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Supplemental Information

Critical Role for GAB2 in Neuroblastoma Pathogenesis through the Promotion of SHP2/MYCN Cooperation

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Supplemental Figure 1

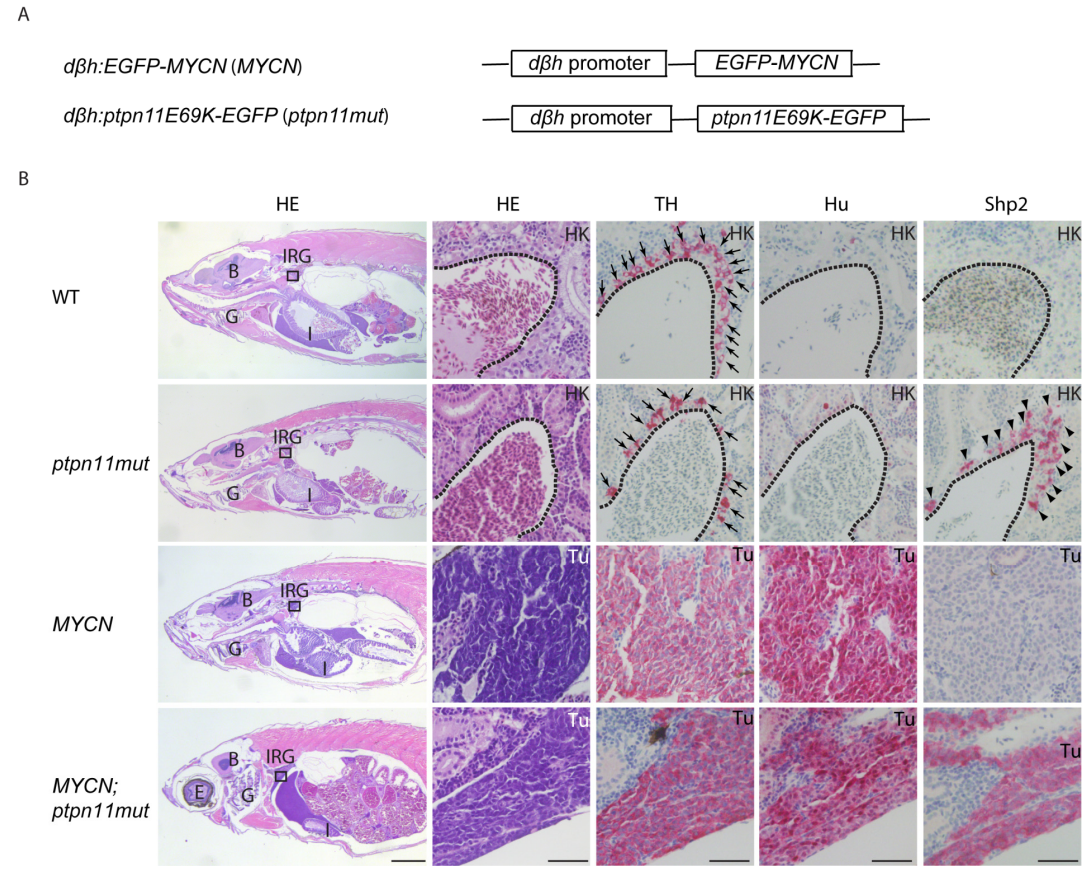

Figure S1. Histopathologic and immunohistochemical analyses of wild-type (WT), *ptpn11mut*, *MYCN*-only and *MYCN;ptpn11mut* fish. Related to Figure 1

(A) Schematic of *dβh:EGFP-MYCN* (*MYCN*) and *dβh:ptpn11mut-EGFP* (*ptpn11mut*) transgenic constructs. *I-SceI* sites are located on each end.

(B) Histopathologic and immunohistochemical analyses for the indicated antigens of the IRG regions of wild-type (WT), *ptpn11mut*, *MYCN*-only and *MYCN;ptpn11mut* fish. Left two columns: H&E-stained sagittal sections. Boxes indicate the interrenal gland (IRG), magnified in panels on the right. The remaining columns show immunohistochemical staining with tyrosine hydroxylase (TH, an enzyme in the catecholamine biosynthesis pathway) (Teitelman et al., 1979), Hu (a pan-neuronal marker), indicative of a PSNS-related neuronal origin (Marusich et al., 1994; Teitelman et al., 1979) and SHP2 antibodies, as indicated. Arrows indicate sympathoadrenal cells expressing TH in the IRG regions of WT or *ptpn11mut* fish (top two panels). Arrowheads indicate sympathoadrenal cells expressing Shp2-EGFP fusion protein in the IRG region of *ptpn11mut* transgenic fish (second panel from top, first column on right). Dotted lines indicate the head-kidney (HK) boundary. Scale bars, 1 mm (first column on left) and 50 μm (all other panels). B, brain; E, eyes; G, gill; I, intestine; IRG, interrenal gland; and Tu, tumors in the IRG of *MYCN* or *MYCN;ptpn11mut* fish.

Supplemental Figure 2

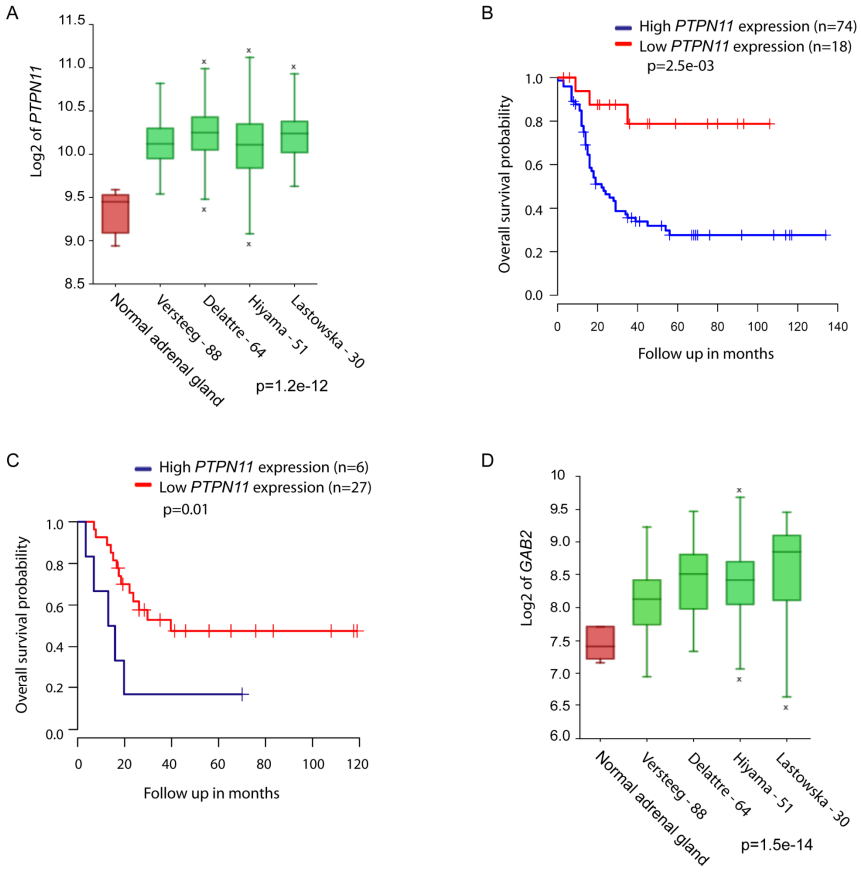

Figure S2. *PTPN11* and *GAB2* are overexpressed in most neuroblastoma cases. Related to Figure 2

(A) Log2-transformed expression of *PTPN11* in normal adrenal gland and human neuroblastomas, as reported in the R2 Neuroblastoma Microarray Database (Tumor Neuroblastoma public - Versteeg - 88 - MAS5.0 - u133p2, Tumor Neuroblastic mixed - Delattre - 64 - MAS5.0 - u133p2, Tumor Neuroblastoma - Hiyama - 51 - MAS5.0 - u133p2, Tumor Neuroblastoma - Lastowska - 30 - MAS5.0 - u133p2). These data show that, compared with levels in normal adrenal, *PTPN11* is overexpressed in most neuroblastomas.

(B-C) Kaplan–Meier curves demonstrating the relationship between patient survival and *PTPN11* gene expression in high-risk cases with *MYCN* amplification. High *PTPN11* expression is associated with worse overall survival in two cohorts, including the GSE49710 dataset (B) and GSE73517 (C).

(D) Log2-transformed expression of *GAB2* in normal adrenal gland and human neuroblastomas, as reported in the R2 Neuroblastoma Microarray Database (Tumor Neuroblastoma public - Versteeg - 88 - MAS5.0 - u133p2, Tumor Neuroblastic mixed - Delattre - 64 - MAS5.0 - u133p2, Tumor Neuroblastoma - Hiyama - 51 - MAS5.0 - u133p2, Tumor Neuroblastoma - Lastowska - 30 - MAS5.0 - u133p2). These data show that, compared to levels in normal adrenal, *GAB2* is overexpressed in most neuroblastomas.

Supplemental Figure 3

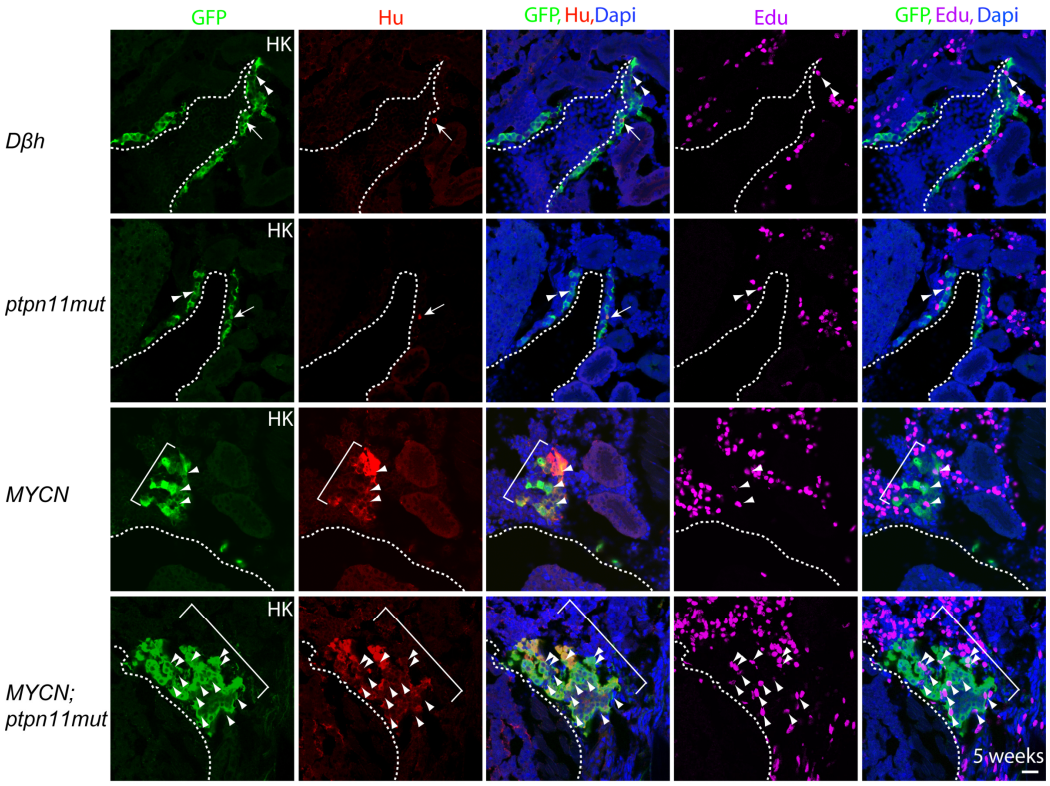

Figure S3. Mutant *ptpn11* promotes proliferation of *MYCN*-induced hyperplastic neuroblasts in the interrenal gland (IRG). Related to Figure 4

Sagittal sections through the IRGs of EdU-labeled *Dβh* control, *ptpn11mut*, *MYCN* and *MYCN;ptpn11mut* transgenic fish at 5 wpf (dorsal up, anterior left). GFP, green; Hu, red; EdU, magenta; Dapi, blue. Arrows indicate Hu+GFP+Edu- neuroblastic cells in the IRG. Arrowheads indicate Hu+GFP+Edu+ neuroblasts. Double arrowheads indicate Hu-GFP+Edu+ proliferating chromaffin cells. Dotted lines outline the head kidney (HK) boundary. Scale bar, 20 μm.

Supplemental Figure 4

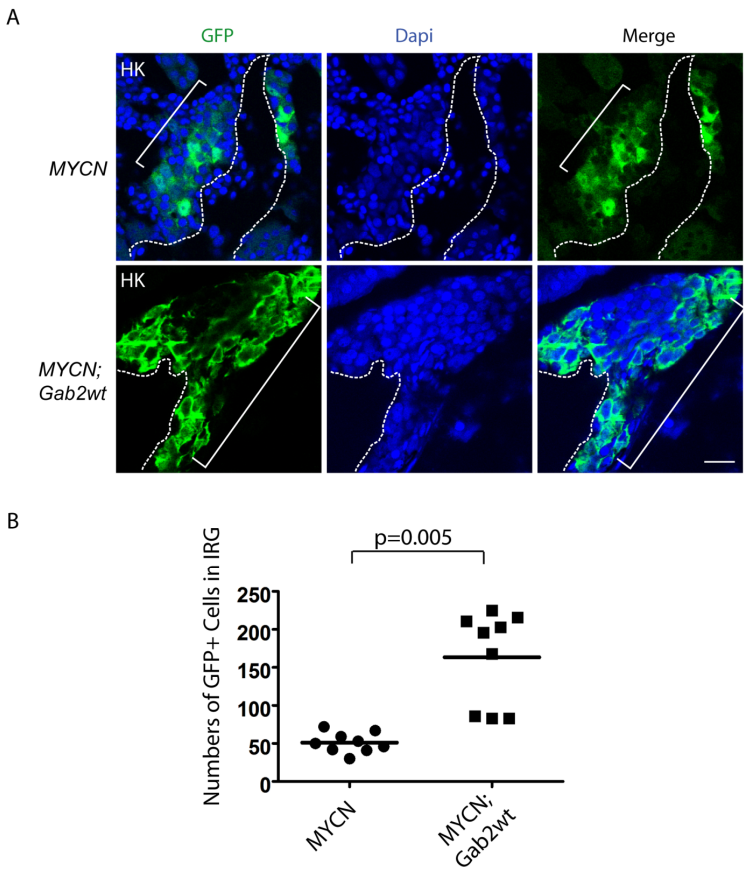

Figure S4. Overexpression of *Gab2wt* increases the number of *MYCN*–induced hyperplastic neuroblasts in the interrenal gland (IRG). Related to Figure 4

(A) Sagittal sections through the IRGs of *MYCN* and *MYCN;Gab2wt* transgenic fish at 5 wpf (dorsal up, anterior left). GFP, green; DAPI, blue; Merge, combined green and blue. GFP+ hyperplastic neuroblasts were indicated by brackets. Dotted lines indicate the head-kidney (HK) boundary. Wpf, weeks postfertilization. Scale bar, 20 μm.

(B) Numbers of GFP+ sympathetic neuroblasts in the IRG regions of *MYCN* and *MYCN;Gab2wt* transgenic fish at 5 wpf. Mean values (horizontal bars) were compared by Welch t-test (two-tailed). GFP+ cell numbers are significantly increased in the *MYCN;Gab2wt* fish as compared to those in the *MYCN*-only fish.

Supplemental Figure 5

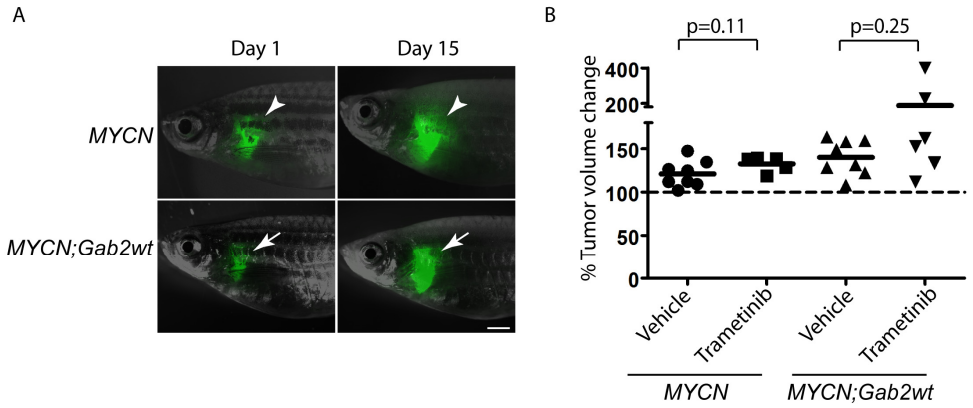

Figure S5. MEK inhibitor treatment does not inhibit tumor growth in *MYCN;GFP* or *MYCN;Gab2wt* transgenic fish. Related to Figure 7

(A) EGFP-expressing tumors in *MYCN;GFP* (arrowhead) or *MYCN;Gab2wt* (arrow) transgenic fish treated with trametinib at days 1 or 15 of treatment. Scale bar, 1 mm.

(B) Changes in tumor volume in animals treated with trametinib on day 15 vs day 1, as measured by the size of EGFP positive tumor masses using Image J software. Mean values (horizontal bars) were compared by Welch t-test (two-tailed).

Supplemental Figure 6

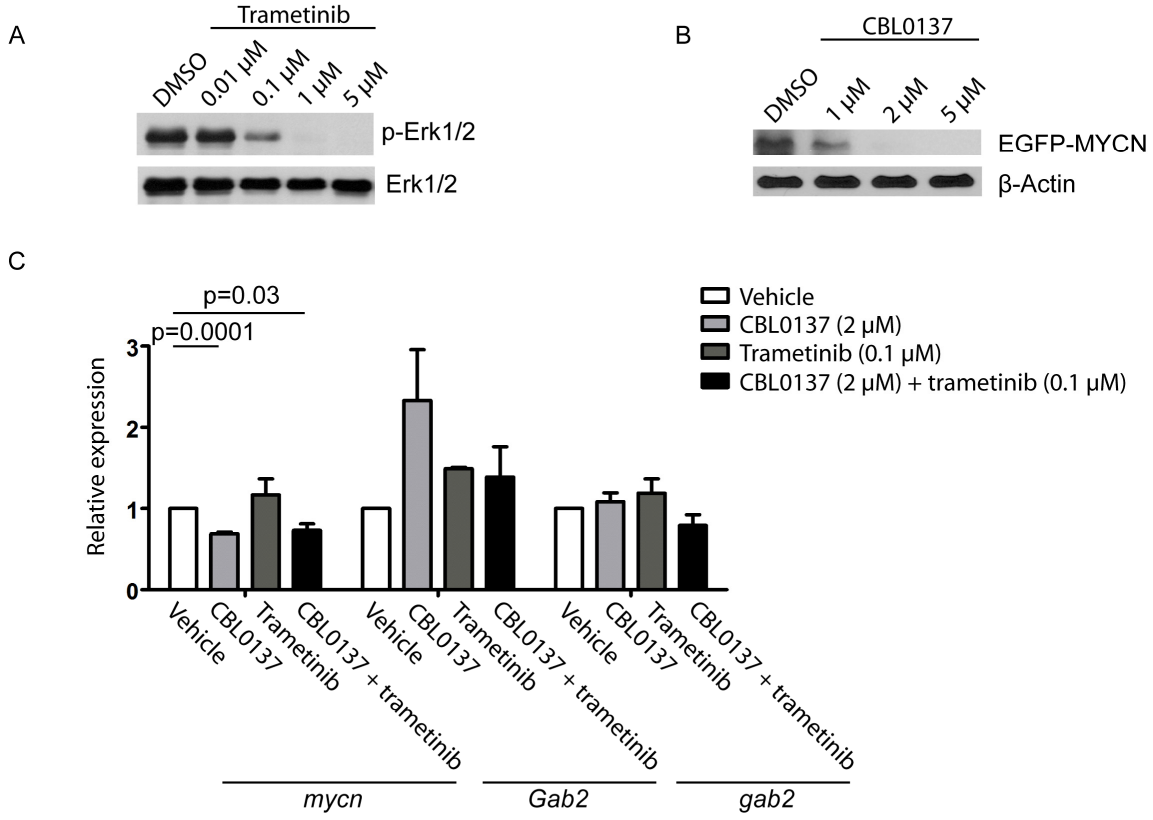

Figure S6. *Gab2* expression is not downregulated by MYCN and/or MEK inhibition. Related to Figure 7
(A) Immunoblot of lysates from 8-day-old wild-type embryos treated with vehicle (DMSO) or trametinib for 1 day. Total levels of Erk1/2 serve as loading controls.
(B) Immunoblot of lysates from 8-day-old *MYCN* transgenic embryos treated with vehicle (DMSO) or CBL0137 for 1 day. Total levels of β-Actin serve as loading controls.
(C) Real-time PCR analysis of relative expression levels of *mycn*, *Gab2* and *gab2* to *β-actin* in 8-day-old *Gab2wt*-expressing embryos treated with vehicle, CBL0137 alone (2μM), trametinib alone (0.1 μM), or CBL0137 (2μM) plus trametinib (0.1 μM) for 1 day. All the values were further normalized to the mean of expression of each given gene in vehicle-treated embryos. The data are means ± SEM of triplicate experiments from pooled embryos. *Mycn* expression levels were significantly downregulated in the CBL0137- or CBL0137 plus trametinib-treated *Gab2wt*-expressing embryos as compared to those in vehicle-treated embryos, p=0.0001 and p=0.03, respectively, by two-tailed t-test. By contrast, there are no significant differences in the expression levels of *Gab2* or *gab2* in the embryos treated with CBL0137- or CBL0137 plus trametinib as compared to those treated with vehicle.

Supplementary Table 1: Association of Expression Levels of Potential Upstream Regulators of SHP2 with Expression levels of *PTPN11* and with Disease Outcome in High-Risk Neuroblastoma with *MYCN* Amplification

	GSE49710 dataset			GSE73517 dataset			TARGET HumanExon cohort for neuroblastoma-Asgharzadeh dataset		
Gene	**Pearson correlation coefficient (PCC)**	**Impact of expression level on outcome**		**PCC**	**Impact of expression level on outcome**		**PCC**	**Impact of expression level on outcome**	
GAB2	r-value=0.491 p-value=6.8e-07	↑= worse	p-value= 0.05	r-value=0.549 p-value=9.4e-04	↑= worse	p-value= 3.5e-03	r-value=0.308 p-value=0.01	↑= worse	p-value= 3.0e-04
GAB1	r-value=0.683 p-value=6.5e-14	None	None	r-value=0.677 p-value=1.5e-05	↑= worse	p-value= 0.029	r-value=0.363 p-value=2.3e-03	↑= worse	p-value= 0.013
FRS2	r-value=0.431 p-value=1.8e-05	None	None	r-value=0.411 p-value=0.02	None	None	r-value=0.382 p-value=1.3e-03	↓= worse	p-value= 1.7e-03
FRS3	r-value=0.513 p-value=1.8e-07	↑= worse	p-value= 7.4e-03	r-value=0.432 p-value=0.01	None	None	r-value=0.199 p-value=0.10	None	None
IRS1	r-value=0.551 p-value=1.3e-08	None	None	r-value=0.628 p-value=9.1e-05	↑= worse	p-value= 9.9e-03	r-value=0.293 p-value=0.02	None	None
IRS2	r-value=0.437 p-value=1.3e-05	None	None	r-value=0.267 p-value=0.13	↑= worse	p-value= 0.036	r-value=-0.092 p-value=0.45	↓= worse	p-value= 0.025
IRS3	r-value=0.222 p-value=0.03	↓= worse	p-value= 0.02	r-value=0.045 p-value=0.80	None	None	r-value=-0.392 p-value=9.6e-04	None	None
FGFR1	r-value=0.480 p-value=1.3e-06	↑= worse	p-value= 0.015	r-value=0.432 p-value=0.01	↑= worse	p-value= 4.2e-03	r-value=-0.138 p-value=0.26	None	None
FGFR2	r-value=0.188 p-value=0.07	None	None	r-value=0.133 p-value=0.46	None	None	r-value=-0.032 p-value=0.80	None	None
EGFR	r-value=0.409 p-value=5.2e-05	None	None	r-value=0.234 p-value=0.19	None	None	r-value=0.009 p-value=0.94	None	None

PDGFRA	r-value=0.039 p-value=0.71	None	None	r-value=-0.088 p-value=0.63	None	None	r-value=-0.059 p-value=0.63	None	None
PDGFRB	r-value=0.250 p-value=0.02	↓= worse	p-value= 0.03	r-value=0.200 p-value=0.26	None	None	r-value=-0.344 p-value=4.1e-03	↓= worse	p-value= 0.028
ALK	r-value=0.271 p-value=8.9e-03	↑= worse	p-value= 9.6e-03	r-value=0.125 p-value=0.49	None	None	r-value=0.014 p-value=0.91	↑= worse	p-value= 2.3e-05
NTRK1	r-value=0.296 p-value=4.1e-03	None	None	r-value=0.323 p-value=0.07	↑= worse	p-value= 0.037	r-value=-0.244 p-value=0.05	None	None
NTRK2	r-value=0.399 p-value=8.1e-05	↑= worse	p-value= 0.026	r-value=0.363 p-value=0.04	None	None	r-value=0.043 p-value=0.73	↑= worse	p-value= 0.023

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Reagents

DNA constructs

The coding region of wild-type (wt) zebrafish *ptpn11* by was reverse transcribed and amplified by RT-PCR with the forward *ptpn11attB1* primer: 5'-GGGGACAGCTTTCTTGTACAAAGTGGACACCATGACATCCCGAAGGTGGTT-3' and the reverse *ptpn11attB2R* primer: 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTATCTGTGACTCTTCTGCTGCT-3'. The PCR product was cloned into the *PDONR221* gateway donor vector by BP recombination reaction. The *ptpn11*^{E69K} mutant (*ptpn11mut*) construct was generated by site-directed mutagenesis, using the *ptpn11wt-PDONR221* construct as the template. PCR was carried out by using the *ptpn11mut FW primer*: 5'-CGACCTGTATGGTGGAAAGAAGTTTGCCACTC-3' and the *ptpn11mut RV* primer: 5'-GAGTGGCAAACTTCTTTCCACCATACAGGTCG-3'. The *ptpn11wt-PDONR221* or *ptpn11mut-PDONR221* construct was combined with the other two entry clones, *dβh-pDONRP4-P1R* (Zhu et al., 2012) and *p3E-EGFPpA*, as well as a modified destination vector containing *I-SceI* recognition sites (Zhu et al., 2012), using the Multisite Gateway System to generate the transgenic constructs, *dβh:ptpn11wt-EGFP* and *dβh:ptpn11mut-EGFP*.

To generate the *dβh:Gab2wt* construct, we first amplified the coding regions of the wild-type *Gab2* gene by PCR using the *mGab2wt-IRES-GFP pmscv* construct (Gu et al., 2001) as templates, and the *Gab2* forward primer: 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTTACCATGAGCGGCGGCGGCGGCGA-3' and *Gab2* reverse primer: 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTTTACAGCTTGGCACCCTTGG-3'. The PCR products were cloned into the modified destination vector containing *I-SceI* recognition sites (Zhu et al., 2012) using the Multisite Gateway System.

The *dβh:mCherry* DNA construct was generated using the Multisite Gateway System (Invitrogen, CA), by combining entry clones of the 5.2-kb *dβh* promoter (Zhu et al., 2012), *pME-mCherry* and *p3E-polyA* into the modified destination vector containing *I-SceI* recognition sites. Further details on the generation of all constructs are available from S.Z. upon request.

Stable or mosaic transgenesis

To generate a stable transgenic line that overexpresses *ptpn11mut-EGFP* in the PSNS, the *dβh:ptpn11mut-EGFP* DNA construct was linearized by digestion with *I-SceI*. Linearized DNA (50-80 pg) was injected into one-cell-stage wild-type embryos. The *Tg(dβh:ptpn11mut-EGFP)* zebrafish line is designated the "*ptpn11mut*" transgenic line in the main body of the text. F1 offspring were screened by fluorescence microscopy for *EGFP* expression, and germline transmission of the *ptpn11mut* gene was confirmed by PCR of DNA from *EGFP*-positive embryos using the primers: *ptpn11 Fw*: 5'- ATCCTGCGAGAGCTGAAGCTGTCTAAAGT -3' and *EGFP Rv*: 5'-ACGCCGTAGGTCAGGGTGGTCACGA -3' and the following reaction conditions: 1 cycle of 94°C for 5 min, 30 cycles of (94°C for 30 s, 55.5°C for 30 s, and 72°C for 60 s). A 900-bp fragment of the *ptpn11mut-EGFP* transgene was amplified and confirmed by sequencing.

The *Tg(dβh:Gab2wt;dβh:EGFP)* zebrafish line, designated the "*Gab2wt*" transgenic line in the main body of the text, was generated by co-injecting linearized *dβh:Gab2wt* DNA and *dβh:EGFP* DNA (3:1 ratio) into one-cell-stage wild-type embryos. Germ line transmission of the *Gab2wt* gene was confirmed by genomic PCR of the EGFP-positive F1 embryos using the primers: *mGab2 Fw1*: 5'- AACTTGTGTGAGCAGGTGGATG -3' and *mGab2 Rv2*: 5'- GTCCTTCTGCTTATGCACTGGT-3' and the following PCR conditions: 1 cycle of 94°C for 5 min, 30 cycles of (94°C for 30 s, 55.5°C for 30 s, and 72°C for 60 s). A 570-bp fragment of the *Gab2wt* transgene was amplified and confirmed by sequencing.

To develop the stable transgenic line overexpressing *mCherry* alone in the PSNS (designated the "*mCherry*" transgenic line in the main body of the text), linearized *dβh:mCherry* DNA was injected into one-cell-stage wild-type embryos.

To mosaically overexpress *ptpn11wt* or *Gab2wt* in *MYCN*-expressing transgenic fish, the *dβh:ptpn11wt* and *dβh:Gab2wt* DNA constructs were coinjected with *dβh:mCherry* (3:1 ratio) into one-cell-stage *MYCN* transgenic embryos. *mCherry*/*EGFP-MYCN*-positive embryos were sorted at day 2-5 and observed for tumor induction.

Tumor monitoring and genotyping of transgenic fish

MYCN and *ptpn11mut or Gab2wt* heterozygous transgenic fish were crossed, and their offspring were raised under

identical conditions and screened every 2 weeks, beginning at 4 weeks postfertilization (wpf), for fluorescent *EGFP*-expressing cell masses. Fish with tumors were separated, genotyped and analyzed further by H&E staining and immunohistochemical assays. For genotyping, genomic DNA was extracted from fin clips and amplified by PCR using *ptpn11* Fw and *EGFP* Rv primers (for *ptpn11* genotyping) or *mGab2* Fw1 and *mGab2* Rv2 primers (for *Gab2* genotyping) and the conditions described above.

EdU labeling, cryosectioning, and paraffin sectioning

For EdU pulse-labeling, 1 μl of EdU solution (2.5 mg/ml) from the Click-iT Alexa Fluor 647 imaging kit (Life technologies, Cat # C10340) was injected retro-orbitally into anesthetized transgenic fish at 5 weeks of age. Two hours after injection, fish were fixed in 4% paraformaldehyde for cryosectioning according to the protocol below or for immunostaining, according to the manufacturer's protocol.

For cryosectioning, embryos or juvenile fish at the indicated stages were fixed in 4% paraformaldehyde at 4°C overnight, washed in phosphate-buffered saline with 0.1% Tween 20 (PBST), embedded in 2% agar/ 5% sucrose, and left in 30% sucrose at 4°C overnight. Sagittal sections (10 μm each) through the whole fish were obtained from embedded specimens by using a conventional cryostat.

Paraffin sectioning, followed by H&E staining, was performed at the Mayo Clinic Histology Core Facility in Arizona with standard protocols.

Immunoblotting and immunostaining

Lysates were prepared from individual tumors dissected from adult fish by immersion in pre-chilled RIPA buffer (150 mM NaCl, 50 mM Tris-HCl pH 8, 5 mM EDTA, 50 mM PMSF, 1% Igepal CA 630, 0.5% sodium deoxycholate, 0.1% SDS and 1×Halt protease inhibitor cocktail). Protein concentrations were determined by Bradford assay (BioRad). Lysates (~50 μg) were separated by gel electrophoresis, transferred to PVDF membranes (Millipore) and probed overnight at 4°C with the following primary antibodies: anti-pERK1/2 (Cell Signaling, 9106; 1:1000), anti-ERK1/2 (Cell Signaling, 9102; 1:1000), anti-pAKT (Cell signaling, 4058; 1:1000), anti-AKT(Cell signaling, 4691; 1:8000), anti-GAB2 (Cell signaling, 3239; 1:1000), anti-SSRP1 (Biolegend, Clone 3E4, 1:800), anti-SPT16 (Biolegend, Clone 8D2, 1:800), and anti-NMYC (Santa Cruz Biotechnology, Cat # sc-53993, 1:1000). Primary antibody binding was visualized on X-ray film using anti-mouse-HRP (Cell Signaling, 7076; 1:5,000) or anti-rabbit-HRP (Cell Signaling, 7074; 1:5,000) secondary antibodies along with Pierce ECL western blotting substrate or the SuperSignal West chemiluminescent substrate (Thermo Fisher Scientific).

For immunohistochemistry of frozen sections, primary antibodies against EGFP (Invitrogen, Cat# A6455, 1:500), HuC/D (Invitrogen, Cat# A-21271, 1:200), and activated caspases-3 (BD Biosciences, Cat# 559565, 1:250) were incubated with slides at 4ºC overnight, washed with PBST, visualized with secondary antibodies conjugated with Alexa 488 and 568 (Invitrogen, 1:500), and counterstained with DAPI.

Immunohistochemistry of paraffin sections was performed using a Leica BOND-MAX instrument and BOND^{TM} reagents with primary antibodies against TH (Pel-Freez, Cat# P40101, 1:500), HuC/D (Invitrogen, Cat# A-21271, 1:200), SHP2 (Cell signaling, 3752; 1:1000), pERK (Cell signaling, 4370s; 1:1000) and PCNA (Santa Cruz Biotechnology, Cat # sc-7907, 1:100)

Cell counts

To analyze the development of the sympathoadrenal cells in the IRG, fish were cryosectioned, stained with primary antibodies against GFP, Hu or activated Caspases-3 and imaged by confocal microscopy at 5 weeks and 5.5 weeks of age. All sections from each individual fish were scanned, a single representative section containing the largest number of sympathoadrenal cells or activated caspases-3 positive sympathoadrenal cells in the IRG was selected, and the resultant cell numbers were quantified (see Figures 2, 3, 5 and S2).

To assess the proliferative capacity of the sympathoadrenal cells in the IRG, fish that had been exposed to a 2 hour EdU pulse were cryosectioned, and stained with primary antibodies against GFP and Hu. A single representative section with the highest ratio of EdU+ (S-phase) sympathoadrenal cells to the total number of GFP+ sympathoadrenal cells in the IRG was selected, and cell number was determined.

Imaging

A Leica MZ10F fluorescence microscope equipped with a Leica EC3 camera was used for capturing bright field and fluorescent images of tumor-bearing fish. Confocal images of the *EGFP*, *mCherry* and antibody staining were collected with a Zeiss LSM 780 laser scanning confocal microscope at the Microscopy and Cell Analysis core facility at Mayo Clinic. For bright-field images of H&E-stained or immunostained paraffin sections (3-5 μm each),

we used an Olympus AX70 compound microscope equipped with an Olympus DP71 camera. Images were processed and compiled with Zeiss ZEN 2012, Adobe Photoshop and Illustrator CS3 (Adobe) software.

Inhibitor treatments and quantification of tumor volume

Tumor-bearing *MYCN*;*GFP* (transgenic for *dβh:EGFP-MYCN* and *dβh:EGFP*) or *MYCN*;*Gab2* transgenic fish were injected intraperitoneally once per day with 10μl of each of the following regimens: i) vehicle (5% dextrose in water); ii) FACT inhibitor CBL0137 (4 mM in 5% dextrose, a generous gift from Incuron); iii) Trametinib (2μM in 5% dextrose from ApexBio Cat No. A3018), or iv) CBL0137 (4 mM) plus trametinib (2μM). After 15 days of treatment, fish were sacrificed for sectioning and pathology analyses or tumors were lysed for immunoblotting analysis.

Tumor-bearing fish were imaged on the 1st day and the 15th day of treatment using identical parameters. The acquired fluorescence images were quantified by measuring the EGFP-covered area using ImageJ software. Means of changes in tumor volume between inhibitor-treated and vehicle-treated groups were compared by the Welch t-test (from GraphPad Prism 6).

Wild-type, *Gab2wt* or *MYCN* transgenic embryos at 7 days postfertilization were immersed in egg water with DMSO, trametinib (0.1μM), CBL0137 (2 μM) or trametinib (0.1μM) plus CBL0137 (2 μM) for 1 day. Then they were lysed for immunoblotting or real-time PCR analyses.

RNA extraction and Real-time PCR analysis

Total RNA were isolated from vehicle- or drug-treated *Gab2wt*-expressing transgenic embryos at 8 days postfertilization with Trizol (Sigma-Aldrich). RNA was reverse-transcribed using Superscript™ III reverse transcriptase (Invitrogen) with oligo(dT) primer. The Real-time PCR was performed using CFX96 Touch™ Real-Time PCR Detection System (BIO-RAD) with the SYBR Supermix (BIO-RAD) with following primers: *β-actin* forward primer: 5'-AGGCTCAGAGCAAGAGAGGTAT-3' and reverse primer: 5'-GTGTCATCTTCTCTCTGTTGGC-3'; *mycn* forward primer: 5'- AGCAGCAGCCAAAGTGTGACA-3' and reverse primer: 5'- TGGTGATGGAACGCCTCTTCT-3'; *Gab2* forward primer: 5'-AACTTGTGTGAGCAGGTGGATG-3' and reverse primer: 5'-TCTCTTCAGCCTGATTGAAGCC-3'; and *gab2* forward primer: 5'-GACATCAAGACAGCAGAGAGG-3' and reverse primer: 5'-CAGAGAGCTGCTCAGCTCATT-3'. All reactions were performed in triplicate. Quantitative data were calculated from the Ct-values for each reaction using the mean reaction efficiency for each primer pair. Data were normalized to the expression level of *β-actin*.

Bioinformatic analyses

To quantitatively assess the correlation between *PTPN11* and *GAB2* expression, we computed the Pearson correlation coefficient (PCC) between *PTPN11* and *GAB2* in high-risk neuroblastomas with *MYCN* amplification from three datasets, including GSE49710, GSE73517, and the TARGET HumanExon cohort for neuroblastoma-Asgharzadeh. Each dataset contains >30 *MYCN*-amplified cases. To generate the plots in Figures 2A-C, we fit a linear model using the stat_smooth function from the ggplot2 R package to highlight the overall trend and association between these genes.

The Kaplan-Meier survival curves were defined as the probability of surviving in a given length of time while considering time in many small intervals. For each time interval, survival probability is calculated as the number of surviving subjects, divided by the number of patients at risk. We focused our analysis on the gene expression data of *MYCN*-amplified high-risk neuroblastoma from the above three datasets. Survival data (death from disease, follow up months) for each group (*GAB2*-low vs. *GAB2*-high expression or *PTPN11*-low vs. *PTPN11*-high expression) were extracted manually from each dataset and imported into the R environment. A scanning procedure was performed (with a minimum group size of 6) and the cutoff value for most significant separation between groups with high or low expression of *GAB2* or *PTPN11* was chosen to generate the curves. Kaplan-Meier analysis was performed using the survival package in R, with rho=1 chosen in the survdiff function to perform the log-rank statistical test.