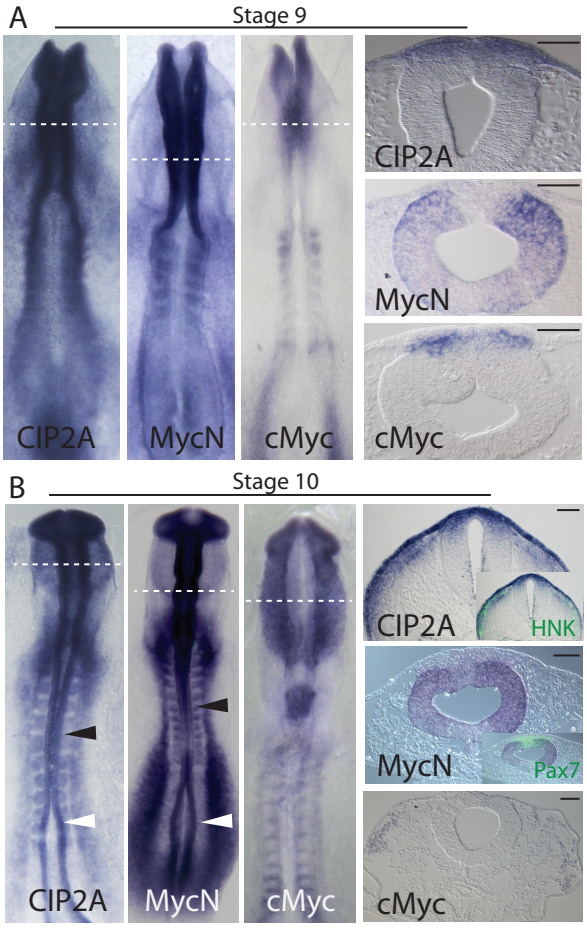
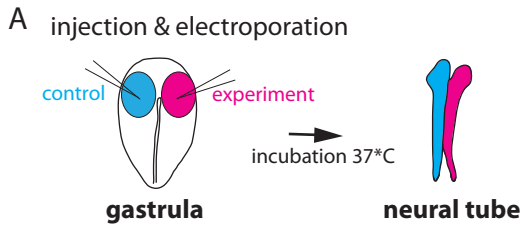
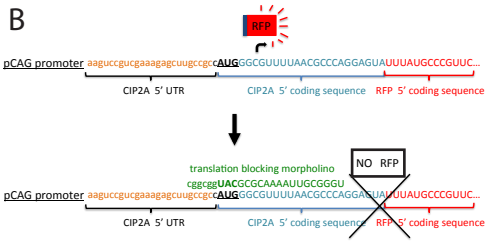
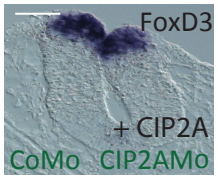
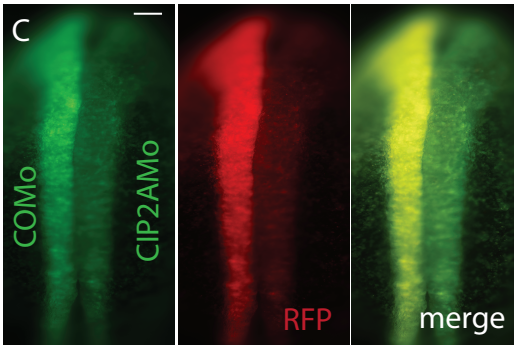

A
Stage 9
CIP2A
MycN
cMyc
CIP2A
MycN
cMyc
B
Stage 10
CIP2A
MycN
cMyc
CIP2A
HNK
MycN
Pax7
cMyc

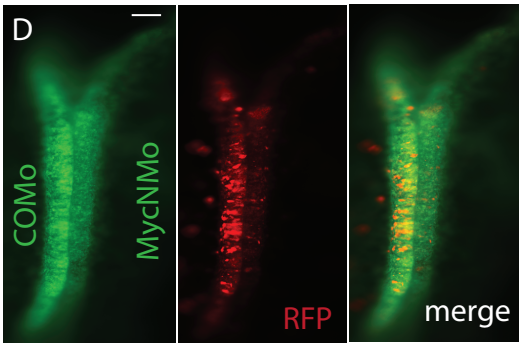
Supplemental Figure 1. Expression pattern of *CIP2A*, *MycN* and *cMyc* mRNA during neural crest migration and in the trunk neural plate/tube. A) At stage HH9, expression of *CIP2A* is visible in the ectoderm and in the neural crest cells that are about to emigrate from the dorsal neural tube. *MycN* is expressed in the rest of the neural tube that will form the central nervous system, and is complementary to *cMyc* that is only expressed in the dorsal neural crest domain. B) At stage HH10, the expression patterns are similar to stage HH9, although very low levels of *MycN* expression is visible in the migrating neural crest cells. Pax7 marks the neural crest at premigratory stage, and HNK1 in the migrating cells. Similar to anterior regions, *MycN* and *CIP2A* expression is seen throughout the body from anterior to posterior in the trunk neural tube (black arrows) all the way to the neural plate in the lumbosacral region (white arrows). Similar to anterior regions, *cMyc* is not expressed before the premigratory neural crest stage in the more posterior parts of the embryo. Scale bar 20μm.

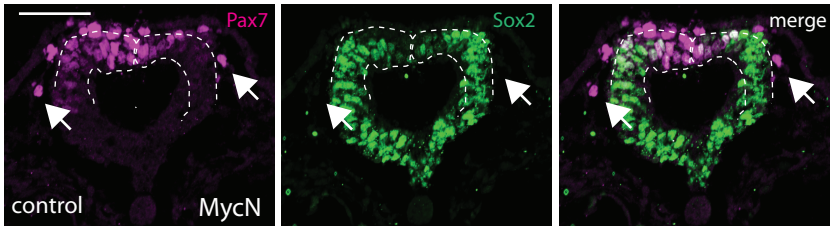

A
injection & electroporation
control
experiment
incubation 37*C
gastrula
neural tube


B
RFP
pCAG promoter
CIP2A 5' UTR
CIP2A 5' coding sequence
RFP 5' coding sequence
translation blocking morpholino
NO RFP


E
FoxD3
+ CIP2A
CoMo
CIP2AMo

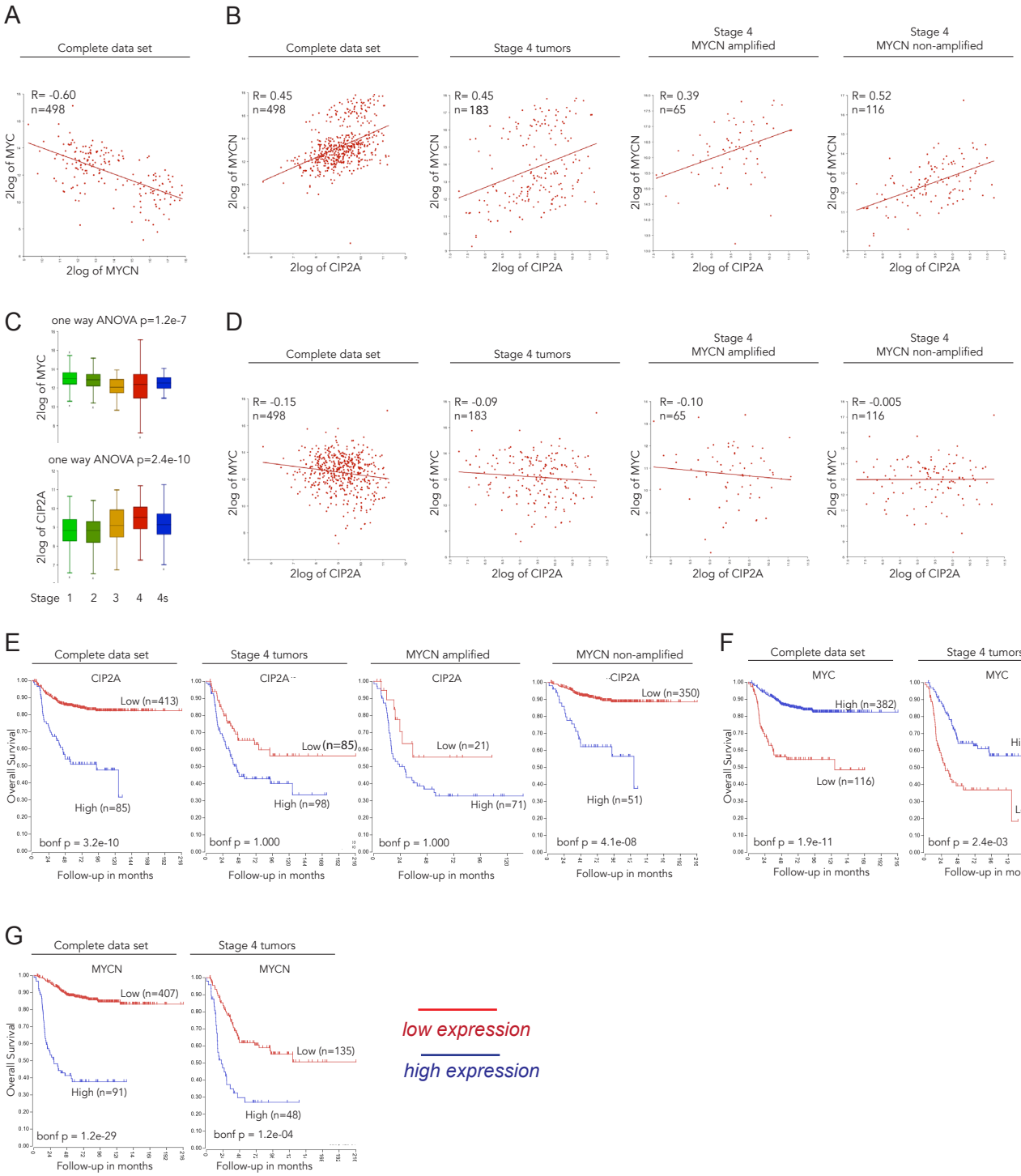

C
COMo
CIP2AMo
RFP
merge


D
COMo
MycNMo
RFP
merge


F
Pax7
control
MycN
Sox2
merge

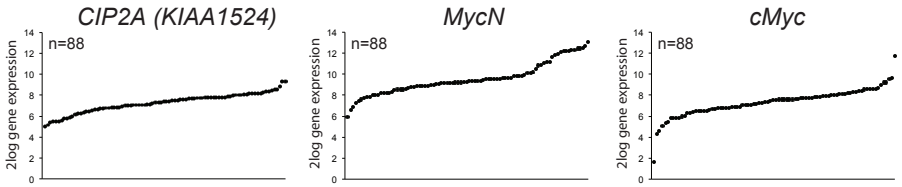
Supplemental Figure 2. Morpholinos specifically block translation of CIP2A and MycN, Sox2 is downregulated in MycN overexpressing cells after emigration.
A) Illustration demonstrating the double sided gene expression perturbation technique for chick embryos. At gastrula stage (HH4), either morpholinos or DNA with two different fluorescent dyes are injected into contralateral sides of the embryo and targeted to the ectoderm using electroporation. The embryos, grown *ex ovo* on filter papers in albumin, are placed in 37^{o}C until they reach the desired stage. B) To verify that the morpholino correctly binds to its target sequence, constructs with about 50bp covering the 5' end / transcription start side region were cloned upstream of RFP coding sequence without ATG. As the morpholino binds, no RFP will be translated. C) CIP2A morpholino and D) MycN morpholino efficiently block translation of RFP and thus bind to their respective specific target sites. E) Loss of neural crest phenotype induced by CIP2AMo is efficiently rescued by co-expression of CIP2A as shown by *FoxD3 in situ* hybridization. F) MycN expressing migrating neural crest cells downregulate their Sox2 expression after emigration, no Sox2-positive migrating cells are seen on either side of the embryo. At early migration, the phenotype seen in the premigratory neural crest persists: fewer early neural crest cells emigrate from the MycN overexpressing side, and the dorsal neural crest domain is smaller as indicated by Pax7 staining (n=3). Scale bar 20μm.

SEQC498 data set

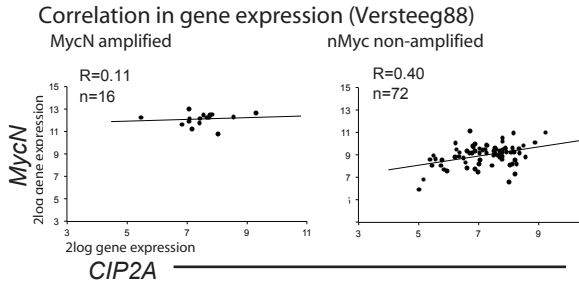


Supplemental Figure 3. Strong correlation between *CIP2A, MycN* and poor prognosis in SEQC498 data set while *cMyc* correlates with better survival probability. A) Expression of *cMyc* and *MycN* show negative correlation in clinical neuroblastoma samples, B) whereas *MycN* correlates positively with *CIP2A* as shown both in the complete data set as well as in Stage 4 tumors. Presumably due to the extremely high expression levels, the correlation is weakest in the MycN amplified stage 4 tumors. C) Expression of *CIP2A* correlates significantly according to INSS stage, and *cMyc* shows an inverse correlation. D) *CIP2A* and *cMyc* display a modest negative correlation in the complete data set but the correlation in this data set is lost in Stage 4 tumors. E) Kaplan-Meier curve showing a significantly lower survival probability for patients with high *CIP2A* in the complete data set and in the MycN non-amplified tumors (with more "physiological MycN expression levels, see Fig 6B), and the correlation is lost in the Stage 4 tumors and the MycN amplified group (bonf p= 1). F) High *cMyc* expression levels (which may reflect rather physiological than overexpressed levels) correlate with better survival probability in clinical neuroblastoma both in the complete and Stage 4 data set, whereas G) *MycN*, as previously known, correlates with the poor outcome.

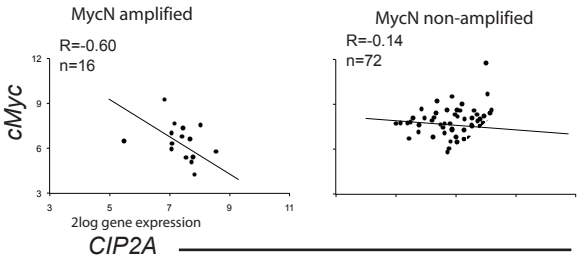
A


Expression in neuroblastoma (Versteeg88)
CIP2A (KIAA1524)
MycN
cMyc
n=88
2log gene expression

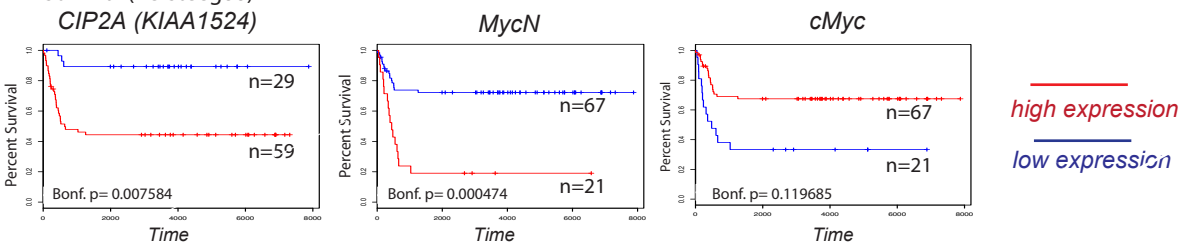
B


Correlation in gene expression (Versteeg88)
MycN amplified
nMyc non-amplified
R=0.11
n=16
R=0.40
n=72
MycN
2log gene expression
CIP2A

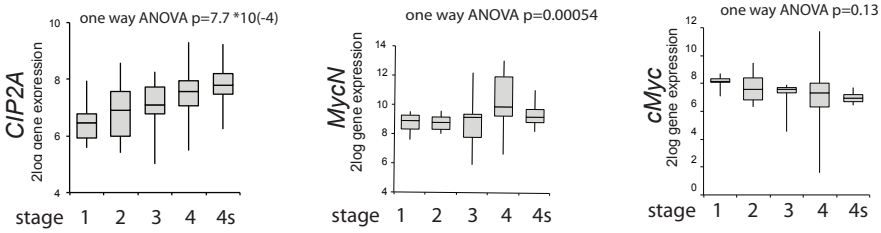
C


MycN amplified
MycN non-amplified
R=-0.60
n=16
R=-0.14
n=72
cMyc
2log gene expression
CIP2A

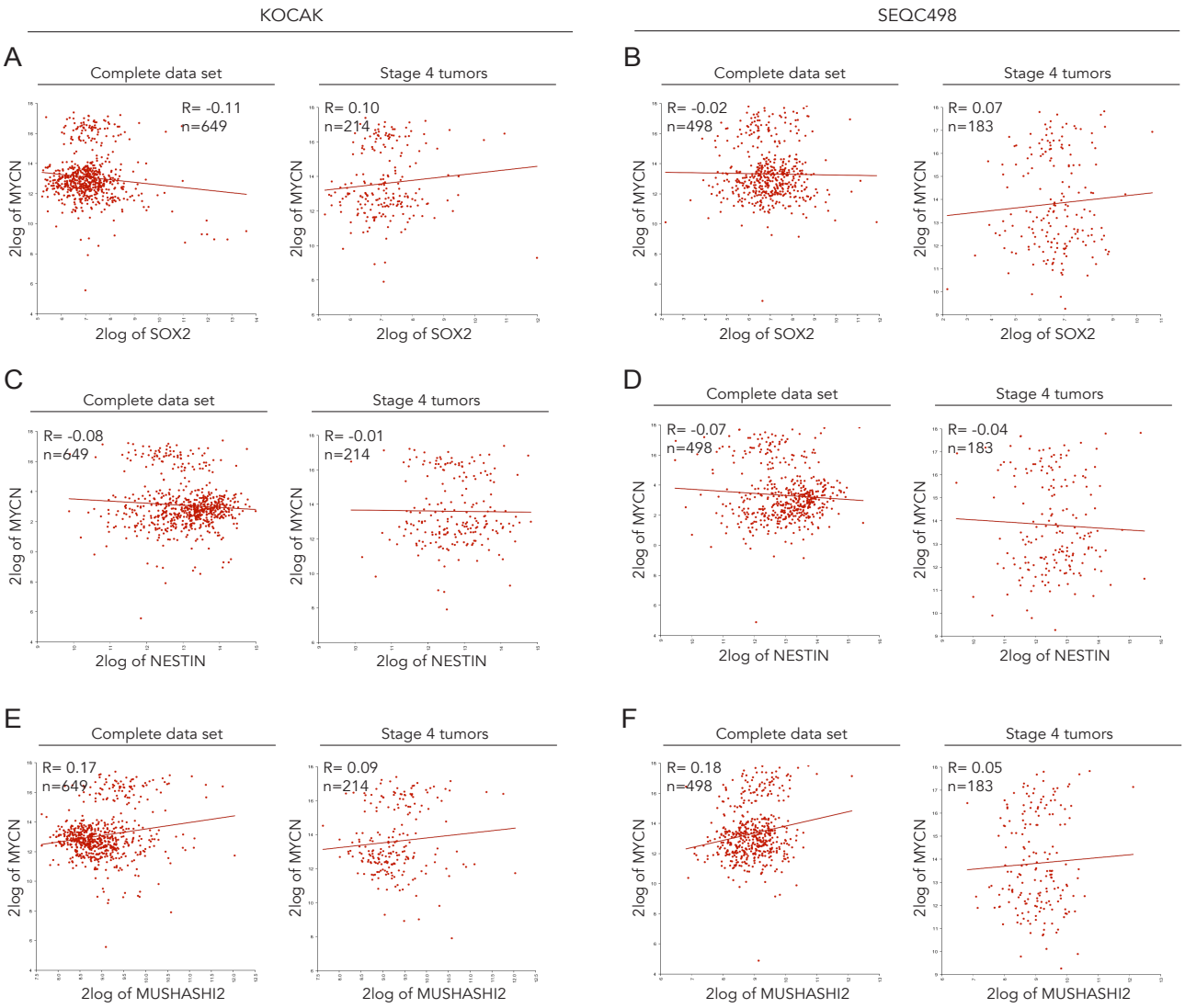
D


Survival (Versteeg88)
CIP2A (KIAA1524)
MycN
cMyc
Percent Survival
n=29
n=59
Bonf. p= 0.007584
n=67
n=21
Bonf. p= 0.000474
n=67
n=21
Bonf. p= 0.119685
Time
high expression
low expression

E


Expression according to stage (Versteeg88)
one way ANOVA p=7.7 *10(-4)
one way ANOVA p=0.00054
one way ANOVA p=0.13
CIP2A
MycN
cMyc
2log gene expression
stage 1 2 3 4 4s

Supplemental Figure 4. Strong correlation between *CIP2A, MycN* and poor prognosis in Versteeg88 data set. A) *MycN*, *cMyc* and *CIP2A* show substantial expression levels in neuroblastoma with *CIP2A* displaying the lowest range. B) *CIP2A* correlates with *MycN* in the complete data set in the MycN non-amplified tumors, C) and shows a strong negative correlation with *cMyc*. D) Kaplan-Meier curves show that both *MycN* and *CIP2A* correlate significantly with poor survival probability, whereas *cMyc* is associated with better survival. The data set may be too small to show statistical significance for *cMyc*. E) Expression of *CIP2A* and *MycN* correlate significantly according to INSS stage, and *cMyc* shows an inverse trend.


KOCAK
SEQC498
A
Complete data set
Stage 4 tumors
R= -0.11
n=649
R= 0.10
n=214
2log of MYCN
2log of SOX2
B
Complete data set
Stage 4 tumors
R= -0.02
n=498
R= 0.07
n=183
2log of MYCN
2log of SOX2
C
Complete data set
Stage 4 tumors
R= -0.08
n=649
R= -0.01
n=214
2log of MYCN
2log of NESTIN
D
Complete data set
Stage 4 tumors
R= -0.07
n=498
R= -0.04
n=183
2log of MYCN
2log of NESTIN
E
Complete data set
Stage 4 tumors
R= 0.17
n=649
R= 0.09
n=214
2log of MYCN
2log of MUSHASHI2
F
Complete data set
Stage 4 tumors
R= 0.18
n=498
R= 0.05
n=183
2log of MYCN
2log of MUSHASHI2

Supplemental Figure 5. Clinical neuroblastomas express low levels of *Sox2*; and *MycN* does not correlate with neural stem cell markers *Sox2, Nestin* or *Mushashi2*. As shown in A) KOCAK and B) SEQC498 data sets, vast majority of the clinical neuroblastoma samples express low levels of *Sox2* (within the log2 range of 5 to 9), and *Sox2* levels don't correlate with *MycN*. C) Expression of the neural stem cell marker *Nestin* does not correlate with *Sox2* in KOCAK or D) SEQC498 data sets. E) Expression of the neural stem cell marker *Mushashi2* shows weak if any correlation with *MycN* in the complete data set that is lost in Stage 4 tumors in KOCAK and F) SEQC498 data sets.

Supplemental Information

MATERIALS AND METHODS

In situ hybridization

Embryos were fixed with 4% paraformaldehyde, washed with PBS/0.1% Tween (PBT), dehydrated in MeOH, and stored at −20°C. The avian *MycN*, *cMyc* and *CIP2A* (KIAA1524) probes were made by using cEST697p20, cEST191o11 and cEST895e1 (www.chick.manchester.ac.uk), respectively, and the *Sox10* and *FoxD3* probes were made by cloning respective genes to DNA vectors from RT-PCR products made by using chicken whole embryo cDNA (HH7-12) as template. Whole-mount *in situ* hybridization was performed in 70^{o}C as described (65, 66). Prior to hybridization, the later stage embryos (HH2-HH7) were bleached in 6% hydrogen peroxide for 1 h, permeabilized with 10 µg/ml proteinase K for 30 min. The embryos were imaged by using Zeiss Axioscope 2 and Zeiss ApoTome.2. Two-dimensional projections of the Z-stacks and contrast enhancement were performed with the Zen Blue software (Zeiss).

Morpholino knockdown, expression constructs and HH4 electroporation of the chicken embryos

FITC-conjugated morpholinos were purchased from Gene Tools LLC (www. gene-tools.com). The CIP2A (GAGACGCGGACGACATACCACATTC), and MycN (TCTTGCTGATCATTCCCGGCATGGC) translation blocking morpholinos were targeted to the respective 5' UTR in close proximity of the ATG and a control morpholino was designed to assure lack of non-specific effects from electroporation (CTGCGATGAAAAACACGGGAGCACA). The MOs were diluted to a 1.5 mM concentration and electroporated together with an empty pGAG vector as carrier DNA (1 µg/ µl). The morpholino was injected as two-sided injections with control morpholino on the contralateral side (Fig S2A). The electroporation to target the ectoderm was carried out as previously described (67). Briefly,

the chicken embryos were collected on Whatman filter papers and electroporated at Hamburger and Hamilton (HH) stage 4 by using 5.3V and 5 pulses (50mA/100mA) and incubated on individual petri dishes (Falcon 1008 35x10mm) in thin albumin until they reached the desired stage. The coding sequence of human CIP2A was cloned into the pcDNA3.1 vector, and the chicken MycN was cloned into the PCI-RFP vector and injected in 3mg/ml concentration. To test the translation blocking ability of the morpholinos, 30bp of the 5' UTR followed by the first 30bp of the coding sequence of the gene of interest (*CIP2A* or *MycN*) were cloned in front of the RFP gene in the PCI-RFP vector backbone that lacked the start codon. Co-electroporation of the construct with the respective morpholino efficiently blocked expression of RFP as compared to injection on the contralateral side with the control morpholino (Figs. S2B-D).

Immunofluorescence and Western Blot

For immunofluorescence, embryos were fixed with 4%PFA for 20 minutes, washed with PBT, blocked with 2.5% goat and 2.5% donkey serum o/n +4^{o}C, and stained with the primary antibody in the block o/n +4^{o}C, washed 5x with PBT and stained with the secondary antibody in block o/n +4^{o}C. The embryos were then embedded in gelatin and sectioned at 12 µm. The sections were imaged by using fluorescence microscopy (Zeiss Axioscope 2 and Zeiss ApoTome.2).

For Western Blot, the neuroblastoma cell line cells were grown in 6-well plates and lysed into 200µl of the SDS lysis buffer (0,19M Tris-HCl, pH 6,8, 3 % SDS (20-%), 30% glyserol, 3 % β-merkaptoethanol, 0,015 % bromphenol blue) after 2 days of RNAi infection and the WB protocol was carried out as previously described (37).

Antibodies

The following antibodies were used for Western Blot: CIP2A (Santa Cruz sc-80659, 1:1000); MycN (Santa Cruz sc-791, 1:500); c-Myc (Abcam y69 1:1000); Sox2 (D6D9, Cell Signalling Technology, 1:500); GAPDH (5G4-6C5 Hytest ,1:200,000). For immunofluorescence: Pax7 and HNK-1 (3H5) from Developmental Studies Hybridoma Bank (1:10); Sox2 (Abcam 97959, 1:2000), and and anti-Tyrosine Hydroxylase (Millipore Cat# AB152 RRID:AB_390204; 1:300). For PLA: MycN (rb) sc-791 and CIP2A (ms) made by The Edward K Chan laboratory at The University of Florida. The respective secondary antibodies were purchased from Alexa Fluor (1/1,000 dilution; http://www.bdbiosciences.com).

Fluorescence quantification on the in vivo chicken embryo sections

Fluorescent images of cross-sections of the embryos that were overexpressing MycN on one side of the embryo were quantified by using ImageJ. The Pax7 or Sox2 expression was normalized to expression of DAPI and the intensity of fluorescence of the mycN injected side was divided by the results on the control plasmid side. For the stage HH8 embryos, the quantified dorsal region was selected based on the area of Pax7 expression, whereas for the migrating neural crest cells stage HH12, an equally large area was manually selected from both sides of the embryo and levels of Pax7 from the MycN overexpressing side were compared with the control side.

RNAi transfections

Neuroblastoma cells were transfected at 30% confluence with 250 pmol of siRNA by using Oligofectamine (Invitrogen 12252011) according to manufacturer's instructions. Three individual siRNAs were used for CIP2A together with a scrambled control (37, 68).

In vitro culture of neuroblastoma cell lines

The SK-N-BE(2) cells were cultured in F12 medium supplemented with 10% FCS, 1% pen/strep, 1% glutamine, and 1% NEAA, and the NGP cells were cultured in DMEM with 10% FBS and 1%pen/strep and 1% glutamine. Finally, the SK-N-AS cells were on grown in DMEM media with 10% FCS, 1% pen/strep, 1% glutamine, 1% NEAA on collagen-coated plates (0.05 mg/mL).

Proximity Ligation Assay (PLA)

PLA was performed according to the manufacturer's instructions for the Duolink kit (DUO92102, Sigma-Aldrich). Briefly, neuroblastoma cell lines were grown 80% confluent on coverslips in 12-well plates, washed once with PBS and fixed in a mix of methanol /acetone (3:1) for 7 minutes. in -20^{o}C. The fixed cells were washed 3x in PBS and the cells were blocked by placing the coverslips on 1 drop (always equals 25 µl) of blocking solution for 37 C for 30 minutes on the PLA dish. Primary antibodies were diluted in antibody diluent, and coverslips were placed on a drop of antibody solution o/N +4^{o}C. The next day, the coverslips were washed 2 times for 5 minutes on nutator at low speed in Buffer A followed by incubation in probe solutions for 1h at 37^{o}C (probes diluted 1:5 in antibody diluent), and washed 2 times for 5 minutes in buffer A. The ligation mix was diluted in H2O (1:5) and the ligase was diluted 1:40 in the mix, and the washed coverslip was placed on a drop of the mix for 30 minutes in 37^{o}C followed by two washes in buffer A. For the light sensitive reaction, the amplification stock was diluted in H2O (1:5) and the polymerase was diluted in the amplification mix (1:80), and the coverslip was placed on a drop of the mix for 100 minutes at 37^{o}C. Finally, the samples were washed twice in buffer B for 10 minutes followed by one wash with 0.01X buffer B for 1minute, and the coverslips were let dry at room temperature in the dark for 20 minutes. Finally, the coverslips were mounted with 8µl of Duolink DAPI mounting media, let dry in the dark for 15 minutes and stored in -20^{o}C.

WST-1 viability assay

To measure cell viability and proliferation we used the WST-1 kit (Roche 5015944001) according to manufacturer's instructions. Briefly, the cells were plated in 96 well plates (5-10 000 cells per well) after 1-2 days of siRNA transfection and the cells were let grow until 70-80% confluent (2-4 days). Absorbance of the formazan dye was measured at 450nm.

Soft agar assay

To measure anchorage independent growth, the cells (5000 cells /well) were seeded in 12-well plates in their respective media mixed with agar (1ml media in 1.2% agar in the bottom, 1.5ml media in 0.4% agar on top and additional 1ml of media on top of the solidified agar) 1-2 days after siRNA transfection. The wells were incubated in 37^{o}C for 10-14 days until colonies were formed. The crystal violet staining and imaging was performed as previously described (37).

Statistical analyses for clinical neuroblastoma samples

Three publically available data sets consisting of 649, 498 and 88 neuroblastomas (KOCAK, SEQC498 and Versteeg88, respectively) were acquired from R2: microarray analysis and visualization platform (http://r2.amc.nl), and used to analyze individual gene expression, gene correlations and survival.