Supporting Information

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SI Materials and Methods

Cell Culture Under Normoxic and Hypoxic Conditions and Reagents. For experiments at normoxia, all cells were grown in a 95% air/5% CO_2 humidified incubator at a temperature of 37 °C. NB12 cells were isolated from relapse bone marrow of a patient with high-risk neuroblastoma. NB88 cells were isolated from bone marrow of a multiple-relapse patient with progressive high-risk neuroblastoma. Cells were treated for 24 h with indicated concentrations of 2,2′-dipyridyl (Sigma). For transient transfections, Accell siRNA (Dharmacon) was used according to the manufacturer's recommendations. Briefly, cells were grown for 72 h with HIF-2a siRNA or a control siRNA before harvest.

Western Blotting. For Western blot experiments, cells were lysed in 10 mM Tris·HCl (pH 7.2), 160 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM EGTA, and 1 mM EDTA (RIPA), supplemented with Complete Protease Inhibitor mixture (Roche Molecular Biochemicals). Protein concentrations were determined by the method of Bradford, and samples were kept at −80 °C in 1× NuPage LDS sample buffer (Invitrogen) with 10% β-mercaptoethanol. Proteins were separated by SDS/PAGE and blotted onto Hybond-C-Extra nitrocellulose membranes (Amersham). Membranes were blocked in 5% milk diluted in 0.010 M phosphate buffer (pH 7.4), 0.0027 M KCl, 0.140 M NaCl, and 0.05% Tween-20 (PBS-Tween). Membranes were incubated with primary antibodies at 4 °C overnight and secondary antibodies for 1 h at room temperature. The following primary antibodies were used: mouse anti-HIF-1α (Novus) diluted 1.5:1,000, mouse anti-HIF-2α (Novus) diluted 1:1,000, and mouse anti-actin (MP Biomedicals) diluted 1:2,000. The following secondary antibodies were used: goat anti-mouse Ig (Jackson Laboratories) diluted 1:10,000 (for HIF-1α and HIF-2α detection) and sheep anti-mouse Ig (GE Healthcare) diluted 1:5,000 (for actin detection).

Nuclear Extracts. For measurements of icNotch-1, cells were harvested in 40 mM Tris·Cl (pH 7.5), 1 mM EDTA, and 150 mM NaCl (TEN), then washed in PBS and 25 mM Tris (pH 7.5), 50 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, and 5 mM DTT (buffer A). Pellets were resuspended in 25 mM Tris (pH 7.5), 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT, and 25% sucrose (buffer NE), and nuclei were extracted for 30 minutes. Cellular debris was removed by centrifugation, and supernatant was loaded on gels for Western blot analyses. icNotch-1 antibody from Cell Signaling Technology was used.

Quantitative Real-Time PCR. Total RNA was extracted and washed by using the Qiashredder and RNeasy mini kits (Qiagen) according to the manufacturer's recommendation before extensive washing and DNase treatment. cDNA synthesis was performed by using random primers and Multiscribe Reverse Transcriptase enzyme (Applied Biosystems). The generated cDNA was used as template with SYBR Green PCR Master Mix (Applied Biosystems). Relative gene expression levels were quantified by use of the comparative Ct method. For normalization of expression levels, three housekeeping genes (*UBC*, *YWHAZ*, and *SDHA*) were used (1). Primer sequences are listed in Table S1.

Patient Material and Immunohistochemistry. Human neuroblastoma specimens were fixed and embedded in paraffin routinely before analysis (ethics approval LU 389-98, Lund University). Human neuroblastoma TICs and cell lines were pelleted and fixed in 4% paraformaldehyde, then routinely embedded in paraffin after dehydration and several rounds of EtOH washing. A tissue microarray consisting of 93 neuroblastomas was described previously (2) and analyzed for HIF-1α expression independently by two pathologists. Immunoreactivity was detected after antigen retrieval using the Envision system and DAKO Techmate 500. Only freshly sectioned tissue was used for HIF-2α stainings. The following antibodies were used: ALDH1 (1:100; PharMingen), CD34 (1:100; Santa Cruz Biotechnology), chromogranin A (1:3,000; DAKO), HIF-1α (1:100; Upstate Cell Signaling), HIF-2α (1:200; Abcam), and TH (1:300; Abcam).

Retrovirus Production and Transductions. The retroviral vectors pRETRO-SUPER-shHIF-2α RS2 and RS9 express shRNA targeting HIF-2α and additionally contain puromycin as a selection marker (*shHIF2A*; kindly provided by W. G. Kaelin, Jr. (3). The retroviral vector rvShRNA$_{SNC}$ expressing a scrambled negative control shRNA in a pRETRO-SUPER vector and both GFP and neomycin as selectable markers (*shC*) was kindly provided by G. L. Semenza (4). Plasmid DNA from the retroviral vectors and the helper plasmids RD114, encoding the feline endogenous virus envelope, and pSV-Mo-MLV-gagpol (5), encoding retroviral Gag and Pol, was amplified by using standard methods. 293T cells were grown in DMEM (Invitrogen) supplemented with 10% FCS (Invitrogen). Retroviral supernatant was produced by calcium phosphate-mediated transfection of 293T cells using 10 μg of retroviral vector DNA, 4 μg of RD114 DNA, and 20 μg of pSV-Mo-MLV-gagpol (5) DNA per 10-cm cell culture plate in the presence of 25 μM chloroquine (Sigma–Aldrich). The transfection medium was removed 8 h later and replaced with fresh DMEM/FCS. Retroviral supernatant was collected 36, 48, 60, and 72 h after transfection, filtered (45-μm pore size), and stored at −80 °C. For retroviral transductions, cells were passaged and seeded in six-well plates in medium consisting of 75% retrovirus-containing supernatant (DMEM) and 25% F12 medium supplemented with 40 ng/mL bFGF and 20 ng/mL EGF. After 12 h, the virus-containing medium was removed and replaced with TIC medium as described above. Four days after the transduction, cells were replated in selection medium consisting of TIC medium with the addition of 2 μg/mL puromycin or 800 ng/mL G418, then used without further cloning.

1. Vandesompele J, et al. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 3:RESEARCH0034.
2. Holmquist-Mengelbier L, et al. (2006) Recruitment of HIF-1alpha and HIF-2alpha to common target genes is differentially regulated in neuroblastoma: HIF-2alpha promotes an aggressive phenotype. *Cancer Cell* 10:413–423.
3. Kondo K, Kim WY, Lechpammer M, Kaelin WG, Jr (2003) Inhibition of HIF2alpha is sufficient to suppress pVHL-defective tumor growth. *PLoS Biol* 1:E83.
4. Okuyama H, et al. (2006) Expression of vascular endothelial growth factor receptor 1 in bone marrow-derived mesenchymal cells is dependent on hypoxia-inducible factor 1. *J Biol Chem* 281:15554–15563.
5. Beyer WR, Westphal M, Ostertag W, von Laer D (2002) Oncoretrovirus and lentivirus vectors pseudotyped with lymphocytic choriomeningitis virus glycoprotein: Generation, concentration, and broad host range. *J Virol* 76:1488–1495.

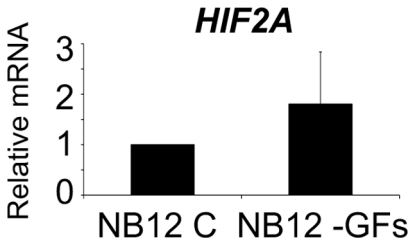

Fig. S1. *HIF2A* mRNA is not affected by growth without EGF and bFGF, as measured by qPCR. Data are mean values of three independent experiments performed in triplicate.

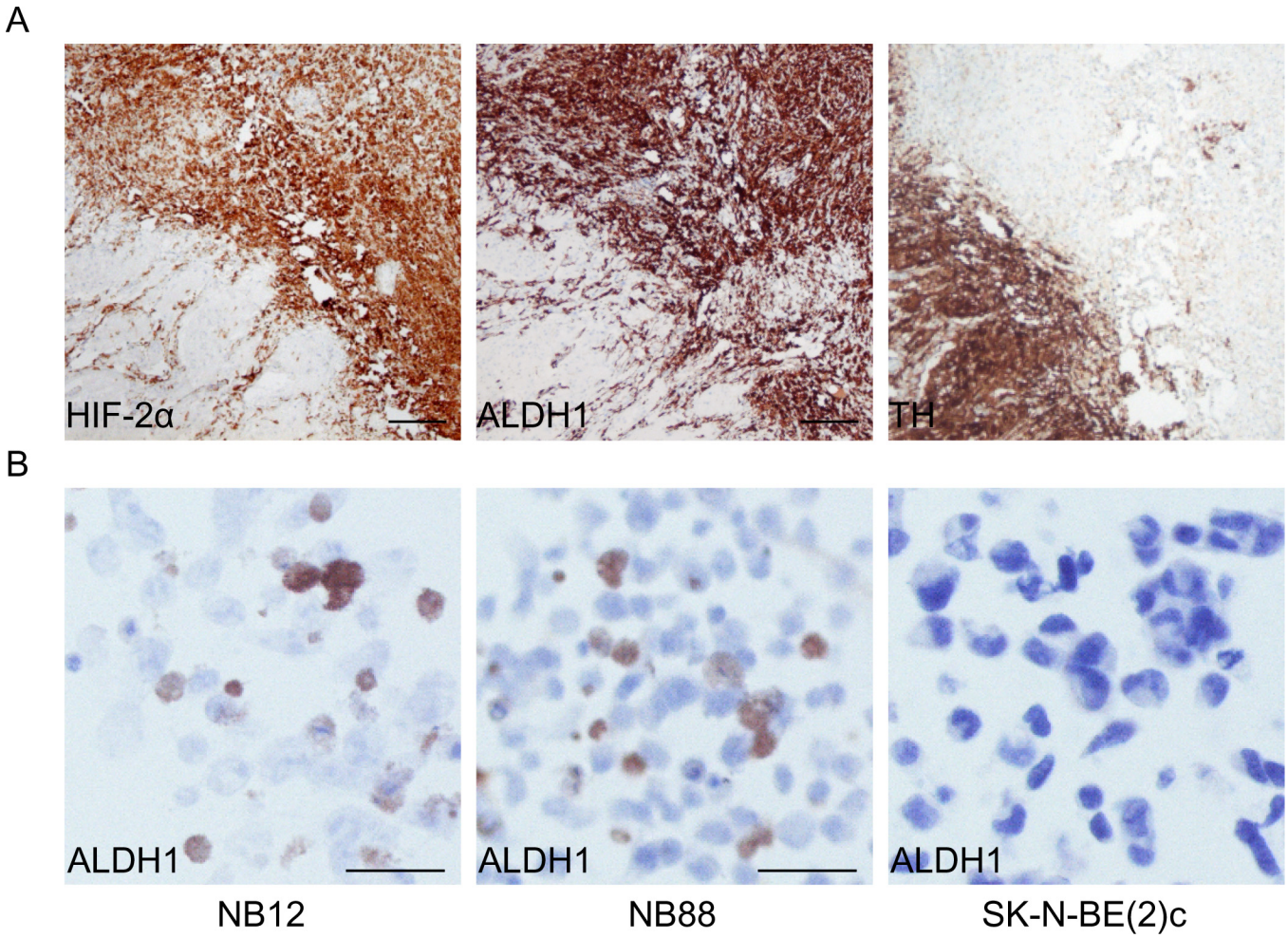

Fig. S2. ALDH1 is expressed in HIF-2α-positive/TH-negative neuroblastoma cells in tumor specimens (*A*) and in neuroblastoma TICs but not in SK-N-BE (2)c cells (*B*), as shown by immunohistochemistry. (Scale bars: *A*, 50 μm; *B*, 10 μm.)

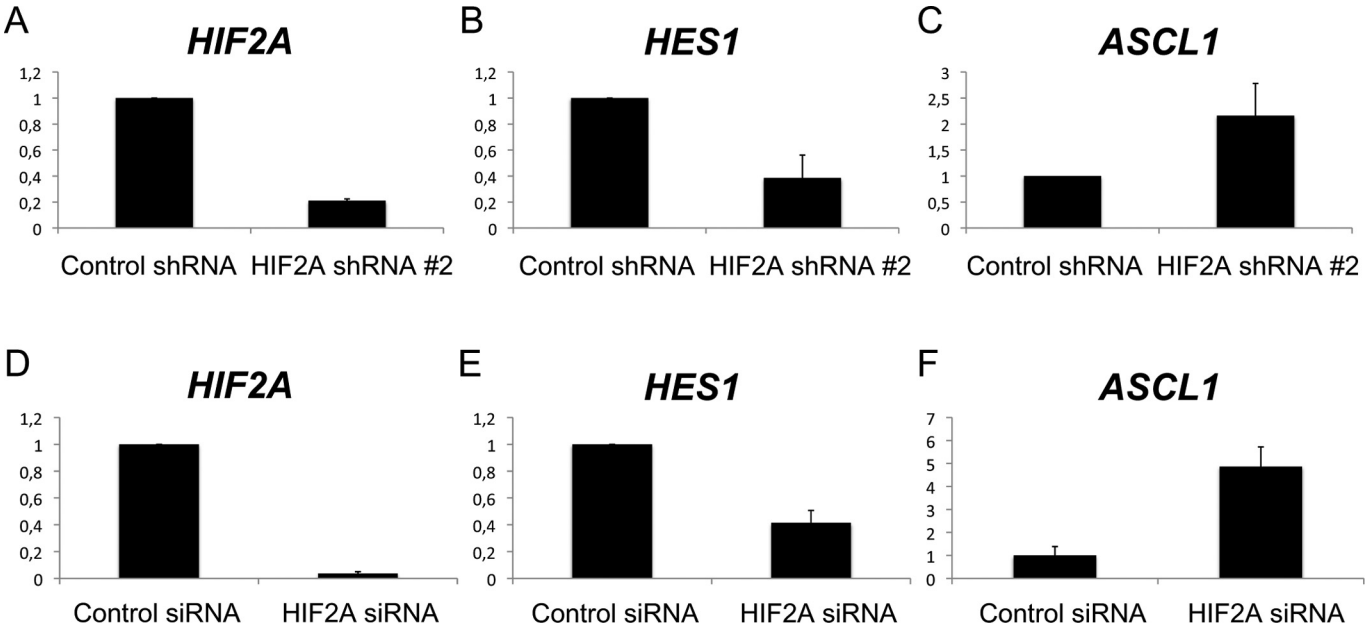

Fig. S3. Induced neuronal differentiation upon HIF2A knockdown with independent sh/siRNA sequences. (*A*–*C*) mRNA levels of *HIF2A* (*A*), *HES1* (*B*), and *ASCL1* (*C*) in shC and shHIF2A-RS2 cells as determined by qPCR. (*D*–*F*) mRNA levels of *HIF2A* (*D*), *HES1* (*E*), and *ASCL1* (*F*) in siC and siHIF2A cells as determined by qPCR. Data are mean values of three independent experiments performed in triplicate.

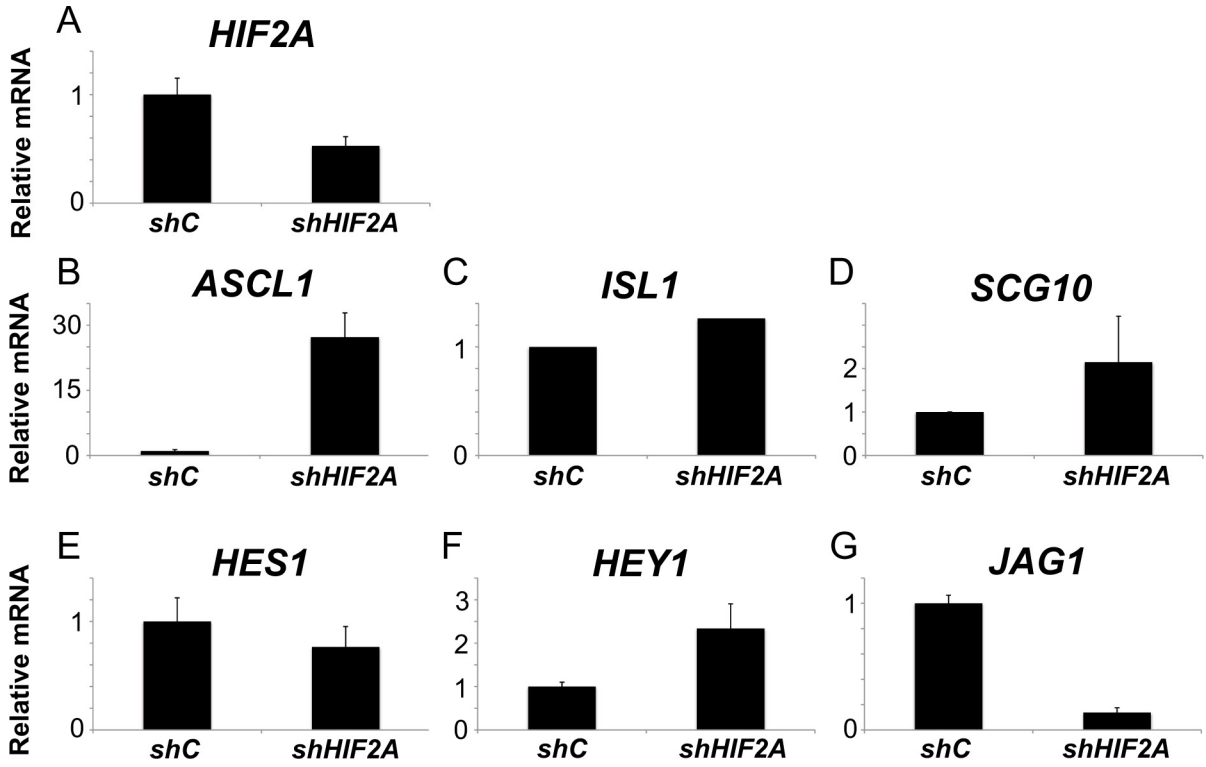

Fig. S4. Induced neuronal differentiation upon HIF2A knockdown in NB88 cells. (*A*) Down-regulation of *HIF2A* mRNA levels upon HIF-2α knockdown in NB88 cells assessed by qPCR. (*B–D*) Up-regulation of sympathetic neuronal differentiation markers at the mRNA level upon HIF-2α knockdown in NB88 cells assessed by qPCR. (*E* and *F*) Down-regulation of Notch pathway components at the mRNA level upon HIF-2α knockdown in NB88 cells assessed by qPCR. Data are representative of three independent experiments performed in triplicate.

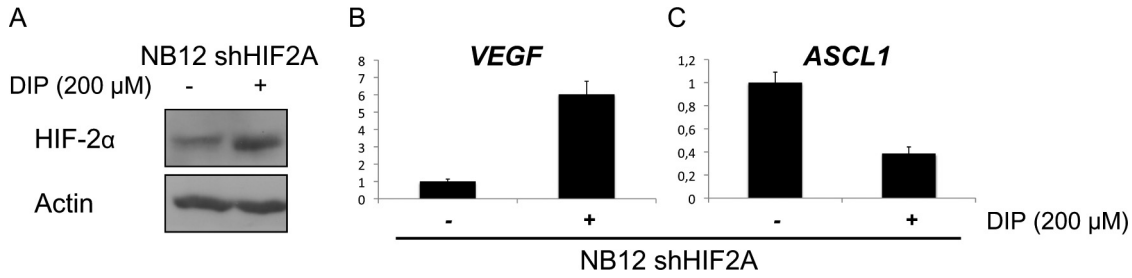

Fig. S5. Differentiation is reverted upon HIF-2α stabilization by DIP. (*A*) HIF-2α protein stabilization upon DIP treatment in NB12 shHIF2A cells as measured by Western blot analysis. (*B*) *VEGF* mRNA up-regulation upon DIP treatment in NB12 shHIF2A cells as measured by qPCR. (*C*) *ASCL1* mRNA down-regulation upon DIP treatment in NB12 shHIF2A cells as measured by qPCR. Data are representative of three independent experiments performed in triplicate.

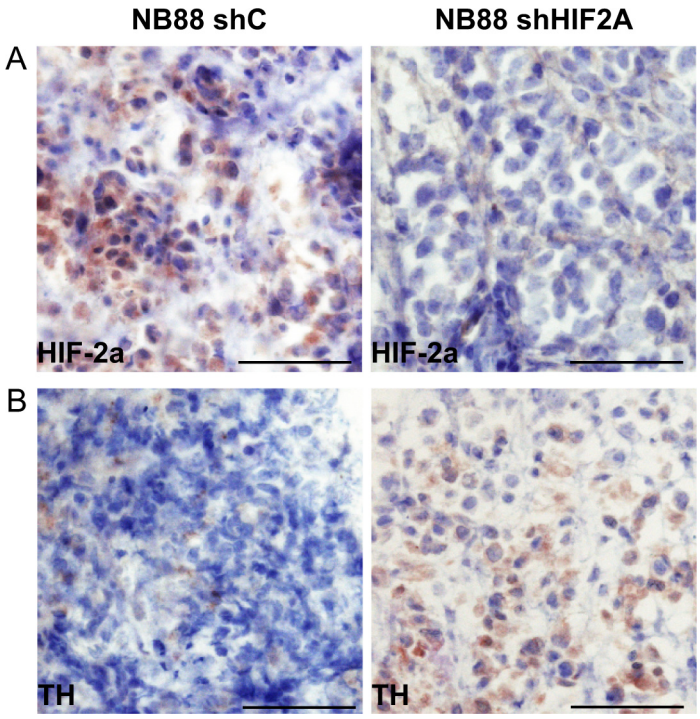

Fig. S6. Immunohistochemistry showing HIF-2α (*A*) and TH (*B*) protein levels in xenograft tumors derived from NB88 shC and shHIF2A cells. (Scale bars: 50 μm.)

Table S1. Primer sequences for quantitative real-time PCR

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
UBC	ATTTGGGTCGCGGTTCTTG	TGCCTTGACATTCTCGATGGT
SDHA	TGGGAACAAGAGGGCATCTG	CCACCACTGCATCAAATTCAT
YWHAZ	ACTTTTGGTACATTGTGGCTTCAA	CCGCCAGGACAAAACAGTAT
BNIP3	AAAATATTCCCCCCAAGGAGTTC	ACGCTCGTCTTCCTCATGCT
Chromogranin A	CGCTGTCCTGGCTCTTCTG	TCACCTCGGTATCCCCTTTATTC
dHAND	AGAGGAAGAAGGAGCTGAACGA	CGTCCGGCCTTTGGTTTT
GAP43	ACGACCAAAAGATTGAACAAGATG	TCCACGGAAGCTAGCCTGAA
Hash-1	GAGCAGCACACGCGTTATAGTAA	GTGAAGGGACCCGAGCAA
Hes-1	AGCGGGCGCAGATGAC	CGTTCATGCACTCGCTGAA
Hey-1	GTGCGGACGAGAATGGAAA	CTGGCCAAAATCTGGGAAGA
HIF1A	TTCCAGTTACGTTCCTTCGATCA	TTTGAGGACTTGCGCTTTCA
HIF2A	GTGCTCCCACGGCCTGTA	TTG TCACACCTATGGCATATCACA
ID2	TCAGCCTGCATCACCAGAGA	CTGCAAGGACAGGATGCTGAT
ISL-1	AGTGCAGCATCGGCTTCAG	CGGAAACACTCGATGTGATACAC
JAG1	CAACACGGTCCCCATCAAG	TACTTCAGAATTGTGTGTCCTTATTTTAGA
Notch-1	CCGCAGTTGTGCTCCTGAA	ACCTTGGCGGTCTCGTAGCT
NPY	TCCAGCCCAGAGACACTGATT	AGGGTCTTCAAGCCGAGTTCT
Oct4	GAGAACCGAGTGAGAGGCAACC	CATAGTCGCTGCTTGATCGCTTG
SCG10	AAGTCCTTCAGAAGGCTTTGGA	TCCATTTTCAGGATCAGCTTTTC
Synaptophysin	TGGTGTTCGGCTTCCTGAA	GCGGCCCAGCCTGTCT
TH	GCGCAGGAAGCTGATTGC	CAATCTCCTCGGCGGTGTAC
VEGF	AGGAGGAGGGCAGAATCATCA	CTCGATTGGATGGCAGTAGCT
Vimentin	ACACCCTGCAATCTTTCAGACA	GATTCCACTTTGCGTTCAAGGT