use.

6.2. Rehydrate sections with 100% ethanol for 3 min, and repeat twice with fresh 100% ethanol. Replace 100% ethanol with 95% ethanol for 3 min and then, 80% ethanol for 3 min.

6.3. Rinse sections with distilled water for 5 min. While the sections are washing in water, make sure to remove oxidized particles from hematoxylin by either filtering or skimming surface of hematoxylin with a lint-free wipe.

6.4. Blot the excess water from the slide holder with lint-free professional grade wipes, and stain slides in 50% hematoxylin (1:1 dilution with distilled water) for 2-5 min depending on desired staining preference and reagent deterioration. Discard hematoxylin when solution color changes from plum to blue/brown or when staining time becomes excessive. Rinse the slides with running tap water for 20 min.

6.5. Decolorize the sections in 1% acid ethanol (3.3 mL of Hydrochloric acid with 50 mL of 70% ethanol) by quickly dipping the slides multiple times. Slides can be dipped up to 3 s, but the longer the slides are dipped, the lighter the sections become.

6.6. Rinse the slides in tap water twice for 1 min each, and once with deionized water. As an option, slides can be left overnight at this stage, soaking in water.

6.7. Immerse the sections in 1.36% lithium carbonate solution (47 g lithium carbonate, 3500 mL water) for 3 s. Make sure not to over incubate the sections since the longer the time in lithium carbonate solution, the more likely the tissue will float.

6.8. Rinse the sections in tap water for 5 min, and blot the excess water from the slide holder with lint-free professional grade wipes.

6.9. Counterstain the slides in 100% ready-to-use eosin for 15-30 s, and immediately dehydrate with 95% ethanol twice for 5 min each. Replace with 100% ethanol twice for 5 min each, discarding the solutions after each use.

6.10. Clear the sections in xylene for 15 min and repeat twice for 3 times in total. Optionally, the slides can be left in xylene overnight at room temperature to better clear excess water.

6.11. Allow the slides to air dry in the chemical fume hood and then mount a cover slip using mounting medium to seal tissue sample.

6.12. Visualize and image the stained tissue sections under microscopy. Carefully examine the tumors at the primary site (the interrenal gland region in head kidney) and the distant sporadic metastases in other tissues and organs of the fish. Send out stained slides for tissue sections to be reviewed by pathologists. Based on the fish model's similar pathological features to human neuroblastoma, expect the tumors that arose in *MYCN*-only or *MYCN;LMO1* fish to be first

diagnosed as neuroblastoma by pathologists.

7. Immunohistochemical Analysis (IHC) with antibodies against NB marker and overexpressed transgenes to further confirm the spread of tumor and their sympathoadrenal lineage property

7.1. Staining with fully automatic staining system

7.1.1. To better compare the IHC result with that of H&E staining, select adjacent slides from step 5.12 for IHC staining.

NOTE: Although an automated staining system allows for faster and less laborious results, a manual protocol for IHC staining can be used in the absence of such by referring to steps of subsection 7.2.

7.1.2. Dilute the primary antibody against tyrosine hydroxylase (TH) (1:500), a neuroblastoma marker, based on desired concentration and total amount needed with the automated system's corresponding Primary Antibody Diluent.

NOTE: Optimal antibody concentrations may vary depending on antibody and tissue.

7.1.3. Since the automated system uses onboard heat-induced antigen retrieval with epitope retrieval solution for 20 min and the system's corresponding detection reagent, ensure the parameters for the machine are set as: Peroxidase Blocking, 5 min; Primary antibody with desired concentration, 15 min; biotinylated anti-rabbit IgG secondary antibody (1:500), 8 min; Streptavidin HRP, 8 min; mixed DAB intense substrate, 5 min; and Hematoxylin, 5 min.

7.1.4. Once the settings are set, place the antibody tray into the machine. Set up the slides by creating a new case, which will label the slides. The machine is now loaded and ready to run (dewaxing, staining, and counterstaining).

7.1.5. Once the slides are dewaxed, stained, and counterstained using the automated machine, dehydrate the slides in ethanol gradients of 70%, 95%, and 100% for 5 min each. Submerge slides of sections in 100% fresh ethanol again for 5 min.

7.1.6. Clear the sections in xylene for 5 min, twice, or leave slides in xylene overnight to better clear excess water.

7.1.7. Allow the slides to air dry in the chemical fume hood and then mount a cover slip using a mounting medium. Visualize and image the stained tissue sections with microscopy.

7.1.8. To firmly confirm metastasis in the fish model, compare the slides stained with antibodies against neuroblastoma marker from step 7 to those stained by H&E from step 6.

NOTE: Expect to see positive staining for neuroblastoma marker, TH, in all tumor cells identified

by H&E staining. Also expect no positive staining for TH in adjacent tissues where the metastatic tumors were identified in *LMO1;MYCN* fish. Make sure to select control WT and *MYCN*-only fish that are of similar age to *MYCN;LMO1* fish for this analysis to rule out the possibility that the metastatic tumors are multifocal primary tumors.

7.2. Manually staining without automated IHC staining system

7.2.1. After the slides are baked, select adjacent slides from step 5.12 to allow better comparison between H&E and IHC staining to deparaffinize. Inside a chemical fume hood, dewax and rehydrate the slides with xylene using previous steps 6.1 and 6.2., respectively.

7.2.2. After deparaffinization and rehydration, soak slides in endogenous peroxide blocking solution (1x PBS containing 0.1% sodium azide and 0.3% hydrogen peroxide) for 5 min at room temperature. Wash slides in fresh 1x PBS for 3 min and repeat twice for a total of 3 times.

CAUTION: Sodium azide is acutely toxic. Ensure to practice precautionary procedures and proper disposal of reagent depending on the regulations of your institution.

7.2.3. Retrieve antigen by incubating slides in solution with proteinase K (1:500 in 1x PBS) for 10 min at room temperature. Wash slides 3 times with 1x PBS for 3 min each.

7.2.4. Block slides by incubating with 5% goat serum in 1x PBS for 30 min at room temperature on rocker. Wash slides with fresh 1x PBS twice for 3 min each.

7.2.5. Add 4 drops of Avidin blocking solution directly onto slide and incubate for 15 min at room temperature. After washing slides with fresh 1x PBS twice for 3 min each, add 4 drops of Biotin blocking solution and incubate at room temperature for 15 min.

7.2.6. After blocking slides, incubate in primary antibody of tyrosine hydroxylase (TH) (1:500) for 45-60 min at room temperature, or overnight at 4 °C.

NOTE: Stain with additional antibodies against transgenes and other relevant markers to address physiology and activity of primary tumor and other metastatic sites. Optimal antibody concentrations may vary depending on antibody and tissue.

7.2.7. Wash slides with fresh 1x PBS for 3 min, repeating twice with a total of 3 times.

7.2.8. Incubate slides in secondary antibody of biotinylated anti-rabbit IgG secondary antibody (1:500) diluted in blocking solution for 45-60 min at room temperature. Repeat previous washing step 7.2.7.

7.2.9. Add HRP conjugated Avidin (1:300 in 1x PBS), and incubate for 20 min at room temperature. Wash slides 3 times with fresh 1x PBS for 5 min each.

7.2.10. Prepare 3,3'-Diaminobenzidine (DAB) solution (2.5 mL distilled water, 1 drop of kit buffer, 1 drop of hydrogen peroxide, and 2 drops of DAB from kit), and place drops of DAB directly onto slides near a microscope. After adding DAB, observe the color change reaction on the slides under microscope. Once desired staining intensity is reached, stop reaction by placing slide sections in cold distilled water.

7.2.11. Counterstain the slides with fresh 50% hematoxylin by submerging or dipping samples for a few short seconds and placing back into distilled water. Repeat as desired, but normally, once should be enough since tissue sections are thin.

7.2.12. Dehydrate tissue samples on slides in alcohol gradient as previously described in step 7.1.5 and continue through the remaining steps (with the last step as 7.1.8) to finish the IHC analysis.

8. Picrosirius red staining of paraffin slides for collagen accumulation in tumors as mechanism study

8.1. Repeat steps 6.1-6.3 to dewax, hydrate, and wash paraffin sections on slides.

8.2. After blotting the excess water from the slide holder with professional grade lint-free wipes, stain in 50% hematoxylin for 10 min. Rinse the slides with running tap water for 10 min.

8.3. Transfer the slides into Picrosirius Red and incubate at room temperature for 1 h.

8.4. Wash the slides in acidified water (5 mL glacial acetic acid in 1 L of tap or distilled water) twice, incubating for 5 min on shaker.

8.5. Decant most of the water from slides first before physically shaking and blotting sections on slides to remove as much water as possible.

8.6. Quickly place slides into three changes of 100% ethanol to dehydrate paraffin sections.

8.7. Repeat steps 6.10 and 6.11 to clear slides in xylene and mount sample. Next, image with compound microscope that is equipped with a camera.

8.8. Using ImageJ, count the picrosirius red-positive collagen fibers in at least three random fields for each section. Compare between slides of MYCN and MYCN;LMO1 fish sections.

REPRESENTATIVE RESULTS:

To determine whether *LMO1* synergizes with *MYCN* to affect NB pathogenesis, transgenic constructs that drive expression of either *LMO1* (*dβh:LMO1* and *dβh:mCherry*) or *MYCN* (*dβh:EGFP-MYCN)* in the PSNS cells under control of the *dβh* promoter were injected into zebrafish embryos[13]. As illustrated in **Figure 1A**, after the development of stable transgenic lines and validation of their genotypes, heterozygous *MYCN* and *LMO1* fish were interbred. Their

offspring were sorted for *MYCN* (EGFP+) or *LMO1* (mCherry+) expression at 1 dpf or 3-4 dpf, respectively. *MYCN* overexpression has been shown to suppress PSNS development[6]. Thus, the EGFP-MYCN expression is more prominent in the non-PSNS dopaminergic neuronal cells at 1 dpf[6], such as the cranial ganglia (CA), arch-associated catecholaminergic neurons (AAC), and medulla oblongata (MO). Due to the instability of *EGFP-MYCN* protein, the EGFP signal becomes dimmer after 2 days. In contrast, the mCherry expression is prominent in both PSNS cells, such as the superior cervical ganglion and non-PSNS dopaminergic neuronal cells, which includes CA, AAC, and MO, from 3 dpf and onwards[6]. Hence, the offspring of MYCN and LMO1 mating are sorted at 1 dpf for *MYCN* (EGFP+) and at 3 dpf for *LMO1* (mCherry+). The sorted fish were separated into different genotypic groups, as follows: i) *MYCN*, ii) *LMO1*, iii) *MYCN;LMO1*, and iv) WT, and raised in the identical conditions. Beginning at 4 wpf, the offspring were screened biweekly for the evidence of tumors by fluorescent microscopy. Fluorescent positive tumor masses were detected in the tissues and organs distant from the primary tumor site in the compound transgenic fish with overexpression of both *MYCN* and *LMO1*, but not in the transgenic fish with expression of *MYCN* alone (**Figure 1B**).

To further verify the metastasis in these transgenic animals, tumor-bearing *MYCN* and *MYCN;LMO1* transgenic fish at 5 to 9 months of age were subjected for paraffin sectioning and immunohistochemical analyses with an antibody against the neuroblastoma marker, TH. The representative results for a *MYCN;LMO1* fish is presented in **Figure 2**. H&E staining of the sagittal sections showed that the primary tumor arose from the interrenal gland region, the zebrafish equivalent of the human adrenal gland, which is the most common site of primary disease in neuroblastoma patient[12,22] (**Figures 2A,B**). Composed of tiny, undifferentiated, and round cancerous cells with hyperchromatic nuclei, the tumor often formed layers that were histologically similar to the human neuroblastomas as described previously[6] (**Figures 2A,B**). Consistent with the observation by fluorescent microscopy (**Figure 1B**), widespread tumor masses distant from the inter-renal gland were detected in multiple regions, including: distal portion of the kidney (the primary adult hematopoietic niche of zebrafish and comparable to the mammalian bone marrow[23]) (**Figure 2A,C**), orbit (**Figures 2A,D**), gill (analogous to the mammalian lung[24]) (**Figures 2A,E**), spleen (analogous to the mammalian lymph node[25]) (Figure 2A and 2F) and inner wall of atrial chamber of heart (**Figures 2G**). Many of these metastases recapitulate the common sites of metastases seen in patients with high-risk neuroblastoma, which include the bone marrow, lymph node, orbit region and lung[13,23,24,25]. Further immunohistochemistry with the antibody against TH confirmed the PSNS neuroblast lineage of tumor cells at the primary tumor and all the metastatic sites (**Figures 2H–M**).

In an effort to better understand the mechanisms underlying the synergy between *MYCN* and *LMO1* in accelerating tumor metastatic spread, it was discovered that the expression levels of a panel of genes involved in tumor cell to extracellular matrix interaction were significantly elevated in the fish tumors with overexpression of both *MYCN* and *LMO1*[13]. To examine whether the extracellular matrix was indeed affected by the altered gene expression in *LMO1*-overexpressing tumors leading to enhanced tumor dissemination or migration, the paraffin sections from *MYCN*-only and *MYCN;LMO1* tumors were stained with Picrosirius red (PSR), a highly selective stain for collagen fibers to assess collagen deposition and extracellular matrix

(ECM) stiffness[26,27]. As demonstrated in **Figure 3A-E**, there were significantly amplified amounts and increased thickness of PSR-stained collagen fibers found in *MYCN;LMO1* tumors, when compared to the tumors only expressing *MYCN* alone. Together, these results demonstrate that the overexpression of *LMO1* can remodel the tumor ECM to increase its stiffness, and therefore, facilitating tumor cell dissemination.

FIGURE AND TABLE LEGENDS:

Figure 1: Illustration of the tumor screening assay on offspring from the breeding of *MYCN* and *LMO1* transgenic zebrafish lines. (**A**) Schematic illustration of sorting and tumor screening of *MYCN* and/or *LMO1*-overexpressing stable transgenic fish for neuroblastoma study. *Upper panels*: Representative pictures of EGFP-MYCN$^+$ embryos at 1 day postfertilization (dpf) (dorsal view on the left and lateral view on the right). *Lower panels*: Representative pictures of mCherry-LMO1$^+$ embryos at 3 dpf (lateral view on the left and ventral view on the right). AAC, arch-associated catecholaminergic neurons; CA, cranial ganglia; and MO, medulla oblongata. Scale bar, 100 μm. Created with BioRender.com (**B**) Coexpression of *LMO1* and *MYCN* promotes neuroblastoma metastasis. *Left*: *T*ransgenic fish overexpressing MYCN alone (*MYCN*) with EGFP-expressing tumor (white arrow) at 36 months of age. *Right*: Transgenic fish overexpressing both *MYCN* and *LMO*1 (*MYCN;LMO1*) with a mCherry-positive tumor mass at the primary site (IRG, white arrow) and multiple metastatic sites (solid arrowheads) at 36 months of age. Scale bar, 1 mm.

Figure 2: Co-overexpression of *MYCN* and *LMO1* promotes distant metastases of neuroblastoma in transgenic zebrafish model. (**A-G**) H&E-stained sagittal sections of *MYCN;LMO1* transgenic fish while at 6 months of age. (**H-M**) Magnified views of immunohistochemical analyses of *MYCN;LMO1* transgenic fish in sagittal tissue sections, using tyrosine hydroxylase (TH) antibody. White box outlines the interrenal gland (b, h), with magnified views in panels **B** and **H**. Disseminated tumor cells were found in kidney marrow (c, i, **C**, and **I** with solid black arrowheads), the sclera of the eye (d, j, **D**, and **J** with black outlined and white filled open arrows), the gill (e, k, **E**, and **K** with black outlined and white filled open arrowheads), the spleen (f, **l**, **F**, and **L** with solid black arrows), and the heart chamber (**G** and **M** with black outlined and white filled double arrowheads). Scale bars, 100 μm (**A**) and 50 μm (**B-M**). This figure has been modified from Zhu, S. et al. LMO1 Synergizes with MYCN to Promote Neuroblastoma Initiation and Metastasis. Cancer Cell. 32, 310–323 (2017)[13].

Figure 3: Increased *LMO1* expression promotes collagen deposition and ECM stiffness leading to facilitated tumor cell dissemination in zebrafish models. (**A–D**) Representative light microscopy images of collagen fibers stained by Picrosirius red (PSR) in *MYCN* only (**A,B**) or *MYCN;LMO1* (**C,D**) transgenic zebrafish. (**B**) and (**D**) are magnified from the boxed areas of (**A**) and (**C**), using arrows (**A** and **B**) and arrowheads (**C** and **D**) to indicate the PSR-positive collagen fibers, respectively. Scale bars, 100 μm (**A** and **C**) and 50 μm (**B** and **D**). (**E**) Quantification of PSR-stained areas on tumor sections of *MYCN* only or *MYCN;LMO1* transgenic fish. Results were normalized to the mean of PSR-stained areas in *MYCN*-only tumors. The statistics present as the mean ± SD of three *MYCN*-only or three *MYCN;LMO1* tumors; $p = 0.02$ by two-tailed t-test. This

figure has been modified from Zhu, S. et al. LMO1 Synergizes with MYCN to Promote Neuroblastoma Initiation and Metastasis. Cancer Cell. 32, 310–323 (2017)[13].

DISCUSSION:

Zebrafish has been commonly used in research for the past few decades, especially in cancer research, for obvious reasons, such as its ease of maintenance, robust reproduction, and clear advantages for *in vivo* imaging[1,28,]. The zebrafish model can be easily manipulated embryonically due to their external fertilization and development, which complements well to mammalian model organisms, such as rats and mice, for large-scale genetic studies[1,2,3]. Moreover, the zebrafish genome has high-level similarities to the human genome. Comparing the detailed annotations of the zebrafish genome to the human reference genome, about 70% of human genes have been shown to obtain a zebrafish orthologue[29]. In addition, other uses of zebrafish in research have been extended to drug discovery and patient avatars for individualized cancer therapy[30,31]. Accumulating studies have shown that tumors induced in zebrafish are similar to human tumors at the histological and molecular levels, which can aid in dissection of tumor initiation, progression, and heterogeneity[32,33]. Together, the zebrafish demonstrates tremendous potential as a valuable animal model that can be used to study metastasis and elucidate possible oncogenic pathways involved in disease pathogenesis.

Using the transgenic zebrafish model with overexpression of both *LMO1* and *MYCN*, the cooperation between these two oncogenes in NB tumorigenesis and metastasis has been clearly demonstrated. With today's availability of powerful live imaging techniques, tumor screens can be easily performed biweekly and metastasis can be traced over time. The widespread metastasis can also be further confirmed by paraffin sectioning and antibody staining. Strikingly, the metastases detected in the *MYCN*;*LMO1* zebrafish correlate well with the common sites of metastasis seen in high-risk NB cases, such as in the bone marrow, lymph nodes, orbit, and lung[34,35], further supporting zebrafish as a feasible and beneficial model for metastasis study.

Moreover, due to relatively small body size, the whole zebrafish can be sectioned though completely, which is yet another clear advantage of this model, allowing thorough characterization of the primary tumor along with the metastases in other regions of the body. The picrosirius red staining of tumor sections has clearly highlighted the collagen networks in fish tumors and demonstrates the increased stiffness of extracellular matrix in tumors with *LMO1* overexpression. Although this technique is not unique to zebrafish, its application together with the high-throughput compound screening on zebrafish embryos that are genetically modified or transplanted with tumor cells might provide a novel means in screening for effective compounds that could target extracellular matrix remodeling, which is a critical process involved in tumor cell metastasis.

Stable transgenic zebrafish models are very helpful for us to understand the contribution of candidate oncogenes to tumor development *in vivo*, although it can be challenging and laborious to develop these lines. Creating a stable transgenic line requires a long period of time since multiple generations must be acquired before line propagation. Furthermore, propagating these lines can be tedious, and strategic mating plans may be needed. For example, the productivity of

a *MYCN* transgenic fish is often markedly reduced once the tumor has developed, and homozygous *MYCN* transgenic fish do not survive well into adulthood. Therefore, to better maintain the *MYCN* transgenic fish line, it is recommended to outcross the heterozygous non-tumor-bearing *MYCN* transgenic fish at a younger age with WT or other genetically engineered fish lines, such as the *LMO1* transgenic fish line. To overcome the challenges in developing and maintaining stable transgenic fish lines, mosaic transient transgenesis may be an alternative approach to rapidly and effectively assess the contribution of a single gene or combination of genes to the tumor initiation and progression in primarily injected fish. In addition, the mosaic pattern of transgene integration in the primary injected fish may better mimic the disease pathogenesis, especially those induced by somatic events[36].

However, like any other animal model used in research to study cancer, the zebrafish also has its disadvantages. For example, antibodies specifically against zebrafish proteins remain largely underdeveloped, although several antibodies against neuroblastoma marker genes—such as tyrosine hydroxylase, synaptophysin, and HuC—are fortunately working well in zebrafish[6,13]. To combat this issue, many vendors have begun to test their products and predict the potential of their antibodies in cross-reacting with zebrafish proteins. More information about validated antibodies can also been found in the zebrafish information network (ZFIN). With these efforts, more and more antibodies that can specifically detect zebrafish proteins will soon become available to the zebrafish community. Another challenge of using zebrafish as a genetic model to dissect the interplay of complex signaling pathways in NB pathogenesis is its partially duplicated genome. Such genome duplication, which occurred in the natural ancestry of zebrafish[37,38], can often lead to more than one variant of zebrafish homologues to humans. This can cause an evolved gain of novel gene functions or unique expression patterns in the animal model[39]. Therefore, when studying genes with potential roles in tumor suppression, it may be necessary for multiple alleles of the duplicated genes to be knocked out at the same time to demonstrate their tumor suppression function, which can be a potentially time-consuming and a technically-challenging endeavor.

Nevertheless, the transgenic zebrafish model can faithfully recapitulate all stages of tumor metastasis *in vivo*, and possesses clear advantages for genetic analysis and real-time imaging of tumor dissemination. This model system, therefore, offers a unique tool for us to address many daunting questions in the field, such as what the molecular and cellular events underlying the multistep process of tumor dissemination and metastasis *in vivo* are, when the NB cells disseminate from primary tumors, and how the tumor microenvironment contributes to NB metastasis. With the robustness of zebrafish for drug screening and testing, the fish model would also provide a valuable means for the evaluation of efficacy of novel agents and inhibitors to prevent or treat metastatic NB.

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DISCLOSURES:

The authors declare that they have no competing financial interests.

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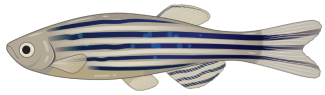
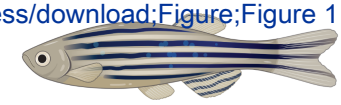


Heterozygous **MYCN** transgenic fish

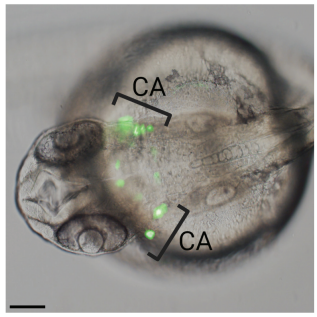
Day 0: set up for breeding

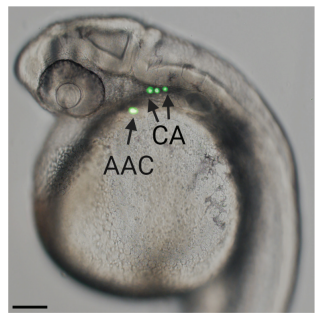
X



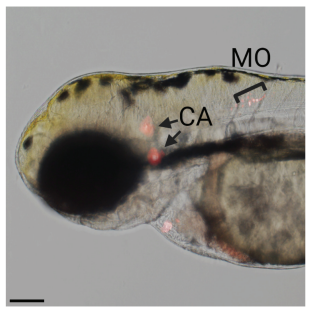
Heterozygous **LMO1** transgenic fish

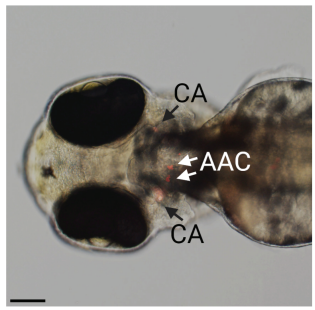
Day 2: sorting for EGFP⁺ (MYCN) embryos at 1dpf



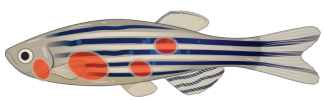


Day 4: sorting for mCherry⁺ (LMO1) embryos at 3dpf

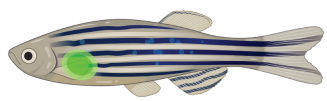




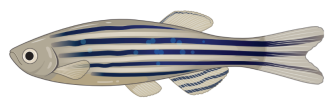
Week 4+: screening for tumor-bearing fish biweekly



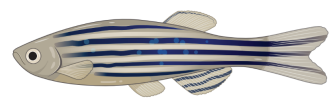
MYCN;LMO1



MYCN



LMO1



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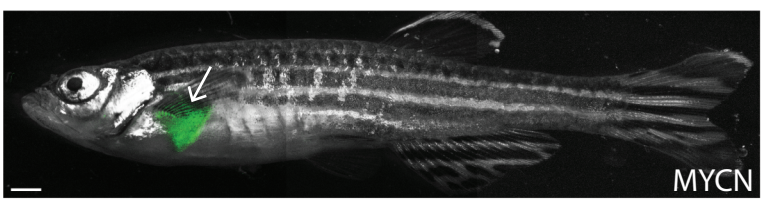

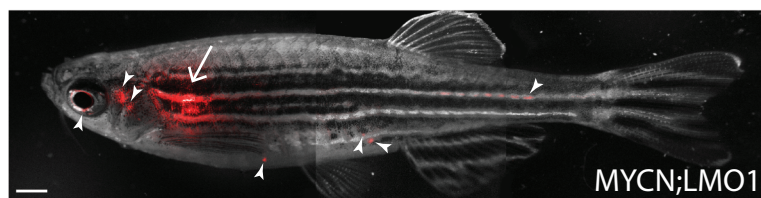

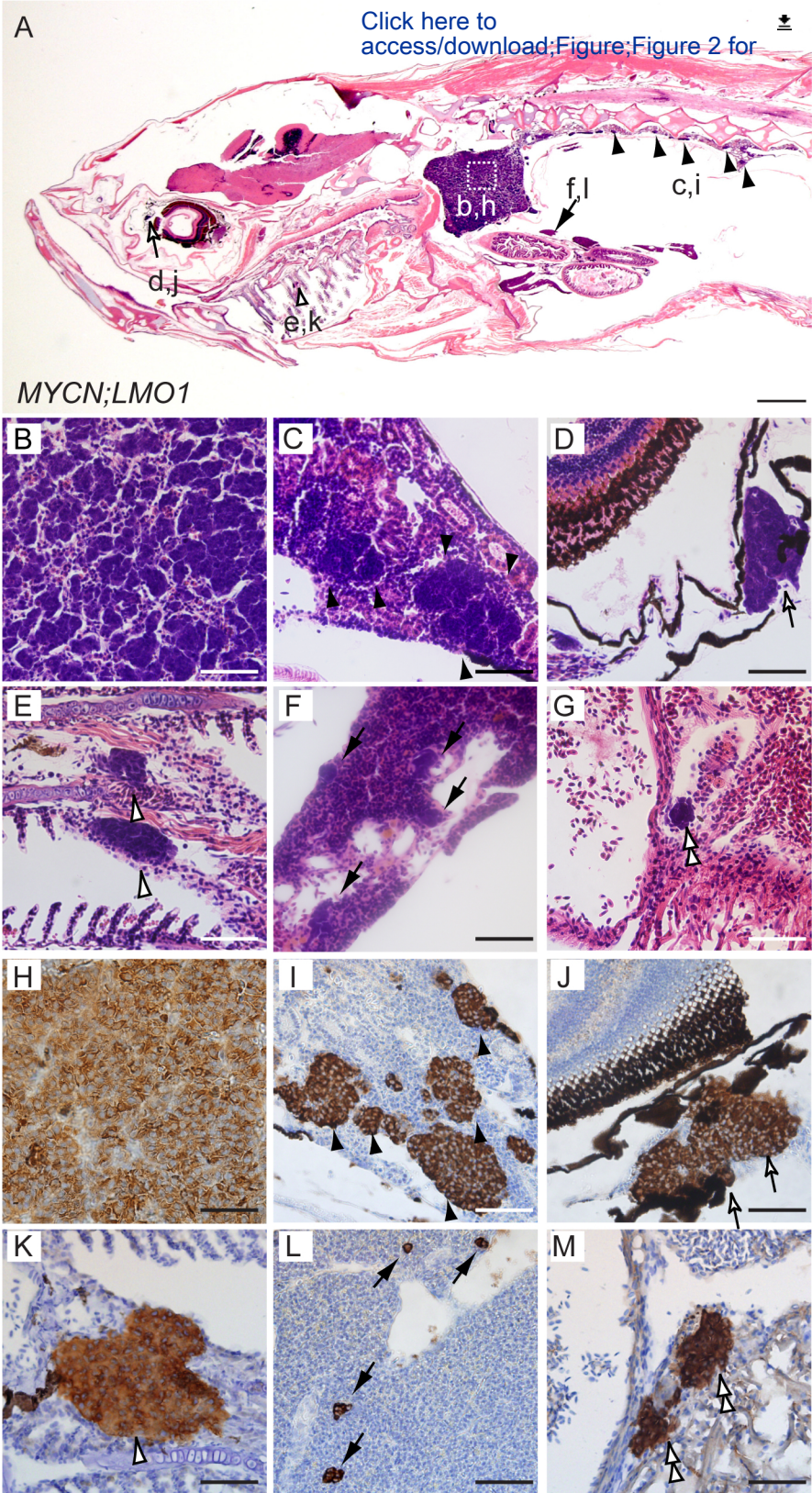
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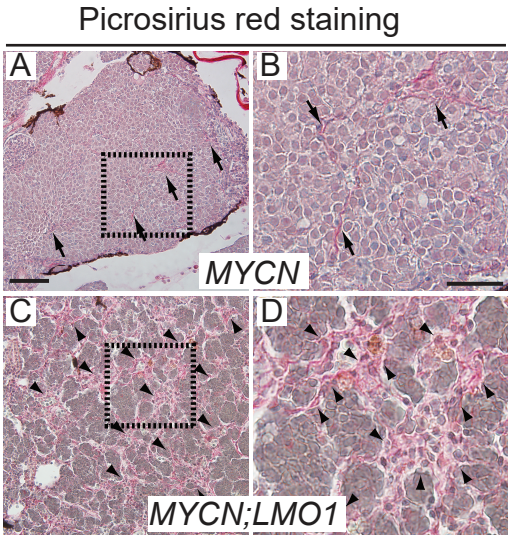

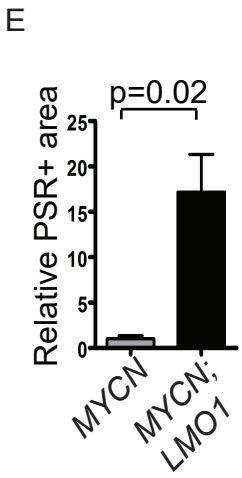

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
3,3'-Diaminobenzidine (DAB) Vector Kit	Vector	SK-4100	
Acetic Acid	Fisher Scientific / Acros Organic	64-19-7	
Agarose GP2	Midwest Scientific	009012-36-6	
Anti-Tyrosine Hydroxylase (TH) Antibody	Pel-Freez	P40101	
Avidin/Biotin Blocking Kit	Vector	SP-2001	
BOND Intense R Detection	Leica Biosystems	DS9263	
BOND primary antibody diluent	Leica Biosystems Newcastle, Ltd.	AR9352	
BOND-MAX IHC instrument	Leica Biosystems Newcastle, Ltd.	N/A	fully automated IHC staining system
CH211-270H11 BAC clone	BACPAC resources center (BRFC)	N/A	
Compound microscope equipped with DP71 camera	Olympus	AX70	

Cytoseal XYL (xylene based mounting medium)	Richard-Allan Scientific	8312-4	
Eosin	Leica	3801601	ready-to-use (no preparation needed)
Ethanol	Carolina	86-1263	
Expand Long Template PCR System	Roche Applied Science, IN	11681834001	
Gateway BP Clonase II enzyme mix	Invitrogen,	11789-020	
Gateway LR Clonase II enzyme mix	Invitrogen, CA	11791-100	
Goat anti-Rb secondary antibody (Biotinylated)	Dako	E0432	
Hematoxylin Solution, Harris Modified	Sigma Aldrich Chemical Company Inc. / SAFC	HHS-32-1L	
HRP Avidin D	Vector	A-2004	
Hydrochloric Acid	Aqua Solutions	4360-1L	
Hydrogen Peroxide, 3%	Fisher Scientific	H324-500	
I-SceI enzyme	New England Biolabs, MA	R0694L	
Kanamycin sulfate	Teknova, Inc.	K2150	
Kimberly-Clark Professional Kimtech Science Kimwipes	Fisher Scientific	34133	

Lithium Carbonate	Sigma Aldrich Chemical Company Inc. / SAFC	554-13-2	
Microtome for sectioning	Leica Biosystems	RM2255	
One Shot TOP10 Chemically Competent E. coli	Invitrogen	C404006	
p3E-polyA	Dr. Chi-Bin Chien, Univ. of Utah	N/A	a generous gift (Please refer to webpage http://tol2kit.genetics.utah.edu/index.php/Main_Page to obtain material, which is freely distrubted as described.)
Parafin wax	Surgipath Paraplast	39603002	Parrafin to parafin
Paraformaldehyde	Alfa Aesar	A11313	
pDEST vector (modified destination vector containing I-SceI recognition sites)	Dr. C. Grabher, Karlsruhe Institute of Technology, Karlsruhe, Germany	N/A	a generous gift
pDONR 221 gateway donor vector	Thermo Fisher Scientific	12536-017	

pDONRP4-P1R donor vector	Dr. Chi-Bin Chien, Univ. of Utah	N/A	a generous gift
Phenol red, 0.5%	Sigma Aldrich	P0290	
Phosphate Buffered Saline (PBS), 10X	BioRad	1610780	
Picrosirrius red stain kit	Polysciences	24901-250	
pME-mCherry	Addgene	26028	(DBH construct)
Proteinase K, recombinant, PCR Grade	Roche	21712520	
QIAprep Spin MiniPrep Kit	Qiagen	27104	
RDO Rapid Decalcifier	Apex Enginerring	RDO04	
Sodium Azide (NaN3)	Sigma Aldrich	26628-22-8	
Stereo fluorescence microscope	Leica	MZ10F	
Stereoscopic fluorescence microscope equipped with a digital sight DS-U1 camera for imaging	Nikon	SMZ-1500	
Taq DNA Polymerase	New England Biolabs, MA	M0273L	
Tissue-Tek VIP® 6 AI Vacuum Infiltration Processor	Sakura	N/A	Model #: VIP-6-A1

Tricaine-S	Western Chemical Incorporated	20513
Xylene	Thermo Fisher Scientific	X3P1GAL

Re: JoVE 62416

Zebrafish Model of Neuroblastoma Metastasis

We would like to thank the editor and each of the reviewers for his/her valuable input. Their comments have provided excellent suggestions to improve our manuscript. We have included a detailed point-by-point response to the editor's and reviewers' comments, including additional supplementary images and changes of writing in response to the comments (such modifications are formatted as underlined in the revised manuscript).

Responses to editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Thank you for your comment. The manuscript has been proofread and reviewed thoroughly as suggested.

2. Please revise the following lines to avoid previously published work: 104-106, 109-112, 116-117, 127-130, 186-187, 418-420, 439-441, 458-466, 470-477.

Thank you, this is a great suggestion. The lines mentioned above have been modified in updated manuscript on Lines 110-114, 130-138, 138-140, 152-161, 217-219, 613-615, 635-637, 657-668, and 672-681 respectively, as follows:

"Use standard Taq DNA Polymerase to prepare a 25 µL reaction following the manufacturer's protocol (2.5 µL 10X standard Taq Reaction Buffer, 0.125 µL Taq DNA Polymerase, 0.5 µL 10 mM dNTPs, 2 µL of cDNA template, 0.5 µL of 10 µM forward LMO1 ATTB1 primer, 0.5 µL of 10 µM reverse LMO1 ATTB2 primer, and 18.875 µL water)."

"To generate a dȏh-pDONRP4-P1R entry clone, obtain dȏh PCR product[6,13] by amplifying the 5.2-kb promotor region using the CH211-270H11 BAC clone as template to prepare a 20 µL reaction as previously described in section 1.1.1, while using the following PCR program parameters: 94 °C, 2 min; 10 cycles of 94 °C, 15 s, 50 °C, 30 s, 68 °C, 8 min; 30 cycles of 94 °C, 15 s, 53 °C, 30 s, 68 °C, 8 min; 68 °C, 4 min (with forward primer 5'GGGGACAACTTTGTATAGAAAAGTTGGCGTACTCCCCCTTTTTAGG-3' and reverse primer 5'-GGGGACTGCTTTTTTGTACAAACTTGTGTTGCTTTGTCGTCTTTTGA-3')."

"… Note: Due to long DNA templates in this step, ensure usage of an appropriate PCR system for accurate PCR amplification."

"Generate the dȏh:LMO1 expression construct by combining 1 µL of each entry clone (dȏh -pDONRP4-P1R, LMO1-pDONR221 and p3E-polyA11) (20 fmole each), 1 µL (60 ng/µL) of modified destination vector with I-SceI recognition sites, and 4 µL of TE buffer (pH 8.0) containing 2 µL of the LR enzyme mix. Incubate this mixture for 1 h at 25 °C. While following the manufacturer's protocol, transform bacteria to chemically competent TOP10 E. coli. Isolate plasmid as described in step 1.1.2 and verify plasmid by sequencing with DBH Forward (5'-GAAGCTGTCACAGGGTTGTG-3') and LMO1 (5'-GGCATTGGACAAGTACTGGCA-3') primers.

"1.2.5 After confirmation of genotype, raise the remaining mCherry-positive F1 embryos in accordance to the standard guidelines of the zebrafish book[21]…"

"Composed of tiny, undifferentiated, and round cancerous cells with hyperchromatic nuclei, the tumor often formed layers that were histologically similar to the human neuroblastomas as described previously[6] (Figures 2A-2B)."

"As demonstrated in Figure 3A-E, there were significantly amplified amounts and increased thickness of PSR-stained collagen fibers found in MYCN;LMO1 tumors, when compared to the tumors expressing MYCN alone."

*"**Figure 2. Co-overexpression of MYCN and LMO1 promotes distant metastases of neuroblastoma in transgenic zebrafish model**.*
(A-G) H&E-stained sagittal sections of MYCN;LMO1 transgenic fish while at 6 months of age. (H-M) Magnified views of immunohistochemical analyses of MYCN;LMO1 transgenic fish in sagittal tissue sections, using tyrosine hydroxylase (TH) antibody. White box outlines the interrenal gland (b, h), with magnified views in panels B and H. Disseminated tumor cells were found in kidney marrow (c, i, C, and I with solid black arrowheads), the sclera of the eye (d, j, D, and J with black outlined and white filled open arrows), the gill (e, k, E, and K with black outlined and white filled open arrowheads), the spleen (f, l, F, and L with solid black arrows), and the heart chamber (G and M with black outlined and white filled double arrowheads). Scale bars, 100 μm (A) and 50 μm (B-M). This figure has been modified from Zhu, S. et al. LMO1 Synergizes with MYCN to Promote Neuroblastoma Initiation and Metastasis. Cancer Cell. 32, 310–323 (2017)[13]."

"(A–D) Representative light microscopy images of collagen fibers stained by Picrosirius red (PSR) in MYCN only (A and B) or MYCN;LMO1 (C and D) transgenic zebrafish. (B) and (D) are magnified from the boxed areas of (A) and (C), using arrows (A and B) and arrowheads (C and D) to indicate the PSR-positive collagen fibers, respectively. Scale bars, 100 μm (A and C) and 50 mm (B and D). (E) Quantification of PSR-stained areas on tumor sections of MYCN only or MYCN;LMO1 transgenic fish. Results were normalized to the mean of PSR-stained areas in MYCN-only tumors. The statistics present as the mean ± SD of three MYCN-only or three MYCN;LMO1 tumors; p = 0.02 by two-tailed t-test. This figure has been modified from Zhu, S. et al. LMO1 Synergizes with MYCN to Promote Neuroblastoma Initiation and Metastasis. Cancer Cell. 32, 310–323 (2017)[13]."

3. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Thank you for your comment. All personal pronouns found in the manuscript have been omitted and revised accordingly.

4. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

An ethics statement was provided in the originally submitted manuscript before step 1 in the protocol section. However, the statement was better elaborated to clarify and address your concerns in Lines 94-95, as follows:

"All research methods using zebrafish and animal care/maintenance were performed in compliance with the institutional guidelines at Mayo Clinic."

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. For example: Clonase, One Shot, Multisite Gateway, Kim Wipe, Bond-Max, etc.

Thank you for addressing this. Commercial products used throughout the manuscript have been omitted as suggested.

6. Please use standard abbreviations for time units preceded by a numeral ("sec" becomes "s", "hr" becomes "h") (Line: 94,139,177,257, etc.).

Thank you for this great comment. The abbreviations have been modified to standard ones throughout the protocol.

7. Line 154-160/ 164-172/200-207: In the JoVE Protocol format, "Notes" should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Please consider moving some of the notes about the protocol to the discussion section.

This is a good point. Thank you for addressing this. Lines 154-160 have been completely omitted since the concept was briefly discussed towards the end of introduction in Lines 77-83, as follows:

"It has been previously demonstrated that coinjected double DNA constructs can be cointegrated into the fish genome; therefore, LMO1 and mCherry are coexpressed in the PSNS cells of the transgenic animals. Once the injected F0 embryos reached sexual maturity, they were then out-crossed with WT fish for the identification of positive fish with transgene(s) integration. Briefly, the F1 offspring were first screened by fluorescent microscopy for mCherry expression in the PSNS cells. The germline integration of LMO1 in mCherry-positive fish was further confirmed by genomic PCR and sequencing."

Lines 164-172, and 200-207 of the original manuscript has been moved and revised to fit context of discussion in Lines 592-596 and 596-599, respectively, of the updated version, as follows:

"MYCN overexpression has been shown to suppress PSNS development[6]. Thus, the EGFP-MYCN expression is more prominent in the non-PSNS dopaminergic neuronal cells at 1 dpf[6], such as the cranial ganglia (CA), arch-associated catecholaminergic neurons (AAC), and medulla oblongata (MO). Due to the instability of EGFP-MYCN protein, the EGFP signal becomes dimmer after 2 days."

"In contrast, the mCherry expression is prominent in both PSNS cells, such as the superior cervical ganglion and non-PSNS dopaminergic neuronal cells, which includes CA, AAC, and MO, from 3 dpf and onwards[6]. Hence, the offspring of MYCN and LMO1 mating are sorted at 1 dpf for MYCN (EGFP+) and at 3 dpf for LMO1 (mCherry+)."

8. Line 227-247: Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

This is a very good comment, thank you. Lines 227-247 of original submission have been modified in the new version's lines of 268-301, as follows:

2.2. "Visualizing Tumor Burden in Transgenic Zebrafish Lines

2.2.1. At 4 weeks-post-fertilization (wpf), anesthetize sorted fish from step 2.1.5 with 0.02% tricaine in a petri dish.

2.2.2. Visualize tumors with a stereoscopic fluorescence microscope by gently flipping fish with a metal spatula onto both lateral sides to view tumor. Expect tumors to present as single EGFP-, single mCherry-, or double EGFP-and-mCherry-positive masses that arise from the interrenal gland region (near head and kidney). Note: It is possible that the initial tumor onset is typically presented with a brighter and larger fluorescence positive mass on one side of interrenal gland, which is why it is crucial to visualize both sides of fish to avoid missing early tumor onset.

2.2.3. After identification of possible tumor-bearing fish, isolate fish into a separate tank with appropriate labels, which includes: date of birth, date when tumor screened, and genotype.

2.2.4. At 6 wpf, repeat previous steps 2.2.1-2.2.3 to screen tumor-bearing fish and non-tumor bearing fish again to confirm presence of tumors for previously screened tumor-bearing fish or identify new possible tumor-bearing fish, respectively. Look for sustained or increased size of fluorescence-positive mass in confirmed tumor-bearing fish.

2.2.5. After identifying tumor-bearing fish, monitor them biweekly for evidence of tumor cell migration, which presents as tiny EGFP- and/or mCherry-positive tumor masses far from the primary site of tumorgenesis (interrenal gland region). Isolate these fish into separate tanks as needed and label appropriately to indicate possible metastasis.

2.2.6. To further confirm the metastasis, continue tracking these fish regularly (every two weeks) until the distant fluorescence-positive tumor masses show clearly increased size, indicating growth of tumors in the metastatic sites."

9. Line 337-342: The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

Thank you for addressing this. The discussion of methods in step 3.3 has been omitted all together, and not further discoursed in the discussion since the concept has been previously explained in the original submitted manuscript, specifically in the Representative Results section in Lines 607-609, as indicated below:

"To further verify the metastasis in these transgenic animals, tumor-bearing MYCN and MYCN;LMO1 transgenic fish at 5 to 9 months of age were subjected for paraffin sectioning and immunohistochemical analyses with an antibody against the neuroblastoma marker, TH."

10. Please include a one-line space between each protocol step and highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

This is a great suggestion and reminder. Changes have been applied to the manuscript as suggested. Thank you!

11. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

Thank you for your comment. Permission to reuse figures from previous publications has been obtained, and such letters/information as described above will be uploaded into the corresponding Editorial Manager account. Figure legends have been updated to include suitable citations in Lines 667-668 and 679-681, as follows:

"This figure has been modified from Zhu, S. et al. LMO1 Synergizes with MYCN to Promote Neuroblastoma Initiation and Metastasis. Cancer Cell. 32, 310–323 (2017)[13]."

12. Figure 1B/ Figure 2: Please insert scale bars in all the images of the panel to provide the reader an understanding of the magnification used.

Thank you, scale bars have been inserted in images as suggested in both Figures 1 and 2.

Responses to the comments from Reviewer 1:

Manuscript Summary:
The manuscript is very well written and appropriately presented. Especially Figure 1A is very informative. I have no major criticisms and only have the following few minor points for authors to consider:

Minor Concerns:

1. The tissues specificity of dȏh promoter should be indicated at its first appearance.

Thank you for your suggestion. The *dȏh* promoter drives downstream gene expression in the peripheral sympathetic nervous system (PSNS). This description is provided in Line 47-49 when the *dȏh* promoter is appeared the first time, as follows:

"Additionally, the first zebrafish model of neuroblastoma (NB) was generated by overexpressing MYCN, an oncogene, in the peripheral sympathetic nervous system (PSNS) under control of the dopamine-beta-hydroxylase (dȏh) promoter"

2. P.4, the section title should be modified to reflect the following two points: 1. The content is restricted to dȏh:LMO1 transgenic zebrafish and thus it is more appropriate to stage KMO1 transgenic zebrafish rather than general stable transgenic line; 2. Section 1.3 also includes crossing with MYCN transgenic line. Alternatively, Section 1.3 could be a separate section, or Section 1.3 and Section 2 (relatively short) are merged.

Thank you for your comment. This is a great suggestion! Changes were made to reflect the specificity of the protocol restricted to the development of the *dȏh:LMO1* transgenic zebrafish line, and sections were merged as suggested. The new title names for the protocol section in Lines 97-100, 184-185, 228-230, and 268 (with steps omitted for ease of visualization). Such changes include the following:

*"**1. Developing Stable, Transgenic LMO1 Zebrafish Line***

- *1.1 Preparation and Microinjection of Transgene Constructs for Development of LMO1 Transgenic Line*
- *1.2 Screen and Verify LMO1 Transgenic Fish Line for Germline Transmission of LMO1 and mCherry*

2. Creating Neuroblastoma Metastatic Zebrafish Model with MYCN and LMO1 Coexpression

- *2.1 Outcrossing LMO1 and MYCN Transgenic Lines to Create Metastatic Model*
- *2.2 Visualizing Tumor Burden in Transgenic Zebrafish Lines"*

3. In 3.1.1, 4C should be 4 oC. Disposal of PFA could be elaborated.

Thank you for your comment. As suggested, 4C has been changed in Line 319. PFA disposal regulations differ between establishments, and proper disposal should be followed per those institutional guidelines. To clarify, the following changes were made to lines 319-322 of Section 3.1 of the Protocols section, as indicated below:

"Caution: PFA is toxic. Since precautionary procedures and proper disposal of reagent depends on the regulations of your institution, seek proper guidelines before using and disposal of reagent."

<u>Responses to the comments from Reviewer 2:</u>

Manuscript Summary:

Her et al. describe a method to generate a metastatic neuroblastoma model in zebrafish and provide staining methods to analyse this model.

Overall, this protocol, although it does not describe a new technique itself, but rather combines standard methods to create a metastasis model in zebrafish and characterize it, will still be valuable for the scientific community.

Major Concerns:
I have no major concerns.

Minor Concerns:
Section 1:
Please describe sections 1.1.1 & 1.1.3 in more detail.
What was the template for the PCR? How much was used in the PCR reaction? Which polymerase was used?

Thank you for your comment regarding Section 1 of the Protocol segment. To develop the stable, transgenic *LMO1* zebrafish line, the *LMO1* gene was amplified with standard PCR protocols using *Taq* DNA Polymerase (New England BioLab) and cDNA extracted from a human cell line as template. Details were added to the section 1.1.1 and 1.1.3, in Lines 102-114 and 130-140, as follows:

"1.1.1 To develop the LMO1-pDONR221 entry clone, amplify coding region of human LMO1 from cDNA obtained from human cell line using PCR with following program: 1 cycle of 94 °C, 2 min; followed by 30 cycles of (94 °C, 30 s, 55 °C, 30 s, 72 °C, 1 min;) and 72 °C, 7 min, (forward LMO1 ATTB1 primer: 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTACACCATGATGGTGCTGGACAAGGAGGA-3' and reverse LMO1 ATTB2 primer: 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTTTACTGAACTTGGGATTCAAAGGT-3'). Use standard Taq DNA Polymerase to prepare a 25 µL reaction following the manufacturer's protocol (2.5 µL 10X standard Taq Reaction Buffer, 0.125 µL Taq DNA Polymerase, 0.5 µL 10 mM dNTPs, 2 µL of cDNA template, 0.5 µL of 10 µM forward LMO1 ATTB1 primer, 0.5 µL of 10 µM reverse LMO1 ATTB2 primer, and 18.875 µL water)."

"1.1.3 To generate a dốh -pDONRP4-P1R entry clone, obtain dốh PCR product[6,13] by amplifying the 5.2-kb promotor region using the CH211-270H11 BAC clone as template and preparing a 20 µL reaction as previously described in section 1.1.1. The following cycle programs for PCR: 94 °C for 2 min, 10 cycles of (94 °C, 15 sec, 50 °C, 30 sec, 68 °C, 8 min), followed by 30 cycles of (94 °C, 15 sec, 53 °C, 30 sec, 68 °C, 8 min), 68 °C, 4 min (forward primer 5'GGGGACAACTTTGTATAGAAAAGTTGGCGTACTCCCCCTTTTTAGG-3' and reverse primer 5'-GGGGACTGCTTTTTTGTACAAACTTGTGTTGCTTTGTCGTCTTTTGA-3'). Note: Due to the long DNA templates in this step, ensure usage of an appropriate PCR system for accurate PCR amplification."

Section 3.
Is there no decalcification step needed prior to sectioning fish?

Great point, there is a decalcification step prior to sectioning fish! The procedure is described in Lines 324-328 of Section 3 of the Protocol Section, as follows:

"3.1.3 Prior to processing, place sample(s) in 100% rapid decalcifier solution for 15-20 min at room temperature. Make sure to use a nonmetal container and to check sample(s) throughout incubation to prevent overdecalclification. If sample tissue looks heavily degraded, place sample in water or at 4 °C to slow down the decalcification process."

Line 296: Should xylene be used under the fume hood?

Yes, the chemical fume hood should be used when using xylene to avoid toxic, unpleasant fumes. Changes were added in Line 374 of Protocol Section 3 as follows:

"3.2.1 Place slides containing paraffin sections from step 3.1.12 into a slide holder, and holder. Inside a chemical fume hood, deparaffinize with xylene three times, 5 min each. Discard solution after each use."

Line 305: What is the stock solution used for hematoxylin, what is the working solution? staining times will greatly depend on how fresh this solution is.

The hematoxylin is diluted in a 1:1 ratio with distilled water. Clarification is described in lines 387-391 as follows:

"3.2.4 Blot the excess water from the slide holder with lint-free professional grade wipes, and stain slides in 50% hematoxylin (1:1 dilution with distilled water) for 2-5 min depending on desired staining preference and reagent deterioration. Discard hematoxylin when solution color changes from plum to blue/brown or when staining time becomes excessive. Rinse the slides with running tap water for 20 min."

Line 311: How can slides be left overnight? In water?

Yes, slides can be left overnight in water, which is clarified in Lines 399-400 and can be seen below as:

"3.2.6 Rinse the slides in tap water twice for 1 min each, and once with deionized water. As an option, slides can be left overnight at this stage, soaking in water."

Line 318: What are stock and working solutions for eosin?

Great point, the eosin can be bought ready-to-use, which is preferred in our lab. This was better explained in Lines 410-413, as indicated:

"3.2.9 Counterstain the slides in 100% ready-to-use eosin for 15-30 s, and immediately dehydrate with 95% ethanol twice for 5 min each. Replace with 100% ethanol twice for 5 min each, discarding the solutions after each use."

The scale bar description in Figure 3 is wrong. Probably 100 micrometer not mm.

Thank you for pointing that out. Changes were made to Figure 3's description in lines 670-681, as follows:

*"**Figure 3. Increased LMO1 expression promotes collagen deposition and ECM stiffness leading to facilitated tumor cell dissemination in zebrafish models.***
(A–D) Representative light microscopy images of collagen fibers stained by Picrosirius red (PSR) in MYCN only (A and B) or MYCN;LMO1 (C and D) transgenic zebrafish. (B) and (D) are magnified from the boxed areas of (A) and (C), using arrows (A and B) and arrowheads (C and D) to indicate the PSR-positive collagen fibers, respectively. Scale bars, 100 μm (A and C) and 50 μm (B and D). (E) Quantification of PSR-stained areas on tumor sections of MYCN only or MYCN;LMO1 transgenic fish. Results were normalized to the mean of PSR-stained areas in MYCN-only tumors. The statistics present as the mean ± SD of three MYCN-only or three

MYCN;LMO1 tumors; p = 0.02 by two-tailed t-test. This figure has been modified from Zhu, S. et al. LMO1 Synergizes with MYCN to Promote Neuroblastoma Initiation and Metastasis. Cancer Cell. 32, 310–323 (2017)[13]."

Some arrowheads are hard to see in Figure 2 e.g. in G & M. Maybe try different color.

Great point, the double arrowheads have been modified as suggested.

Mention full name for TH earlier, before using the abbreviation.

Thank you for pointing this out; tyrosine hydroxylase, is spelled out at it first occurrence in Line 444-445, as follows:

"3.3.1.2 Dilute the primary antibody against tyrosine hydroxylase (TH) (1:500), a neuroblastoma marker, based on desired concentration and total amount needed with the automated system's corresponding Primary Antibody Diluent. Note: Optimal antibody concentrations may vary depending on antibody and tissue."

The authors state that transgenic fish are created in an AB background. In figure 1B, the fish seem to have no pigment. Are they really AB?

We are sorry for the poor quality of the images included in the previously submitted manuscript. As we have stated, both *MYCN* and *LMO1* transgenic fish lines are in the AB background. To avoid the confusion, we took new images, which are updated in the revised version as Figure 1B. Now the pigments of both tumor-bearing fish are clearly seen in these updated images.

There is many typos throughout the text, e.g. line 66 metastasis, ….please proofread carefully.

Thank you for your comment regarding typos; the document was proofread thoroughly and mistakes were corrected as needed throughout the manuscript. As suggested, the typo has been corrected in Line 72 in the Introduction section, as follows:

"This paper will introduce how the transgenic fish line that overexpresses both MYCN and LMO1 in the PSNS was created and how it was demonstrated that the cooperation of these two oncogenes accelerate the onset of NB tumorigenesis and metastasis[13]."

<u>Responses to comments from Reviewer 3:</u>

Manuscript Summary:
In their manuscript, Her et al describe a protocol for generation of a metastatic neuroblastoma model in zebrafish. Additional protocols cover sectioning and staining of zebrafish tissues to confirm metastatic lesions and to examine the tumor microenvironment. Overall, this protocol will be useful to researchers with access to zebrafish who want to exploit the benefits of zebrafish cancer models in assessing tumor dissemination. There are several concerns that the authors should address before publication.

Major Concerns:

1. **Introduction: The authors made a good case for tumor and metastasis models in zebrafish.**

a. What might some drawbacks be to this system?

This is a great point, thank you! We have added a new paragraph to discuss the potential drawbacks of zebrafish model for cancer and metastasis study in the Discussion on Lines 736-752, as follows:

"However, like any other animal model used in research to study cancer, the zebrafish also has its disadvantages. For example, antibodies specifically against zebrafish proteins remain largely under developed, although several antibodies against neuroblastoma marker genes—such as tyrosine hydroxylase, synaptophysin, and HuC—are fortunately working well in zebrafish. To combat this issue, many vendors have begun to test their products and predict the potential of their antibodies in cross-reacting with zebrafish proteins. More information about validated antibodies can also been found in the zebrafish information network (ZFIN). With these efforts, more and more antibodies that can specifically detect zebrafish proteins will soon become available to the zebrafish community. Another challenge of using zebrafish as a genetic model to dissect the interplay of complex signaling pathways in NB pathogenesis is its partially duplicated genome. Such genome duplication, which occurred in the natural ancestry of zebrafish[37,38], can often lead to more than one variant of zebrafish homologues to humans. This can cause an evolved gain of novel gene functions or unique expression patterns in the animal model[39]. Therefore, when studying genes with potential roles in tumor suppression, it may be necessary for multiple alleles of the duplicated genes to be knocked out at the same time to demonstrate their tumor suppression function, which can be a potentially time-consuming and a technically-challenging endeavor."

b. Also, are there other metastasis models in zebrafish, either genetic or transplant? Or is the NB model presented the only one so far?

Thanks for the good point. Zebrafish model of melanoma with metastasis have also been reported previously. These models have been briefly described in Lines 44-47, as follows:

"During the past few years, many zebrafish lines have been developed to study tumorigenesis and metastasis of a variety of human cancers, including but not limited to leukemia, melanoma, rhabdomyosarcoma, and hepatocellular carcinoma[2,3,4,5]."

2. Several of the plasmids used to construct the zebrafish lines are listed in the materials table as a "generous gift". How should researchers wanting to replicate the protocol obtain this material? Could the plasmids used be placed in Addgene and made available to the research community?

Thank you for the comment. Regarding your concern for obtaining the materials, we may not be authorized to share the constructs to other labs specifically under the MTA (Material Transfer Agreement). However, the information for such constructs is available via webpage http://tol2kit.genetics.utah.edu/index.php/Main_Page, in which it states that the constructs are freely distributed from the original lab as described. The materials table was adjusted to clarify, as follows:

p3E-polyA	Dr. Chi-Bin Chien, Univ. of Utah	N/A	a generous gift (Please refer to webpage http://tol2kit.genetics.utah.edu/index.php/Main_Page to obtain material, which is freely distributed as described.)

3. Similarly, since the MycN;LMO1 zebrafish already exist, could they be deposited in ZIRC or somehow made available to researchers?

This is a valid point, thank you! This specific line was developed at the Dana-Farber Cancer Institute (DFCI) in Boston, MA. We will work with the institution to deposit this line into the ZIRC inventory. Indeed, we have been freely distributing our MYCN transgenic fish line to many labs in the past, once they signed MTAs with DFCI. We will continue to do so for the MYCN;LMO1 line if there is a request.

4. Since the zebrafish line already exists, it is not clear what the goal of part 1.1 is? Generating fish or plasmids that have already been made? It seems a lot easier to request the materials. Maybe it would be better to highlight this section as a way to generate either LMO1 or any transgene of interest with a role in metastasis?

Thank you for addressing this. The section has been modified to better highlight the goal of this methods section, which is the development of the LMO1 transgenic line, made specifically to discourse its role in metastasis. The changes were made to the title sections (Lines 97-100, 184-185, 228-230, and 268), as follows:

*"**1. Developing Stable, Transgenic LMO1 Zebrafish Line***
- *1.1 Preparation and Microinjection of Transgene Constructs for Development of LMO1 Transgenic Line*
- *1.2 Screen and Verify LMO1 Transgenic Fish Line for Germline Transmission of LMO1 and mCherry*

2. Creating Neuroblastoma Metastatic Zebrafish Model with MYCN and LMO1 Coexpression
- *2.1 Outcrossing LMO1 and MYCN Transgenic Lines to Create Metastatic Model*
- *2.2 Visualizing Tumor Burden in Transgenic Zebrafish Lines"*

5. A useful figure in figure 1 would be images of MycN fish at 1dpf and the LMO1;mCherry at 3-4dpf, when they need to be screened/sorted.

This is a great consideration, and we have modified Figure 1 to better address your concern.

6. The Picosirius red staining seems like something that could be done with any mouse model—it would be great to highlight some benefit that zebrafish provide, such as visualizing/sorting early metastatic cells, or visualizing the effects of a drug treatment on metastatic spread. This might be beyond the scope of what can fit in a JoVE article though?

Thank you for your comment and great idea to use picosirius red staining for visualizing/sorting early metastatic cells. Unfortunately, picosirius red staining can only be applied to the tissue sections after fixation. However, it is a great idea to use it as an inexpensive histological approach to evaluate effect of drug treatment on the remodeling of collagens and subsequent tumor metastasis. This great idea has been implemented in the Discussion, in Lines 712-718, as follows:

"The picrosirius red staining of tumor sections has clearly highlighted the collagen networks in fish tumors and demonstrates the increased stiffness of extracellular matrix in tumors with LMO1 overexpression. Although this technique is not unique to zebrafish, its application together with the high-throughput compound screening on zebrafish embryos that are genetically

modified or transplanted with tumor cells might provide a novel means in screening for effective compounds that could target extracellular matrix remodeling, which is a critical process involved in tumor cell metastasis.

Minor Concerns:
1. Part 1.1.2, the authors skipped over how clones were selected and verified after transformation.

This is a great point, thank you. This comment was better addressed in Step 1.1.2 (lines 121-128) of the Protocol section, as follows:

"Next, spread 50-200 µl from the transformation vial on a Luria Broth (LB) agar plate containing 50 µg/mL kanamycin and incubate at 37°C overnight. Select clones by inoculating a single colony into 2-5 mL of LB with 50 µg/mL kanamycin and culturing overnight (16-18h) at 37 °C. Use 2 mL of overnight bacterial culture for plasmid isolation according to the manufacturer's protocol. To verify LMO1 plasmid, send sample for sequencing with M13 forward primer5- GTAAAACGACGGCCAG-3'."

2. It seems like part 1.3.2, screening the MycN line, is a critical step since transgene can only be visualized at 1dpf. This should somehow be better highlighted.

Thank you for your comment. This point has been highlighted in the Representative Results section, Lines 592-596, as follows:

"*MYCN overexpression has been shown to suppress PSNS development[6]. Thus, the EGFP-MYCN expression is more prominent in the non-PSNS dopaminergic neuronal cells at 1 dpf[6], such as the cranial ganglia (CA), arch-associated catecholaminergic neurons (AAC), and medulla oblongata (MO). Due to the instability of EGFP-MYCN protein, the EGFP signal becomes dimmer after 2 days."*

In addition, although screening at 1 dpf is preferred, unsorted MYCN embryos can still be raised to adulthood and finclipped for genotyping as well. This alternative has been added to the paper in step 2.1.2 (lines 236-245) of the Protocol section, as follows:

"2.1.2. At 1 dpf, sort the progeny of outcross for EGFP expression with stereoscopic fluorescence microscope, which presents as EGFP-positive points in the hindbrain region. Note: Alternatively, if not all embryos are sorted for MYCN at 1 dpf, embryos can still be raised to adulthood and genotyped by finclipping and using formerly stated guidelines from steps 1.2.3-1.2.4 for gDNA isolation and PCR genotyping, with primers: MYCN-F (5'-ATT CAC CAT CAC TGT GCG TCC-3'); MYCN-R (5'-TGC ATC CTC ACT CTC CAC GTA-3', and the following program with standard Taq polymerase: 1 cycle of 94 °C for 3 min, 35 cycles of (94 °C for 30 s, 60 °C for 30 s, and 68 °C for 3 min), and 68 °C for 7 min with expected amplicon size of 145 bp.

3. Section 2 should be retitled "Visualizing Tumor Burden in Transgenic Zebrafish Lines" or something less colloquial than Tumor Watch.

Great suggestion, thank you! The title has been modified as suggested in Line 268.

4. Tumor fish should be written as tumor-bearing fish.

Thank you, changes were made throughout the manuscript to address this comment.

5. Part 3.3 use a BondMax system. I am not familiar with that—is there an alternate if researchers don't have access to this system?

This is a good point. Yes, a manual alternative protocol has been added to the manuscript as its own subsection (3.3.2) from Lines 488-554 in the Protocol segment, as follows:

"3.3.2 Manually Staining Without Automated IHC Staining System

3.3.2.1 After the slides are baked, select slides from step 3.1.11 that are adjacent to allow better comparison between H&E and IHC staining to deparaffinize. Inside a chemical fume hood, dewax and rehydrate the slides with xylene using previous steps 3.2.1 and 3.2.2., respectively.

3.3.2.2 After deparaffinization and rehydration, soak slides in endogenous peroxide blocking solution (1X PBS containing 0.1% sodium azide and 0.3% hydrogen peroxide) for 5 min at room temperature. Wash slides in fresh 1X PBS for 3 min, and repeat twice for a total of 3 times. Caution: Sodium azide is acutely toxic. Ensure to practice precautionary procedures and proper disposal of reagent depending on the regulations of your institution.

3.3.2.3 Retrieve antigen by incubating slides in solution with proteinase K (1:500 in 1X PBS) for 10 min at room temperature. Wash slides 3 times with 1X PBS for 3 min each.

3.3.2.4 Block slides by incubating with 5% goat serum in 1X PBS for 30 min at room temperature on rocker. Wash slides with fresh 1X PBS twice for 3 min each.

3.3.2.5 Add 4 drops of Avidin blocking solution directly onto slide and incubate for 15 min at room temperature. After washing slides with fresh 1X PBS twice for 3 min each, add 4 drops of Biotin blocking solution and incubate at room temperature for 15 min.

3.3.2.6 After blocking slides, incubate in primary antibody of tyrosine hydroxylase (TH) (1:500) for 45-60 min at room temperature, or overnight at 4 °C. Note: Antibodies against transgenes and other relevant markers can additionally be used to address physiology and activity of primary tumor and other metastatic sites. Optimal antibody concentrations may vary depending on antibody and tissue.

3.3.2.7 Wash slides with PBS for 3 min, repeating twice with a total of 3 times.

3.3.2.8 Incubate slides in secondary antibody of biotinylated anti-rabbit IgG secondary antibody (1:500) diluted in blocking solution for 45-60 min at room temperature. Repeat previous washing step 3.3.2.7.

3.3.2.9 Add HRP conjugated Avidin (1:300 in PBS), and incubate for 20 min at room temperature. Wash slides 3 times for 5 min each.

3.3.2.10 Prepare 3,3'-Diaminobenzidine (DAB) solution (2.5 mL distilled water, 1 drop of kit buffer, 1 drop of hydrogen peroxide, and 2 drops of DAB from kit), and place drops of DAB directly onto slides near a microscope. After adding DAB,

observe the color change reaction on the slides under microscope. Once desired staining intensity is reached, stop reaction by placing slide sections in cold distilled water.

3.3.2.11 Counterstain the slides with hematoxylin by submerging or dipping samples for a few short seconds and placing back into distilled water. Repeat as desired, but normally, once should be enough since tissue sections are thin.

3.3.2.12 Dehydrate tissue samples on slides in alcohol gradient as previously described in steps 3.3.1.5 and continue through the remaining steps (with the last step as 3.3.1.8) to finish the IHC analysis."

6. It might be useful to note that besides screening the human transgenes, care should be taken in choosing antibodies that are specific for zebrafish tissue, since not all available antibodies cross-react well with zebrafish.

Thank you, this is great comment. Changes as suggested have been made, as seen in Lines 737-744 in the Discussion section:

"For example, antibodies specifically against zebrafish proteins remain largely under developed, although several antibodies against neuroblastoma marker genes—such as tyrosine hydroxylase, synaptophysin, and HuC—are fortunately working well in zebrafish[6,13]. To combat this issue, many vendors have begun to test their products and predict the potential of their antibodies in cross-reacting with zebrafish proteins. More information about validated antibodies can also been found in the zebrafish information network (ZFIN). With these efforts, more and more antibodies that can specifically detect zebrafish proteins will soon become available to the zebrafish community."

7. It would be helpful to see an example of fluorescent visualization of a metastatic lesion in figure 1.

Thank you for your comment. As suggested, metastatic tumor-burden fish for both *MYCN* only and *MYCN;LMO1* genotypes were reimaged as Figure 1B.

8. Do the transgenic zebrafish succumb to disease? A survival curve is given related to tumor development but since overall survival is not given it is unclear whether these MycN;LMO1 fish can be incrossed or need to be the result of an MycN x LMO1 line each time.

Thanks to the reviewer for this great question. The tumor-bearing fish can survive for weeks up to months after tumor development. However, the progeny output of tumor-bearing fish does decrease. In addition, compared to the heterozygous *MYCN* transgenic fish, the homozygous fish do not survive. Therefore, to maintain the *MYCN;LMO1* line, we prefer to cross heterozygous *MYCN* fish with either heterozygous or homozygous *LMO1* fish. Such mating plans are illustrated in Lines 725-729, as follows:

"For example, the productivity of a MYCN transgenic fish is often markedly reduced once the tumor has developed, and homozygous MYCN transgenic fish do not survive well into adulthood. Therefore, to better maintain the MYCN transgenic fish line, it is recommended to outcross the heterozygous non-tumor-bearing MYCN transgenic fish at a younger age with WT or other genetically engineered fish lines, such as the LMO1 transgenic fish line."

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February 14, 2021

Dear Dr. Lyer,

Thank you for your review of our manuscript entitled: "**Zebrafish Model of Neuroblastoma Metastasis**". We appreciate the insights provided by the reviewers' comments and suggestions, and have included the updated figures with additional images that substantially strengthen our work. We are submitting a revised version of our manuscript that addresses all comments of the reviewers, as described in detail in the attached document. We hope that our manuscript will now be acceptable for publication in *JoVE*.

Sincerely yours,

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