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Supplemental Information

Activated ALK Collaborates with MYCN in Neuroblastoma Pathogenesis

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Inventory of Supplemental Information

Supplemental Data

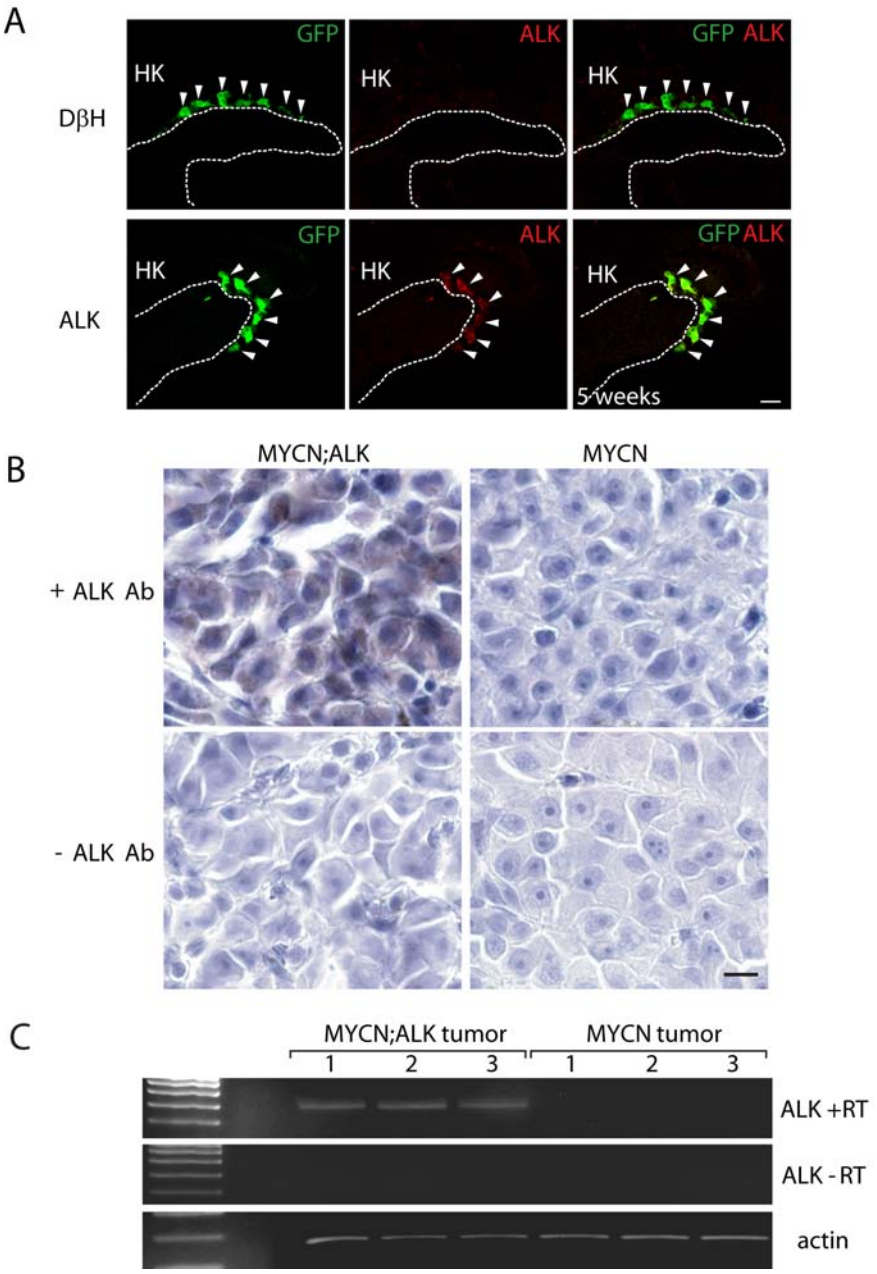

Figure S1, related to Figure 1. ***ALK* is expressed in the ALK transgenic fish and MYCN;ALK tumors**.

(A) *Top*: DβH transgenic fish. *Bottom*: ALK transgenic fish. ALK protein is detected in the EGFP-expressing sympathoadrenal cells in the interrenal gland of the ALK transgenic fish (arrowheads in lower panels), but there is no detectable *ALK* expression in the EGFP-expressing sympathoadrenal cells in the control DβH transgenic fish (arrowheads in top panels) at 5 weeks postfertilization. EGFP, green; ALK, red. Dotted lines indicate the head kidney (HK) boundary. Scale bar, 10 μm.

(B) ALK protein is detected in the MYCN;ALK tumor (left panels) but not in the MYCN-only tumor (right panels) by immunohistochemistry on paraffin sections through tumors of *MYCN*-expressing fish at 4 months postfertilization. Control sections with no primary antibody (lower panels) show no staining in either the MYCN;ALK tumor or the MYCN-only tumor. Scale bar, 50 μm.

(C) RT-PCR assays show that *ALK* RNA is expressed in the MYCN;ALK tumor but not in the MYCN-only tumor. *ALK* is not amplified in the control reaction without reverse transcription (ALK-RT), indicating the lack of genomic DNA contamination. *β-actin* RNA amplification serves as a loading control.

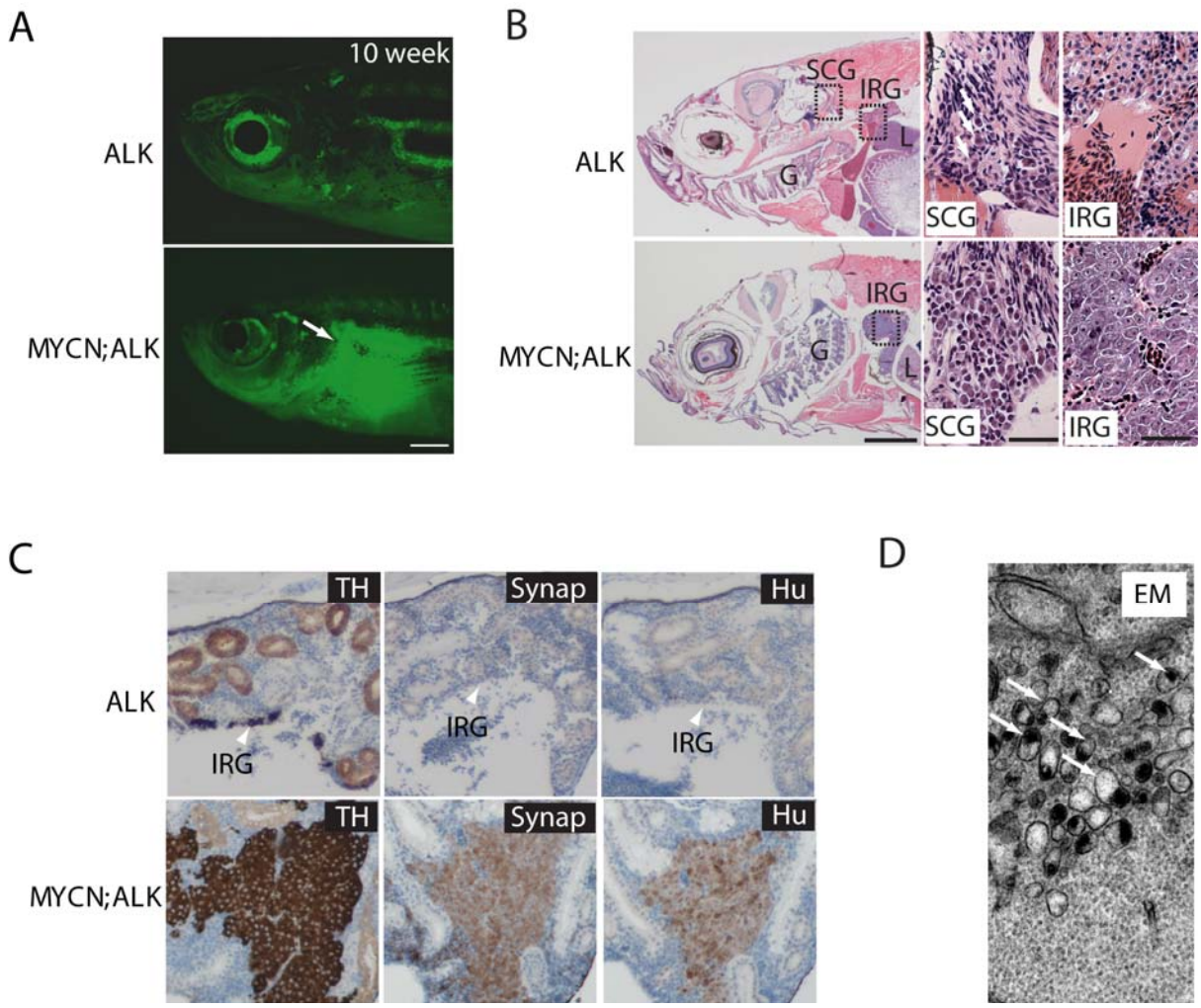

Figure S2, related to Figure 2. Features of tumors arising in MYCN;ALK compound transgenic fish.

(A) 10-week-old ALK stable transgenic fish (top panel) and MYCN;ALK compound transgenic fish with a tumor in the IRG (lower panel). The tumor is EGFP-positive, as indicated by the arrow. Scale bar, 1 mm.

(B) H&E stained sagittal paraffin sections of ALK fish (top left panesl). Boxes indicate the SCG and IRG, and are magnified in the right panels respectively (top right panels). H&E stained, sagittal paraffin sections of MYCN;ALK compound transgenic fish (lower left panels). Boxed region shows a tumor in the IRG, with magnified views in the lower right panels. G, gill; L, liver. Scale bars, 50 μm.

(C) Immunohistochemical analysis of different neuroblastoma markers on sagittal sections through the IRG of the ALK transgenic fish (top panels) and through a tumor in the MYCN;ALK compound transgenic fish (lower panels). T*yrosine hydroxylase (TH) (left panels), Synatophysin (Synap)* (middle panels) and Hu (right panels) expression. Scale bar, 100 μm.

(D) Electron microscopic (EM) image of a tumor in the IRG region of the MYCN;ALK compound transgenic fish. Arrows indicate neurosecretory granules. Scale bar, 500 nm.
IRG, interregnal gland; SCG, superior cervical ganglion.

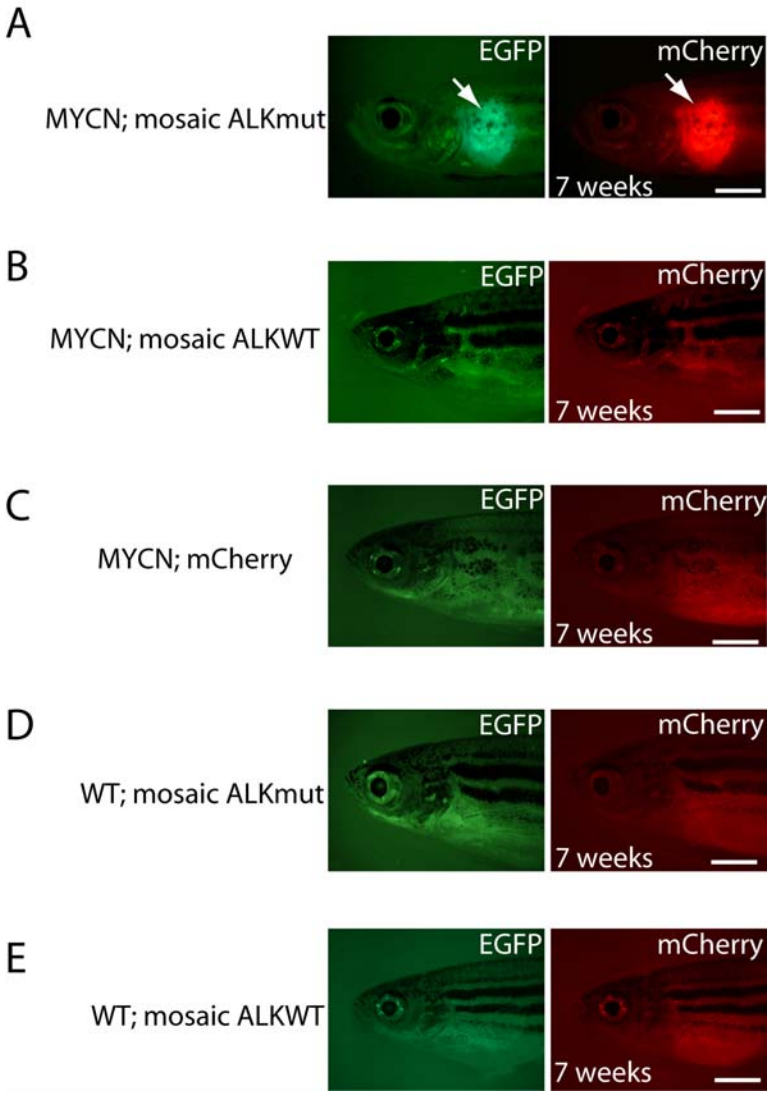

Figure S3, related to Figure 3. Mosaic expression of activated ALK accelerates the onset of MYCN-induced neuroblastoma.

(A) MYCN fish coinjected with *dβh-ALKF1174L* and *dβh-mCherry* constructs (MYCN;mosaic ALKmut). EGFP and mCherry positive tumor arose at 7 weeks (arrows). Scale bars, 1 mm.
(B) MYCN fish coinjected with *dβh-ALKWT* and *dβh-mCherry* constructs (MYCN;mosaic ALKWT). Scale bars, 1 mm.
(C) MYCN fish injected with *dβh-mCherry* construct alone (MYCN;mCherry). Scale bars, 1 mm.
(D) Wild-type (WT) fish coinjected with *dβh-ALKF1174L* and *dβh-mCherry* constructs (WT; mosaic ALKmut). Scale bars, 1 mm.
(E) Wild-type (WT) fish coinjected with *dβh-ALKWT* and *dβh-mCherry* constructs (WT; mosaic ALKWT). Neuroblastomas were not identified in the MYCN fish coinjected with *dβh-ALKWT* and *dβh-mCherry* or *dβh-mCherry* alone at 7 wpf, or in any of the siblings that did not inherit the *MYCN* transgene and were injected with either the *ALKWT* gene or the *ALKF1174L* gene.

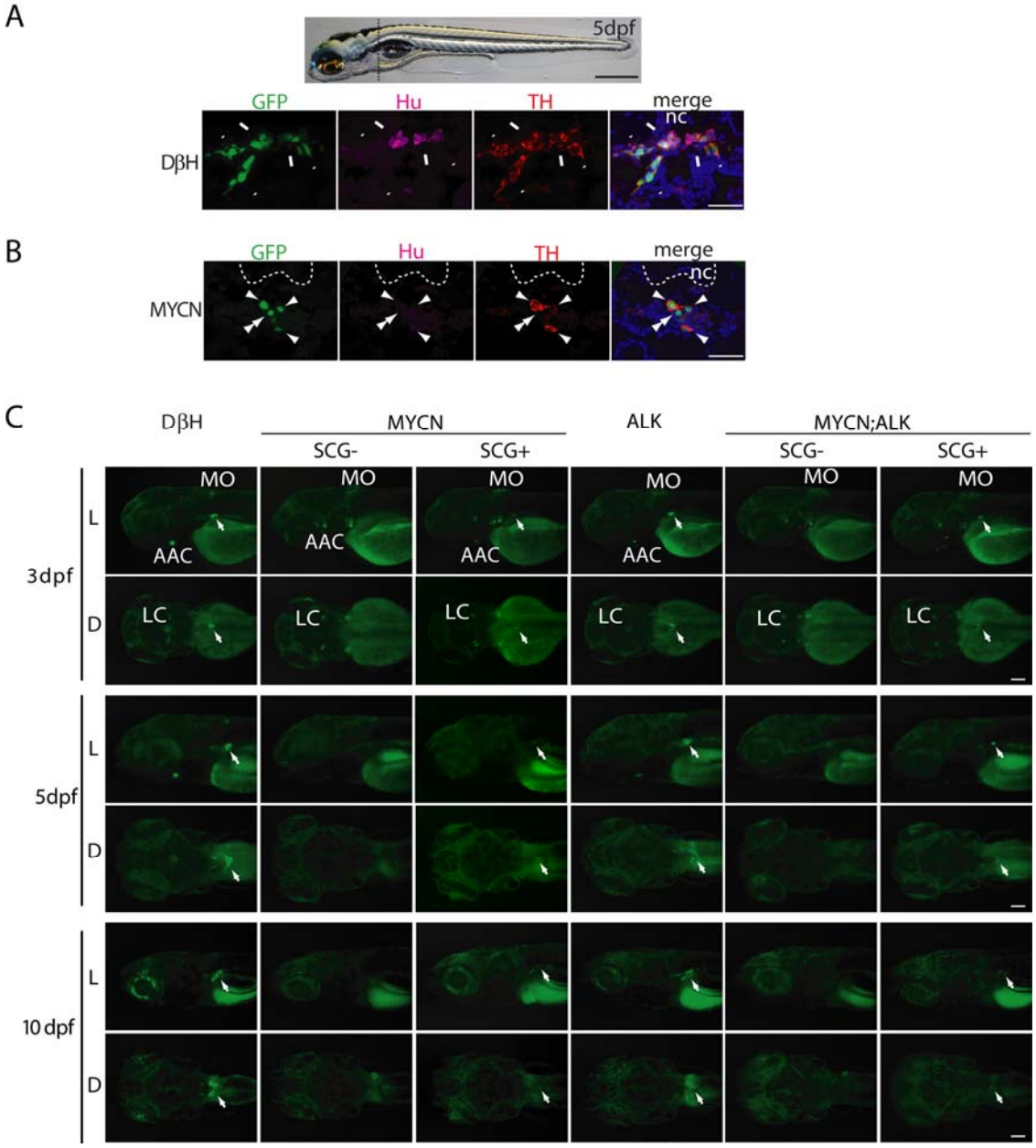

Figure S4, related to Figure 4. A subpopulation of sympathoadrenal cells evades MYCN-induced cell loss.

(A) *Top*: 5 days postfertilization (dpf) embryo, dorsal up, anterior to left. The dotted line indicates the approximate anterior-posterior level where transverse sections were taken. *Bottom*: transverse sections show sympathoadrenal cells in the superior cervical ganglia of DβH transgenic animals at 5dpf. EGFP, green; Hu, magenta; TH, red; DAPI-stained nuclei, blue. EGFP+ sympathoadrenal cells in the DβH fish include Hu+/TH+ sympathetic neurons (arrows) and Hu-/TH+ chromaffin cells (arrowheads). nc, notochord. Scale bar, 500 μm (top panel) and 20 μm (lower panels).

(B) 5dpf MYCN transgenic fish. Surviving EGFP+ cells in MYCN fish can be Hu-/TH+ (arrowheads) or Hu-/TH- (double arrowheads). nc, notochord. Scale bar, 20 μm.

(C) Expression of EGFP driven by the *dβh* promoter in stable transgenic zebrafish during early development. DβH (left panels), MYCN (middle left panels), ALK (middle right panels) and MYCN;ALK transgenic fish (right panels) at 3 dpf, 5 dpf and 10 dpf. L, lateral view (top panels); D, dorsal view (lower panels). While the expression of *MYCN* often ablated the normal development of the SCG in approximately 80% of embryos, EGFP-positive cells did persist in 20% of the MYCN-expressing animals (arrows). AAC, arch-associated catecholaminergic cells; dpf, day postfertilization; LC, locus coeruleus; MO, medulla oblongata; SCG, superior cervical ganglia. Arrows indicate SCG. Scale bar, 100μm.

Table S1, related to Figure 4. The onset of MYCN-induced neuroblastoma is independent of EGFP+ sympathoadrenal cells.

	SCG+		SCG-		
Genotype	Total number of fish at 11 weeks	Number of tumor fish identified by 11 weeks	Total number of fish at 11 weeks	Number of tumor fish identified by 11 weeks	Fisher exact test p-value (two-tailed)
MYCN+ALK-	31	0	64	2	1.00
MYCN+ALK+	35	6	69	14	0.80

To determine whether the presence or absence of EGFP+ sympathoadrenal cells in the region of the superior cervical ganglia (SCG) of *MYCN*-expressing fish affects tumor onset, heterozygous MYCN and ALK transgenic fish were crossed and the offspring were sorted at 5 dpf based on the presence of EGFP+ cells (SCG+ MYCN+ and SCG- MYCN+). These embryos were raised and monitored for the onset of tumorigenesis as described in the Methods. By 11 wpf, no significant differences in tumor formation were detected in MYCN transgenic fish or MYCN;ALK compound transgenic fish with respect to the presence or absence of EGFP+ cells in the SCG at 5 dpf. The ratio of SCG+ to SCG- embryos at 5dpf was 1:4, but was reduced to 1:2 by 11 wpf. It is likely that the lack of sympathoadrenal cells in the SCG- group affects animals with relatively more severe defects in the PSNS development, leading to more death in the SCG- group over time.

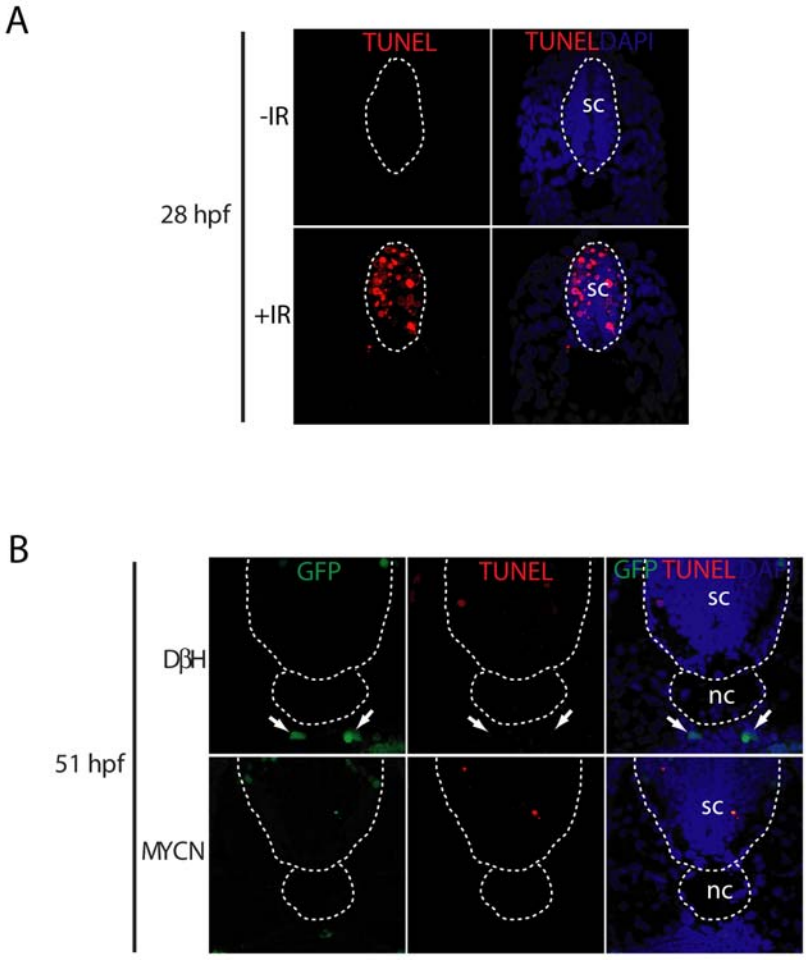

Figure S5, related to Figure 5. Apoptosis does not occur in sympathoadrenal cells of MYCN transgenic embryos during early development.

(A) Transverse sections through the spinal cord (sc) of wild-type fish stained for apoptosis using the TUNEL assay. TUNEL-positive cells were not detected in the fish without irradiation (top panels), but were observed in the fish irradiated (at 12.5 Gy) at 28 hpf (red in lower panels), which serves as a controls for TUNEL assay. IR, irradiated.

(B) Transverse sections through the spinal cord of DβH (top panels) and MYCN (lower panels) transgenic animals stained for TUNEL at 51 hpf. TUNEL-positive EGFP+ sympathoadrenal (SA) cells of the DβH transgenic embryo was rarely observed. In MYCN transgenic embryos, EGFP+ SA cells were scarce, and TUNEL+ cells were not observed in the SA cells. Arrows point to the EGFP+ sympathoadrenal (SA) cells.

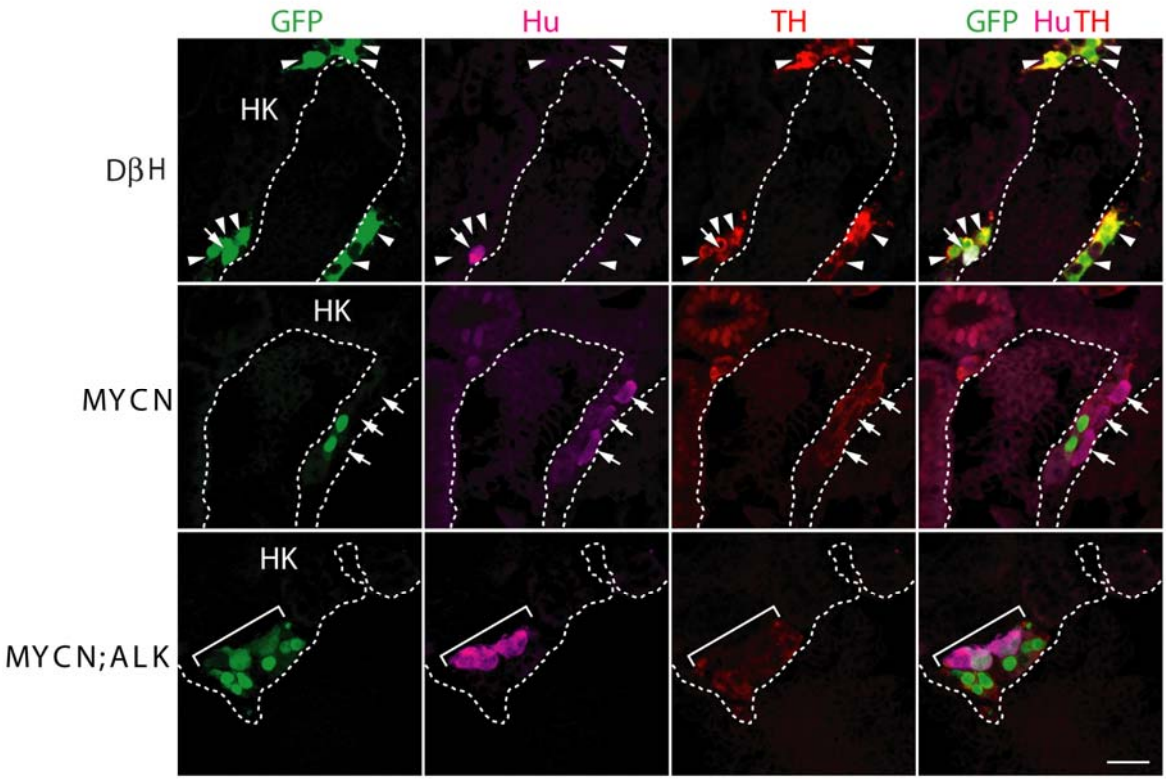

Figure S6, related to Figure 6. MYCN causes Hu+ cell hyperplasia in the lateral interrenal gland.

Sagittal sections through the lateral interrenal gland in DβH (top panels), MYCN (middle panels) and MYCN;ALK (lower panels) transgenic fish at 5wpf (dorsal up, anterior left). EGFP, green; Hu, magenta; TH, red. A few (1-3) GFP+,Hu+,TH+ sympathetic neuroblasts were observed in the lateral interrenal gland of the DβH transgenic fish (arrows) together with multiple GFP+,Hu-,TH+ chromaffin cells (arrowheads). In MYCN and MYCN;ALK transgenic fish, Hu+ cells were expanded within comparable locations of the lateral interrenal gland (arrows and brackets, respectively. The dotted lines indicate the head kidney (HK) boundary. Scale bar, 20 μm.

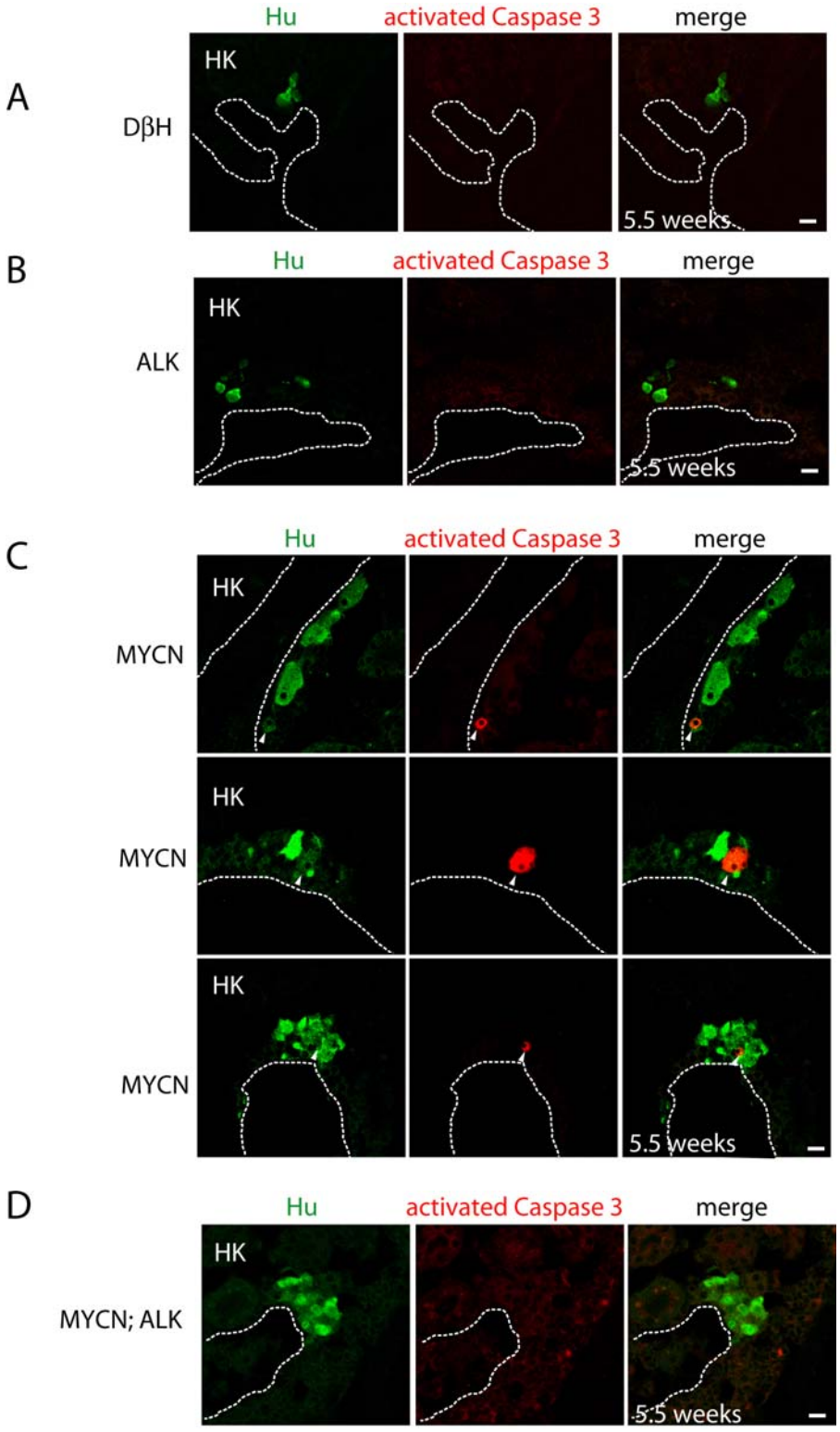

Figure S7, related to Figure 8. ALK inhibits a developmentally-timed apoptotic response triggered by *MYCN* overexpression in the interrenal gland.

More examples of sagittal sections through interrenal gland of DβH (A), ALK (B), MYCN (C) and MYCN;ALK transgenic fish (D) at 5.5 weeks (dorsal up, anterior left). Hu, green; activated Caspase-3, red. Hu+, activated Caspase-3+ apoptotic cells were detected in the majority of MYCN transgenic fish containing Hu+ hyperplastic neuroblasts (C, arrowheads), but only rarely in the MYCN;ALK transgenic fish. Scale bars, 10 μm.

Supplemental Experimental Procedures:

Zebrafish: Embryos were staged according to Kimmel *et al*. (Kimmel et al., 1995). All zebrafish studies and maintenance of the animals were in accord with Dana-Farber Cancer Institute IACUC-approved protocol #02-107.

DNA constructs for transgenesis: A 5.2-kb *dβh* promoter region was cloned using the CH211-270H11 BAC as a DNA template and the Expand Long Template PCR System (Roche Applied Science, IN), with the following cycle times: 94°C, 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'-GCG TAC TCC CCC TTT TTA GG-3' and reverse primer 5'-TGT TGC TTT GTC GTC TTT TGA-3'). The PCR product was first cloned into a pCR-TOPO vector (Invitrogen, CA), subcloned into *XhoI-ClaI* restriction sites of a I-SceI-pBSII-SK+ vector (GI:111054490, a generous gift from Dr. C. Grabher, Karlsruhe Institute of Technology, Karlsruhe, Germany) and injected into one-cell embryos to generate the *Tg(dβh:EGFP)* transgenic line. The expression of EGFP in the *Tg(dβh:EGFP)* zebrafish line recapitulated endogenous expression of *dβh* and is designated the "DβH" transgenic line in the main body of the text.

The DNA construct for the *Tg(dβh:EGFP-MYCN)* transgenic line was made using the Multisite Gateway System (Invitrogen, CA), by combining entry clones of the 5.2-kb *dβh* promoter and *EGFP* lacking a stop codon, and human *MYCN* cDNA (a generous gift from Dr. Hogarty at the Children's Hospital of Pennsylvania) into the modified destination vector containing *I-SceI* recognition sites. The PNP-mitf vector (a generous gift from Dr. D. Raible, Univ. of Washington) was used to release a 2.65-kb DNA fragment from *NotI* and *SalI* restriction sites, containing the promoter and the coding sequence of the zebrafish *mitf* gene, which was cloned into the *MluI* and *NotI* sites of a modified pBluescript vector containing flanking *I-SceI* recognition sites. To generate transgenic animals overexpressing *MYCN* under control of the *dβh* promoter, *dβh:EGFP-MYCN* and *mitf:mitf* DNA constructs were coinjected with fresh *I-SceI* enzyme into embryos of the pigmentation mutant *nacre* zebrafish at the one-cell stage, and the injected embryos were grown to adulthood. The adult fish were incrossed to identify stable transgenic animals based on pigmentation and EGFP expression; once identified, the lines were maintained and expanded by outcrossing into the wild-type AB strain. The *mitf* promoter is specific for cells of the melanocyte lineage and is not expressed in the sympathoadrenal cells. The *Tg(dβh:EGFP-MYCN)* line is designated the "MYCN" transgenic line in the main body of the text.

To generate the *Tg(dβh:EGFP;dβh:ALKF1174L)* transgenic line, we subcloned the human *ALKF1174L* gene from the PCDNA3 vector (George et al., 2008) into *EcoRI* and *NotI* sites of a pENTRY1A vector and constructed the expression clone by combining three entry clones, *dβh*-pDONRP4-P1R, *ALKF1174L*-pENTRY1A and p3E-polyA, with a pDest I-SceI destination vector using the multisite Gateway system. To generate a stable line, we linearized this *dβh:ALKF1174L* DNA construct and the *dβh:EGFP* DNA constructs (3:1 ratio), used to create the DβH line, with the *I-SceI* enzyme. 50-80 pg of total DNA was then injected into one-cell stage wild-type embryos. The *Tg(dβh:EGFP;dβh:ALKF1174L)* zebrafish line is designated the "ALK" transgenic line in the main body of text. F1 offspring were first screened by fluorescent microscopy for EGFP expression, and the germline transmission of the *ALK (F1174L)* gene was confirmed by genomic PCR of the EGFP-positive embryos with the following primers: *ALK* P7: 5'-AGG CCA GGT GTC CGG AAT GC-3' and *ALK* P18: 5'-TGT CTT CAG GCT GAT GTT GC-3' and the following PCR reaction: 1 cycle of 94°C for 5 min, 30 cycles of (94°C for 30 s, 55°C for 30 s, and 72°C for 60 s). A 721-bp fragment of the *ALK* transgene fragment containing the *F1174L* mutation was amplified and confirmed by sequencing.

Tumor watch and genotyping of transgenic fish: MYCN and ALK heterozygous transgenic fish were crossed and the offspring were grown under identical conditions. The zebrafish line carrying both *MYCN* and *ALK* transgenes is designated the "MYCN;ALK" transgenic line in the main body of text. Starting at 5 weeks postfertilization, offspring were anesthetized with tricaine and examined every two weeks for EGFP-expressing tumor masses. Once fish with strong EGFP-positive tumors were identified, they were genotyped, fixed, sectioned, and stained with H&E. All of the screened fish were genotyped at 6 months of age to provide data for determining tumor incidence over time. For genotyping, genomic DNA extracted from finclip was PCR-amplified with the following primers: *ALK* P9: 5'-GAA ACA AGC CCA CCA GCT TG-3'; *ALK* P20: 5'-CCA

GGC TGG TTC ATG CTA TT-3'; *MYCN*-test F1: 5'-CTG CTT GAG AAC GAG CTG TG-3'; *MYCN*-R3: 5'-AGG CAT CGT TTG AGG ATC AG-3'. *ALK* genotyping PCR was performed with Taq DNA Polymerase (New England Biolabs, MA) using the program described above, while *MYCN* genotyping was performed with the GC-RICH PCR System (Roche Applied Science, IN) using the following program: 1 cycle of 95°C for 3 min, 25 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 3 min.

In addition, for Figure 3B, either activated human *ALK(F1174L)* or wild-type human *ALK* (*ALKWT*) were overexpressed in the MYCN fish as mosaics by coinjecting the following constructs into the one-cell stage of MYCN transgenic and control embryos: i) *dβh-ALKF1174L* with *dβh-mCherry*, ii) *dβh-ALKWT* with *dβh-mCherry* or iii) *dβh-mCherry* alone. The primary injected embryos were raised and monitored for the onset of tumorigenesis, as described above.

Semiquantitative and quantitative RT-PCR: To detect the *ALK* transgene expression, we extracted total RNA from tumors dissected from individual MYCN;ALK and MYCN only transgenic fish using TRIZOL (Invitrogen, CA) and treated RNA with TURBO™ DNase (Applied Biosystems, CA). CDNA was produced using the SuperScript® III First-Strand Synthesis System for RT-PCR (Invitrogen, CA). For controls, no reverse transcriptase was added to the same mRNA samples. *ALK* was amplified using *ALK* P9 and P20 primers for 32 cycles, while *β-actin* was amplified as a loading control using primers (actin F2: 5'-AAT CCC AAA GCC AAC AGA GA-3'; actin R2: 5'-TCA CAC CAT CAC CAG AGT CC-3') for 25 cycles. To assess the expression of zebrafish endogenous *caspase-8* expression, total RNA was extracted from EGFP-positive tumor cells sorted from three individual MYCN tumor and pooled EGFP-expressing cells sorted from DβH control fish. The quantitative PCR was carried on ViiA™ 7 real-time PCR machine (Life Technologies Corporation, CA) using primers for amplification of *caspase-8* (z-casp8-F 5´-GGGCAAAGCTGGGAAGATC-3´**;** z-casp8-R 5´-CTTCTTCTAGAGGAAGTCTGC- 3´**)** and *β-actin* (see above).

RNA *in situ* hybridization: Whole-mount RNA *in situ* hybridization assays were performed according to Thisse and Thisse (Thisse and Thisse, 2008). DNA of *dβh, th, phox2b and tfap2a* for making RNA probes have been described (Stewart et al., 2006).

Cryosectioning, paraffin sectioning and immunostaining: For cryosectioning, embryos or juvenile fish at indicated stages were fixed with 4% paraformaldehyde at 4°C overnight, washed with phosphate-buffered saline with 0.1% Tween 20 (PBST), embedded in 1.5% agar / 5% sucrose, and sunk in 30% sucrose at 4°C overnight. 14-μm sections were taken from the embedded specimens with a conventional cryostat. For immunofluorescence assays, primary antibodies against EGFP (Invitrogen, Cat# A6455, 1:500), TH (Pel-Freez, Cat# P40101, 1:500), HuC/D (Invitrogen, Cat# A-21271, 1:200), Synaptophysin (Millipore, Cat# MAB5258, 1:200), ALK/p80 antibody (Epitomics, Cat# 4204-1, 1:100**)** and activated Caspase-3 (BD Biosciences, Cat#559565, 1:250) were incubated on slides at 4°C overnight, washed with PBST, visualized with secondary antibodies conjugated with Alexa 488, 568, 647 (Invitrogen, 1:500), and counterstained with DAPI for nuclear staining.

Paraffin sectioning followed by H&E staining and immunohistochemistry was performed at the DF/HCC Research Pathology Core with primary antibodies against GFP, TH, HuC/D, Synaptophysin and ALK/p80 antibody using standard protocols (Macdonald, 1999).

Electron microscopy: Transmission electron microscopy (TEM) was carried out at the Harvard Medical School EM Facility. Briefly, the dissected tumor tissues were fixed in 2% formaldehyde/2.5% glutaraldehyde, postfixed with 1% osmiumtetroxide, and embedded in epon; 80-nm sections were then collected. Images were captured by a Tecnai™ G² Spirit BioTWIN electron microscope with an AMT 2k CCD camera.

Imaging: An Olympus stereoscopic microscope and a Leica M420 microscope equipped with a Nikon digital sight DS-U1 camera were used for capturing the bright field and fluorescent images. A Zeiss LSM 510 META confocal microscope at the Harvard Neurodiscovery Imaging Center and a Leica SP5X laser scanning confocal microscope at the Confocal and Light Microscopy core facility at Dana-Farber Cancer Institute were used for

collecting confocal images of the native EGFP and antibody staining. Images of optical sections (3-5 μm each) acquired with a 20X water immersion lens (N. A. = 0.7), were taken of whole-mounted embryos, while 40X/63X/100X oil immersion lenses (N. A. = 1.4) were used for viewing sections. For brightfield DIC images, a Zeiss Axio Imager.Z1 compound microscope equipped with an AxioCam HRc was used. The acquired images were processed and compiled with Leica LAS AF Lite, Openlab v5 (Improvision) and Adobe Photoshop and Illustrator CS3 (Adobe) software.

Cell counts: To identify the cells that give rise to neuroblastoma, the DβH, ALK, MYCN and MYCN;ALK transgenic fish at 3 wpf, 5 wpf, 5.5 wpf and 7 wpf were cryosectioned, stained with primary antibodies against HuC/D, GFP, TH and activated Caspase-3, and imaged using confocal microscopy. All sections from each individual fish were scanned for three types of cells: i) neuroblast cells that were Hu-positive, ii) chromaffin cells that were Hu-negative/GFP-positive, and iii) apoptotic neuroblast cells that were coexpressed activated Caspase-3-positive and Hu. For each cell type/condition a single representative section was selected that contained the largest number of each respective cell type and the numbers of those cells were quantified.

Statistical analysis: Kaplan-Meier analysis was applied to assess the rate of tumor development. Fish that died prior to evidence of external EGFP-positive masses were censored. When tissue from the dead fish was available, the genotype was determined; otherwise, genotypes for the fish that were lost or died were based on the following assumptions. The offspring from the cross of MYCN and ALK heterozygous stable transgenic fish contain each of the four genotypes in equal proportions. However, based on the genotypes of available fish, *MYCN*+ fish died during development more often than *MYCN*-fish. Thus, the fish that could not be genotyped were likely to have the *MYCN*+ genotype and were designated as such for this study. Since roughly equal numbers of *ALK*+ and *ALK*- fish were observed among both the *MYCN*- and *MYCN*+ groups, the total number of dead, but ungenotyped, fish was divided equally between the Alk+ and Alk- cohorts within the *MYCN*+ group. The Fisher exact test was used to assess the difference between tumor onset by 9 wpf in the MYCN fish coinjected with *dβh-ALKF1174L* and *dβh-mCherry* and that in the MYCN line coinjected with *dβh-ALKwt* and *dβh-mCherry* or *dβh-mCherry* alone. The Wilcoxon signed-rank test was used to assess the difference between the numbers of Hu-positive neurons or Hu-negative/GFP-positive chromaffin cells in the DβH, ALK, MYCN and MYCN;ALK transgenic fish at 3 wpf, 5wpf, 5.5 wpf and 7 wpf. The numbers of MYCN transgenic fish with Hu-positive apoptotic cells were compared to those of DβH, ALK and MYCN;ALK transgenic fish at 5.5 wpf using Fisher exact test. The Fisher exact test was used to assess the difference between tumor onset by 11 wpf in the MYCN transgenic fish with residual superior cervical ganglia cells (SCG+) and that in the MYCN transgenic fish lacked superior cervical ganglia cells (SCG-) at 5 dpf.