The Transcription Factor Runx3 Represses the Neurotrophin Receptor TrkB during Lineage Commitment of Dorsal Root Ganglion Neurons

Ken-ichi Inoue, Kosei Ito, Motomi Osato, Bennett Lee, Suk-Chul Bae, and Yoshiaki Ito

From the Institute of Molecular and Cell Biology, Singapore 138673, the Oncology Research Institute, National University of Singapore, and Bioinformatics Institute, Singapore, and the Department of Biochemistry, School of Medicine, Institute for Tumor Research, Chungbuk National University, Cheongju 361–763, South Korea

Runx3, a Runt domain transcription factor, determines neurotrophin receptor phenotype in dorsal root ganglion (DRG) neurons. Molecular mechanisms by which Runx3 controls distinct neurotrophin receptors are largely unknown. Here, we show that RUNX3 abolished mRNA induction of TRKB expression, and concomitantly altered the neurotrophin response in a differentiating neuroblastoma cell line. In contrast, RUNX3 did not play a significant role in TRKC regulation even under the relevant BMP signaling pathway. We identified putative regulatory elements of Ntrk2/NTRK2 (a gene that codes for TrkB) using an unbiased computational approach. One of these elements was a highly conserved intronic sequence that contains a cluster of Runx binding sites. In a primary culture of DRG neurons, endogenous Runx3 bound to the consensus cluster, which had repressor activity against the Ntrk2 promoter under the control of NT-3 signaling. Consistent with these findings, Runx3-deficient embryos showed an increased number of trkB+ DRG neurons and failed to maintain trkC expression. Taken together, Runx3 determines TrkC positive sensory neuron identities through the transcriptional repression of TrkB when TrkB/TrkC double positive neurons differentiate into TrkC single positive neurons.

Neurotrophins and their specific receptor-tyrosine kinases (the Trk family) exert their diverse functions in the developing nervous system (1–3). Nerve growth factor (NGF) is the preferred ligand for TrkA (Ntrk1), both brain-derived neurotrophic factor (BDNF) and neurotrophin-4 (NT-4) bind to TrkB (Ntrk2) and neurotrophin-3 (NT-3) has a high affinity for TrkC (Ntrk3). Trk receptors are specifically expressed in functionally distinct neurons of the dorsal root ganglion (DRG). TrkA is expressed in both nociceptive and thermoreceptive sensory neurons, and TrkB and TrkC are expressed in a subset of mechano-receptive and proprioceptive neurons, respectively (4). The significance of neurotrophin signaling goes far beyond classical cell survival function of trophic factors. For example, it plays an important role in the control of axonal projections (5–7). It is also a strong determinant of neuronal cell fate specification (8). Therefore, for distinct sensory neurons to acquire appropriate neuronal traits, expression of Ntrk genes must be tightly regulated. Several studies have revealed that Ntrk genes are transcriptionally regulated during embryonic development. In the absence of the Brn-3a POU domain transcription factor, all Ntrk genes are deregulated in the trigeminal ganglion (9). Parada and colleagues (10–13) identified the minimal enhancer of Ntrk1 (TrkA) and its binding transcription factors. However, little is known about the regulatory mechanism of Ntrk2 (TrkB) and Ntrk3 (TrkC), probably because of the large sizes of their genomic loci.

Runt domain transcription factors, Runxs, are known to act as tissue-specific transcription factors. Runx1 and Runx2 control the formation of hematopoietic stem cells and maturation of osteoblasts, respectively (14, 15). They are also important as oncogenes or tumor suppressors in various cancers (16). We generated a Runx3 knock-out mouse and described the causal relationship between RUNX3 and gastric cancer (17). Runx3 knock-out mice also have severe motor discoordination due to the phenotype of proprioceptive DRG neurons (18, 19). Arber and colleagues (20) recently clarified the genetic interaction between Ntrk2 and Runx3. Other groups also described the positive/negative regulation of TrkA by Runx1 (21, 22).

Here we investigated potential roles for Runx3 in the transcriptional regulation of TrkB and TrkC. In cultured neuroblastoma cells, RUNX3 per se abrogated TRKB induction and desensitized cells to trophic factors for survival. In contrast, the contribution of RUNX3 to the regulation of TRKC appeared to be minimal. We identified one of the putative cis-regulatory elements of the gene of TrkB as an element for the mediation of Runx3 repression. These results clarified the molecular mechanism for previously described genetic interaction between Runx3 and Ntrk2 and suggest that lineage-specific transcription factors control neuronal fate by transcriptional repression.
Transcriptional Repression of TrkB by Runx3

EXPERIMENTAL PROCEDURES

Cell Culture—DRGs were dissected from rat embryos at E15.5 (corresponds to mouse E13.5). 0.125% trypsin/verse was added to the ganglions and incubated at 37 °C for 8 min. The cells were then centrifuged at 200 × g and 4 °C for 3 min. Cells were re-suspended in L-15 (10% fetal bovine serum, 400 units of DNase I) and triturated with micropipette until the solution was homogeneous. To enrich for neuronal cells, non-adhering cells were plated on poly-L-ornithine/laminin-coated dishes (BD Biosciences). Primary DRG cells were cultured in high-glucose Dulbecco’s modified Eagle’s medium (Invitrogen), which was supplemented with ITS media supplement (Sigma) and GlutaMax (Invitrogen) in the presence of 10–100 ng/ml NGF (Chemicon), NT-3 (Sigma), or BDNF (Peprotech). The neuroblastoma cell lines TGW (ICR80618, Health Science Research Resources Bank), SK-N-DZ (CRL-2149, ATCC), and LA-N-2 (RCB0484, RIKEN Bioresource Center) were grown in RPMI1640 (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone) and GlutaMax (Invitrogen). SK-N-FI (CRL-2142, ATCC) was grown in minimal essential medium α (Invitrogen). Cells were plated on collagen I-coated dishes (Asahi Techno Glass). Differentiated TGW cells were maintained in similar conditions to those of DRG cells in a serum depletion assay. All-trans-retinoic acid (Sigma) was dissolved in absolute ethanol, stored in −80 °C, and used within 2 weeks. Medium was refreshed for every 2 days during the differentiation assay.

Plasmids—pcDNA3-driven FLAG-tagged human RUNX3 was described elsewhere (23). A point mutation (R178Q) was introduced by site-directed mutagenesis according to the instructions in the QuikChange XL manual (Stratagene). Although this mutation has not been found in human disease, the corresponding point mutation R174Q in the conserved Runt domain was reported for both RUNX1 and RUNX2 in experimental cell population, the results critically depended on transfection efficiency. Random-primed cDNA was synthesized at 37 °C from 1.5 μg of total RNA with Omniscript (Qiagen) following the manufacturer’s protocol. Mx3000P (Stratagene) was used for the real-time PCR analysis. The mRNA level of each gene was normalized using glyceraldehyde-3-phosphate dehydrogenase as a reference. Gene-specific fluorescent probes (Applied Biosystems) were generated from the following genes: human RUNX3 (Hs 00231709_m1), human TRKB (Hs 00178811_m1), human TRKC (Hs 00176797_m1), and human glyceraldehyde-3-phosphate dehydrogenase (Hs 99999905_m1). Flow Cytometry Analysis—Cell death was quantified by flow cytometry (LSRII; BD Biosciences) using the Annexin V PE-detection Kit I (BD Biosciences). Bioinformatics—The Enhancer Element Locator (EEL) program was obtained (from www.cs.helsinki.fi/u/ukpalin/EEL) and installed onto a Personal Computer running Linux. Sequences used for the analysis were obtained from Ensembl (26) and include the following accession numbers, ENSG00000148053 (human NTRK2), ENSG00000140538 (human NTRK3), ENSG00000140538 (dog Ntrk2), ENSCAGF00000011457 (dog Ntrk3), ENSMUSG0000052524 (mouse Ntrk2), ENSMUSG0000059146 (mouse Ntrk3), ENSMODG0000008224 (opossum Ntrk2), and ENSMODG0000019593 (opossum Ntrk3). The entire genomic sequence including the Ntrk2/Ntrk2 or Ntrk3/NTRK3 gene coding regions and covering 100 kb upstream and downstream of each gene region was used for the analysis. The EEL program was run with the following parameters to return the top 500 cis modules: A2.0, μ0.12, ν200, ξ200, and 10.4 nucleotides per rotation. The cutoff used for the binding site detection phase was an absolute score of 8.8, which was chosen to include some of the transcriptional factors of interest. All mammalian transcriptional factor binding sites in the JASPAR data base (mordor.cgb.ki.se/cgi-bin/jaspar2005/jaspar_db.pl) were included in the analysis. In addition to the information from JASPAR, the binding site for Brn-3 (POU-homeodomain) was also included (27). Subsequently, a score cutoff of 200 was applied to the cis modules, and only cis modules having scores greater than or equal to 200 were used for later analysis. A summary of the final EEL results is provided in the supplementary materials.

Chromatin Immunoprecipitation Assay—pcDNA Myc-RUNX3 was transfected to the TGW cell line by electroporation. After 1 day of treatment with retinoic acid, formaldehyde was added to the culture medium (final conc. 1%) for 10 min

BamHI-Sall sites. Point mutations of Runx binding sites were introduced by using Pfu-Ultra (Stratagene).

Transient Transfection by Nucleofector—Gene delivery by electroporation was carried out using Nucleofector (AMAXA). Experimental conditions were optimized for rat primary DRG (program O-03), TGW (T-16), SK-N-DZ (T-16), LA-N-2 (X-05), or SK-N-FI (X-05). The efficiencies of gene delivery were 30–50 and 70–90% for DRG and neuroblastoma cells, respectively.

Quantitative Reverse Transcriptase-PCR—Total RNA was purified from cultured cells using the RNeasy kit (Qiagen). Because cells that did not express transgenes remaining in the experimental cell population, the results critically depended on transfection efficiency. Random-primed cDNA was synthesized at 37 °C from 1.5 μg of total RNA with Omniscript (Qiagen) following the manufacturer’s protocol. Mx3000P (Stratagene) was used for the real-time PCR analysis. The mRNA level of each gene was normalized using glyceraldehyde-3-phosphate dehydrogenase as a reference. Gene-specific fluorescent probes (Applied Biosystems) were generated from the following genes: human RUNX3 (Hs 00231709_m1), human TRKB (Hs 00178811_m1), human TRKC (Hs 00176797_m1), and human glyceraldehyde-3-phosphate dehydrogenase (Hs 99999905_m1).
followed by quenching with 0.125 M glycine for 5 min. For carrier ChIP, dissociated DRGs from E15.5 rat embryos were cultured in the presence of 100 ng/ml NT-3. Sf9 insect cells were mixed together with a smaller amount of DRG cells and harvested for formalin cross-link as above. DNA was sheared by sonication to generate fragments with a mean length of around 500 bp. Immunoprecipitation was performed using the following antibodies: 1 μg of normal rabbit or mouse IgG (Santa Cruz), 2 μg of polyclonal anti-c-Myc (A-14; Santa Cruz), 2 μg of monoclonal anti-RUNX3 (R3–3F12; see supplemental materials Fig. S), or 1 μl of polyclonal anti-RUNX3 (from Dr. Groner, The Weizmann Institute of Science) and the immune complexes including the target chromatin were pulled down with protein A-agarose beads (Upstate). After the washing and elution steps, the protein-chromatin complex was reverse cross-linked by incubating it with sodium chloride at 65 °C for 8 h, and subsequently treating it with RNase A at 37 °C for 1 h and proteinase K at 45 °C for 2 h. DNA was purified using a PCR purification kit (Qiagen) and amplified by PCR. The enrichment of the target sequence was quantified using SYBR Green (Qiagen) for TGW cells. Primer sequences for the detection of the genomic locus are human NTRK2 promoter, 5′-TTCCCCTTCTCTTTAATGACTGC-3′ and 5′-GGCTCGGGGTTCTACTAGGGACAA-3′; human NTRK2 intron 7, 5′-GGGATATCTTGAGGGGATCA-3′ and 5′-CATAGACTCTGCGTTGCCATTAACC-3′; human NTRK2 intron 14-1, 5′-TAACTACCATGCGGAAATG-3′ and 5′-CCAGGCCACGAGTTTACCTATCAG-3′; human NTRK2 intron 14-2, 5′-GGTGTGGACCACTCCGTTTGC-3′ and 5′-GGGTCTCAAGCATTCTATCAGGA-3′; human NTRK3 promoter, 5′-GGGATGTCATGGCATAAATACTCTCTCTGGTTGATGC-3′; human NTRK3 intron 13, 5′-TTCCCCCTAGTTCTACCTGACC-3′ and 5′-CTTAGATCTGCGATTTCCGACAAGC-3′; rat Ntrk2 intron 7, 5′-CCCTCCGAACTACAGAGAGAA-3′ and 5′-CTTGGGCTCCTAAGTACTACGTTG-3′. Optimized PCR conditions and primer concentrations are available on request.

Electrophoretic Mobility Shift Assay (EMSA)—DRGs were dissected from rat embryos (E15.5) and Dounce homogenized. The nuclear extract was purified using the NE-PER (Pierce) for the luciferase kit (Pierce) following the manufacturer's instructions. Briefly, binding reactions on ice were performed for 20 min before adding the antibodies (Runx3; R3–3F12). 4–7 μl of the reaction mixture was electrophoresed and transferred to a Nylon membrane (Hybond-N°, Amersham Biosciences). Biotin-labeled DNA was detected by chemiluminescence. Sequences of the Biotin end-labeled or unlabeled double-stranded DNA are as follows: R1, 5′-AGTCTCATTGACCACTTCC-3′; R2, 5′-CAGTCAAAAAACACAGGATTAAT-3′; R3, 5′-CTTAAATTTGCTTTAGGCTAGG-3′; R4, 5′-TTTACCCCTGTTGTTTGGTTT-3′.

Reporter Assay—Dissociated rat DRG cells from E15.5 embryos were transfected by electroporation with 50 ng of pRL-SV40 (Promega) luciferase, as an internal control, and 5 μg of each of the designated Firefly reporter plasmid containing the sequence of interest. Cells were split into 24-well plates and cultured with or without different neurotrophins. Luciferase activities were measured 24–48 h after transfection. Firefly luciferase activity was normalized with respect to that of hRenilla.

Animals—Animal studies were done in accordance with guidelines set by National Advisory Committee for Laboratory Animal Research (NACLAR). Runx3+/− mice or Sprague-Dawley rats were mated in the evening and the next morning was considered embryonic day 0.5. Pregnant animals were euthanized by carbon dioxide and embryos were removed by Cesarean section. Embryos were fixed in 4% paraformaldehyde/PBS from 4 h to overnight at 4 °C and were rinsed in PBS, cryoprotected in 10–30% sucrose/PBS overnight, and embedded in OCT (Sakura).

In Situ Hybridization—Samples were cryosectioned with a thickness of 16 μm. Slides were rehydrated and postfixed in 4% paraformaldehyde/PBS for 10 min, briefly rinsed twice in PBS, and immersed in 0.1% diethyl pyrocarbonate/PBS at room temperature for 15 min. Slides were dehydrated by passing them through a succession of chambers increasing ethanol concentration, and subsequently incubated with riboprobe in mRNA in situ hybridization buffer (DAKO). To synthesize DIG riboprobes, we used a rat trkC cDNA (1–311) and a rat trkB cDNA (632–1048) (from Dr. Matsumoto, University of Tokyo). Hybridization was carried out for 40 h at 63 °C. After hybridization, slides were washed twice in 60% formamide, 2× SSC, and 0.1% 2-mercaptoethanol for 15 min at 63 °C, and then treated with RNase A (4 μg/ml) in 0.5 M NaCl and 10 mM Tris-HCl (pH 7.4) for 30 min at 37 °C. Slides were washed twice in 60% formamide, 2× SSC, and 0.1% 2-mercaptoethanol for 15 min and twice in 0.1× SSC containing 1% 2-mercaptoethanol for 15 min. Detection procedures were performed with a DIG system according to the manufacturer’s instructions (Roche), except that 10% polyvinyl alcohol was also included in the alkaline phosphatase reaction mixture.

Immunohistochemistry—Slides were prepared as described above, rehydrated in PBS for 5 min, placed in target retrieval solution (pH 6.0, DAKO) and heated for 1 min at 105 °C. A rabbit antibody raised against mouse ER81 (1:8000, from Dr. Jessell, Columbia University) was used and visualized with a Cy3-conjugated anti-rabbit IgG antibody (1:400, Chemicon).

RESULTS
Runx3 Abolishes TRKB Induction during Retinoic Acid-induced Differentiation of a Neuroblastoma Cell Line—Recently, Kramer et al. (20) used a battery of mouse strains to investigate the function of Runx1/Runx3 in DRG neurons. Genetic evidence clearly showed that Runx3 contributes to selective repression of TRKB synthesis within TrkB-positive sensory neurons (20). To investigate this phenomenon further, we first used an accessible in vitro model. Retinoic acid (RA) induces both TRKB expression and differentiation in neuroblastoma cell lines (28, 29). We observed that the neuroblastoma cell line TGW responded to RA and changed morphology 24 h following treatment (Fig. 1A). TGW lacked endogenous RUNX3 and its expression was not increased after differentiation (data not shown). We monitored the relative expression level of TRKB...
Transcriptional Repression of TrkB by Runx3

A. morphological change of a neuroblastoma cell line by RA. Left, neuroblastoma cell line TGW in proliferation. Right, TGW cells 7 days after RA treatment. B, quantitative reverse transcriptase-PCR analysis for TRKB or TRKC in RA-treated TGW cells. Expression plasmids were delivered by electroporation, and all-trans-retinoic acid (10 μM) was administered after cells had attached on the plate. The expression of TRKB/TRKC was monitored in each designated time point (days) after RA treatment. The relative expression level was calibrated by defining proliferating TGW cells as the standard. Note that RUNX3 abrogated the induction of TRKB and slightly enhanced TRKC expression. Data are shown as mean ± S.E. (n = 2, non-repeated measures analysis of variance, p < 0.05; Bonferroni, p < 0.01). C and D, 2 days after RA treatment, serum was depleted from the medium and differentiating cells were further incubated with RA in the presence of 20 ng/ml BDNF, 40 ng/ml NT-3, or 10% serum. Cells were harvested at the indicated time periods and cell death was assessed by annexin V staining. Homogeneous populations with respect to cell size (forward scatter-side scatter (FSC-SSC) window) and enhanced green fluorescent protein-positive cells were gated and analyzed. C, representative data from BDNF culture for 1 day after serum depletion. The percentages of living cells (Annexin V−/7AAD−), apoptotic cells (Annexin V+/7AAD−) and dead cells (Annexin V+/7AAD+) are shown in the fluorescence-activated cell sorter profiles. D, summary of annexin V staining. Representative data are shown. Each experiment was carried out at least three times and displayed a similar trend.

FIGURE 1. A, morphological change of a neuroblastoma cell line by RA. Left, neuroblastoma cell line TGW in proliferation. Right, TGW cells 7 days after RA treatment. B, quantitative reverse transcriptase-PCR analysis for TRKB or TRKC in RA-treated TGW cells. Expression plasmids were delivered by electroporation, and all-trans-retinoic acid (10 μM) was administered after cells had attached on the plate. The expression of TRKB/TRKC was monitored in each designated time point (days) after RA treatment. The relative expression level was calibrated by defining proliferating TGW cells as the standard. Note that RUNX3 abrogated the induction of TRKB and slightly enhanced TRKC expression. Data are shown as mean ± S.E. (n = 2, non-repeated measures analysis of variance, p < 0.05; Bonferroni, p < 0.01). C and D, 2 days after RA treatment, serum was depleted from the medium and differentiating cells were further incubated with RA in the presence of 20 ng/ml BDNF, 40 ng/ml NT-3, or 10% serum. Cells were harvested at the indicated time periods and cell death was assessed by annexin V staining. Homogeneous populations with respect to cell size (forward scatter-side scatter (FSC-SSC) window) and enhanced green fluorescent protein-positive cells were gated and analyzed. C, representative data from BDNF culture for 1 day after serum depletion. The percentages of living cells (Annexin V−/7AAD−), apoptotic cells (Annexin V+/7AAD−) and dead cells (Annexin V+/7AAD+) are shown in the fluorescence-activated cell sorter profiles. D, summary of annexin V staining. Representative data are shown. Each experiment was carried out at least three times and displayed a similar trend.

and TRKC in the presence or absence of RUNX3. In the mock control (green fluorescent protein transfection), TRKB expression was dramatically enhanced by RA, whereas the expression of TRKC did not change (Fig. 1B). However, transient transfection with RUNX3 inhibited the induction of TRKB and slightly enhanced TRKC (Fig. 1B). Taking into account the transfection efficiency (around 70%, see “Experimental Procedures”), TRKB induction was completely inhibited in cells that received the expression vector. Unlike wild-type RUNX3, the point mutant R178Q did not prevent TRKB induction (Fig. 1B). Because R178Q is predicted to disrupt DNA binding activity (see “Experimental Procedures”), the data suggest that TRKB repression is regulated through direct DNA binding by RUNX3. We next confirmed the functional status of neurotrophin receptors in a cell survival assay. The TGW cell line required culture serum for survival because the cells started to die soon after serum depletion (data not shown). Sufficient TrkB or TrkC protein should protect cells from serum-depleted cell death in the presence of their preferred ligands, BDNF or NT-3, respectively. In the mock control (green fluorescent protein was transiently expressed), BDNF supported the survival of RA-treated cells (apoptotic cells; Annexin V+/7AAD− in Fig. 1, C and D), which was consistent with the high level of induced TRKB expression. We observed that NT-3 was also sufficient to protect the mock transfectant from cell death (Fig. 1D), presumably because NT-3 binds to TrkB, albeit with less affinity compared with BDNF (2). In contrast, few of the RUNX3 transfectants survived in the presence of BDNF or NT-3 (Fig. 1, C and D). The point mutation R178Q completely eliminated the effect of wild-type RUNX3 (Fig. 1D), indicating that the massive cell death was not a consequence of excessive protein synthesis following transient expression. Cells transfected with mock plasmid, RUNX3, or R178Q survived in continuous culture with 10% serum (Fig. 1D). Despite the drastic change in neurotrophin receptor status, similar neurite extensions were observed in RUNX3 transfectant (data not shown). Although RUNX3 increased TRKC expression slightly (Fig. 1B), we observed cell death even in the presence of NT-3 (Fig. 1D). This discrepancy may be due to the fact that the induction of TRKC was not high enough to support cell survival in the RUNX3 transfectant (see below). Taken together, RUNX3 alone is sufficient to repress TRKB expression in vitro, whereas the regulatory mechanism of TRKC expression needs further investigation.

RUNX3 Fails to Enhance TRKC under the Control of BMP, a Potential Upstream Signaling Pathway—Because the effect of RUNX3 on TRKC induction was not so prominent, it seemed possible that an upstream signal of RUNX3 might have been missing in the experimental setting of Fig. 1. One possible candidate is the BMP signaling pathway. The RUNX protein is a nuclear target of the transforming growth factor-β superfamily signaling pathways (23, 30). Moreover, BMP signaling induces TrkC in sympathetic neurons (31) and enteric neurons (32), both of which have the same developmental origin as DRG neurons. Therefore, we compared the effect of RUNX3 on TRKC expression with or without BMP signaling. For this purpose, a constitutive active BMP receptor (Alk3-CA) (33) or a dominant negative form (Alk3-KN) (33) was transiently expressed with RUNX3 and the change in TRKC expression was monitored in
neuroblastoma cell lines. To see the immediate early effects by exogenous protein synthesis, we harvested cells 12–16 h after transfection. The basal level of TRKC was varied among the different cell lines; TGW and SK-N-DZ neuroblastoma cells had a lower level of TRKC expression than the other two cell lines, LA-N-2 and SK-N-Fi (Fig. 2A). However, none of these cell lines synthesized a detectable level of RUNX1 or RUNX3 (data not shown). This suggests that transcription regulators other than RUNX determine the basal level of TRKC. Similar to the results of the RA assay, RUNX3 slightly increased TRKC expression in both TGW and SK-N-DZ cells with either Alk3-CA or Alk3-KN BMP receptor (Fig. 2B). This suggests that the function of RUNX3 does not depend on BMP signaling. The other cell lines, LA-N-2 or SK-N-Fi, did not show any significant change in TRKC expression by RUNX3, irrespective of BMP signaling (Fig. 2B). Because the basal level of TRKC was lower in TGW and SK-N-DZ, the slight induction in TRKC expression by RUNX3 was unlikely to be significant. Therefore, we decided to examine the effect of other transcription factors on BMP signaling. The POU-homeodomain factor Brn-3A and Kruppel-like zinc finger Klf7, which are both abundantly expressed in DRG neurons, are known to control Trk receptors (9, 11, 13). Co-expression of Brn-3A and Alk3-KN resulted in variable change of TRKC expression in the four cell lines tested (Fig. 2C), but the combination of KLF7 and Alk3-KA enhanced TRKC expression in all cell lines except for TGW cells (Fig. 2D). Co-expression of KLF7 and Alk3-KN did not affect TRKC expression (Fig. 2D), suggesting that KLF7 activates TRKC under the control of BMP signaling. Taken together, these results indicate that KLF7, but not RUNX3, plays a significant role in TRKC regulation in neuroblastoma cell lines.

Prediction of cis-Regulatory Modules for Ntrk2/NTRK2 and Ntrk3/NTRK3 by the Enhancer Element Locator—To investigate the direct transcriptional regulation of trkB/TRKB expression by Runx3/RUNX3, we used the EEL, which is publicly available. The software uses the entire genomic information available in the Ensemble Genome Browser (26) to predict cis-regulatory modules throughout the genome (34). Based on comparative genomics and clustering algorithms, this allowed us to determine tissue-specific enhancer/silencer that are often positioned at distal regions from the promoter. The scoring scheme of EEL takes into account transcription factor binding site clustering, affinity, and conservation (34). We performed pairwise alignments of human NTRKs (genes that code Trk receptors) to orthologous mouse, dog, opossum, or chicken Ntrks using mammalian transcription factor binding profiles downloaded from the data base (see “Experimental Procedures”) (34). We defined the cut-off value of EEL prediction score as 200, and chose the common loci that represented both human-mouse and human-dog alignments (supplemental materials table). EEL processed a region over 500 kb containing each gene (NTRK2; 555 kb and NTRK3; 580 kb), resulting in the identification of seven Runx consensus sites in the Ntrk2/NTKR2 (TrkB) gene (Fig. 3A and supplemental materials table). Notably, four of seven Runx sites were clustered together in the seventh intron (Fig. 4A and supple-
Transcriptional Repression of TrkB by Runx3

This element contains four Runx binding sites in a highly conserved 400-bp sequence (Fig. 4A). Exactly the same cluster was observed in the human, mouse, rat, dog, and cow Ntrk2 gene, but not in opossum or chicken (data not shown). This conservation among closely related organisms suggests the potential regulatory role for transcription of the TrkB gene (35). Our data suggests that Runx3/RUNX3 controls Ntrk2/NTRK2 through direct binding to distal regulatory sequences.

**Repressor Activity of Highly Conserved Runx Sites in Ntrk2 Intron7**—To further validate the significance of intron 7 of the Ntrk2/NTRK2 gene, we first performed the EMSA using nuclear extracts from dissected DRG cells. All four Runx consensus sequences displayed a shifted band in the presence of nuclear extract (Fig. 4B, lane 2 in all panels). The addition of a Runx3-specific antibody resulted in a supershift of this complex (Fig. 4B, lane 3 in all panels), indicating that endogenous Runx3 protein was bound to the consensus sequences. An excess amount of each competitor inhibited the binding (Fig. 4B, lane 4 in each panel). Next, we confirmed whether endogenous Runx3 resides on Ntrk2/NTRK2 intron 7 using the ChIP assay. Cultured rat DRG neurons were used in the study to enrich for Runx3+ neurons. We used the ChIP technique, which made it possible to perform ChIP assay with much fewer cells than needed conventionally (36). Due to a limited amount of template material, the consensus binding sites of Smad, a downstream target of BMP signaling, was over-represented both in Ntrk2/NTRK2 and Ntrk3/NTRK3 genes (Fig. 3A). Notably, three Kruppel-like zinc finger sites coexisted in the Ntrk3/NTRK3 (TrkC) promoter region together with one Smad site, and the EEL score was the highest for the entire gene locus (Fig. 3A and supplemental materials table). Our data suggests that BMP signaling and/or Klf/KLF proteins potentially regulate the Ntrk3/NTRK3 gene. To validate the Runx sites predicted by EEL, we chose five representative loci and performed the ChIP assay. A neuroblastoma cell line TGW was transfected with the Myc-tagged RUNX3 expression vector and treated by RA. Target sequence enrichment (αMyc/ IgG) was observed in several loci examined (Fig. 3B). In particular, an exceptionally high level of enrichment was observed for NTRK2 intron 7 (Fig. 3B), and the presence of carrier DNA, PCR priming efficiency was not high enough to carry out real time PCR quantification (data not shown). Thus, the relative amount of target sequence was quantified by calculating the frequency of PCR amplification in several different tubes (37). The conserved sequence of Ntrk2 intron 7 was enriched in the precipitated fraction by either different RUNX3-specific antibodies, compared with a normal mouse IgG control (PCR amplification frequency for individual ChIPs: IgG, 1/6, 1/6, 0/3; monoclonal anti-RUNX3, 2/6, 2/6, and 2/3; polyclonal anti-RUNX3, 2/3 and 2/4; Fig. 4, C and D). This indicates that endogenous Runx3 was bound to this region of the Ntrk2 gene in cultured DRG neurons. Finally, we examined the cis-regulation of the Ntrk2 gene by the conserved Runx cluster in vitro. We initially constructed the SV40 promoter-driven luciferase reporter with or without the intron 7 sequence...
depends not only on Runx protein complex, but also on silencer of the "Discussion". These data suggest that intron 7 contains a conserved Ntrk2 promoter activity was estimated by firefly luciferase activity in cultured DRG neurons. Cells were cultured in the presence of the designated neurotrophins. R1–4mut, all four Runx sites were disrupted by site-directed mutagenesis. Relative luciferase activity with 100 ng/ml NGF (right gray bar), BDNF (white), NT-3 (black) or without neurotrophin (dark gray). Triplicate analyses of independent samples were performed and each experiment was carried out at least four times. Data are shown as mean ± S.E. and the statistical tests were carried out among the conditions treated with each neurotrophin (arbitrary units; n = 4, non-repeated measures analysis of variance, * p < 0.01, Bonferroni, † p < 0.01). Note that the Runx site-specific repression effect was observed only in the presence of NT-3.

and estimated the cis-effects in neuroblastoma cell lines. However, a conventional overexpression-based approach failed to recapitulate the repressor activity by RUNX3 (data not shown). To mimic the physiological conditions as much as possible, a primary culture of DRG neurons was used with distinct neurotrophin receptor phenotypes. For the same purpose, we constructed the native Ntrk2 promoter-driven luciferase reporter with or without the intron 7 sequence (Fig. 5A). In the absence of neurotrophin, the promoter activity was below the detection limit (Fig. 5B). In the presence of NT-3, the seventh intronic sequence displayed a repressive effect on the Ntrk2 promoter (Fig. 5B, asterisk). This effect was not observed when all four Runx binding sites were disrupted in intron 7 (R1–4mut, from ACCACA to GTCACA; Fig. 5B), suggesting that endogenous Runx-DNA binding is necessary for the repression. However, neither NGF nor BDNF altered the reporter activity (Fig. 5B). We tested the various culture conditions, but a clear dependence on NT-3 for repression of the Ntrk2 promoter activity was observed, especially at higher doses of each neurotrophin (data not shown, see "Discussion"). These data suggest that intron 7 contains a silencer of the Ntrk2 promoter and that the repressor activity depends not only on Runx protein complex, but also on upstream NT-3 signaling. Taken together, endogenous Runx3 is bound on the conserved seventh intron of Ntrk2 and is likely to function as a silencer of TrkB gene expression.

**Runx3 Blocks trkB Induction in DRG Neurons**—To validate the biological significance of our data, we examined whether proprioceptive neurons display derepression of trkB in Runx3−/− DRG. DRG starts to express trkB around E10.5 and the number of trkB-positive neurons increases through E11.5 (data not shown). In Runx3−/− embryos, the number of trkB-expressing neurons was twice as high as in wild-type embryos at E11.5 and E12.5 (white bar, wild type; black bar, Runx3−/−). Scale bars, 100 μm in A and B; 50 μm in C–F.

**Failure to Maintain trkC Expression and Loss of Proprioceptive Traits in Runx3−/− DRG**—Previously, we and another group described the drastic phenotype of Runx3−/− proprioceptive DRG neurons (18, 19). Although we arrived at essentially the same conclusion as that of the other group, some
discrepancy exists in the observation of the TrkC phenotype in Runx3−/− DRG. Because NT-3-TrkC signaling is essential for appropriate axonal projection (6), we re-estimated the expression of trkC at different developmental stages of wild-type and Runx3−/− DRG. In wild-type embryos, most DRG neurons start to express trkC immediately after migration from the neural crest (38, 39) (Fig. 7, A and C). At embryonic day 11.5 (E11.5), we observed no difference in trkC expression between wild-type and Runx3−/− DRG (Fig. 7, A–D). Thus, Runx3 was not required for the de novo expression of trkC. At a later embryonic stage, the percentage of the wild-type DRG population that was trkC positive declined (Fig. 7, E and G) (38–40). In clear contrast to wild-type DRG (Fig. 7, E and G), few neurons in Runx3−/− DRG maintained trkC expression at E12.5 (Fig. 7, F and H). Failure to maintain trkC expression continued through to E13.5 (data not shown), but the expression started to recover from E15.5 (Fig. 7), albeit in much fewer neurons than in wild-type DRG. To confirm that NT-3-TrkC signaling is abrogated in the mutant DRG, we investigated the protein synthesis of its critical downstream target, ER81 (6, 41). At E13.5, we observed that the levels of ER81 protein in Runx3−/− and wild-type DRG were similar (Fig. 7, K and L). This suggests that in the absence of Runx3, proprioceptive neurons survive and develop normally at this stage. However, at E15.5, ER81 was no longer synthesized in Runx3−/− DRG neurons (Fig. 7, M and N). The behavior of ER81 lagged behind 2–3 days that of trkC expres-
Transcriptional Repression of TrkB by Runx3

represses the native Ntrk2 promoter (Fig. 5). It is an exciting possibility, therefore, that the Runx cluster acts as an essential silencer element during the lineage commitment of DRG neurons. Interestingly, repression of reporter activity was observed only when neurons were cultured in media containing NT-3, but not in media containing NGF or BDNF (Fig. 5). This observation was not trivial to interpret because in dissociation culture conditions, the expression of distinct neurotrophin receptors is not clearly segregated (45), and less-preferred ligand-receptor combinations could affect the overall results. Nevertheless, our data suggest the interesting possibility that the signaling cascade NT-3-TrkC-Runx3 intron 7 effectively inhibits TrkB expression to develop TrkC single positive neurons (Fig. 7). Overall, the approach used in this study (computational prediction, EMSA/ChIP validation, reporter analysis) narrowed down the potential cis-element candidates within the large gene locus.

In contrast to the unequivocal effect of Runx3 on TrkB repression, elucidating the regulatory role, if any, of Runx3 in TrkC expression is more complex. In Runx3−/− DRG, we showed that Runx3 was not required for the initial induction of trkC (Fig. 7). However, without Runx3 trkC is not maintained in prospective TrkC positive neurons (Fig. 7). Because the NT-3-TrkC signaling is crucial for the proper maturation of proprioceptive neurons (6), the loss of TrkC signaling is likely to be the basis of the drastic phenotype observed in Runx3−/− DRG (18, 19). However, our data suggest that RUNX3 has little contribution to direct TRKC gene activation (Fig. 2). We deduce that the loss of trkC in Runx3−/− DRG could be a secondary consequence of trkB derepression. In DRG neurons, ectopic synthesis of TrkC receptor in TrkA−/− neurons resulted in repression of the TrkA promoter and mis-expression of endogenous TrkC (8). So far, a similar study has not been carried out between TrkB and TrkC, but in the NT-4 knock-out DRG, the number of TrkC−/− neurons was increased at the expense of TrkB−/− neurons (46), suggesting that NT-4-TrkB signaling represses trkC expression. Thus, the activation of NT-4-TrkB signaling in Runx3−/− DRG neurons is likely to be the reason for trkB loss in prospective TRkC positive neurons (Fig. 7). Whereas further investigation is needed to confirm the speculation, the strategy “to repress alternate traits” appears to be common in neuronal lineage commitment (47). Particularly in DRG neurons, “insulation” against alternate Trk signaling is a crucial problem. Distinct neurotrophins guide growth cones to appropriate targets (48). To insulate each sensory population from irrelevant neurotrophins, each Trk receptor might be under mutual regulation (Fig. 70). The Runx protein cooperates with several repressor proteins, including the Groucho corepressor (reviewed in Ref. 49). However, a recent study indicated that the VWWRPY motif of Runx3, which is critical for binding to Groucho, is dispensable for the normal function of DRG neurons (50).

There are interesting parallels between Runx3 function in DRG neurons and the immune system. In T cell development, targeted excision of a CD4 silencer abrogates lineage commitment of CD4CD8 double positive cells into CD8 single positive cells (51). The CD4 silencer contains two evolutionally conserved Runx binding sites and Runx3 is responsible for CD4 silencing to generate CD8+ cytotoxic T cells (52). Runx3−/− mice display derepression of trkB and concomitant loss of pro-pripioceptive neuronal traits in the DRG. Runx3, as a transcriptional repressor, appears to control lineage commitment both in the immune system and in sensory neurons.

Finally, we would like to point out the relevance of our observations in carcinogenesis. RUNX3 has been shown to be a tumor suppressor of gastric and many other cancers (53). On the other hand, TrkB is considered to be an oncogene in several tumors including neuroblastoma (54–58). Moreover, TrkB was recently identified as a “suppressor of anoikis” in an unbiased genetic screening (59). The strong link between Runx3 and TrkB observed in DRG neurons, therefore, implies that one of the tumor suppressor activities of RUNX3 could be to repress the expression of the TrkB oncogene.

Acknowledgments—We thank Dr. T. M. Jessell for the gift of the ER81 antibody, Dr. I. Matsumoto and K. Abe for the trkB and trkC plasmids, Dr. E. J. Huang for the expression vector of Brn-3a, Dr. K. Miyazono for the expression vectors of ALK3-CA or ALK3-KN, Dr. Y. Groner for the polyclonal RUNX3 antibody, M. Inoue for optimization of qChIP assay, Drs. S. Ozaki, S. Shiga, and T. Yokomizo for critical reading of the manuscript, E. Tai and J. N. Goh for technical assistance, and the staffs at the Biological Resource Center, Biopolis, for the animal husbandry.

REFERENCES

1. Bibel, M., and Barde, Y. A. (2000) Genes Dev. 14, 2919–2937
2. Huang, E. I., and Reichardt, L. F. (2001) Annu. Rev. Neurosci. 24, 677–736
3. Segal, R. A. (2003) Annu. Rev. Neurosci. 26, 299–330
4. Snider, W. D. (1994) Cell 77, 627–638
5. Patel, T. D., Jackman, A., Rice, F. L., Kucera, J., and Snider, W. D. (2000) Neuron 25, 345–357
6. Patel, T. D., Kramer, I., Kucera, J., Niederkoller, V., Jessell, T. M., Arber, S., and Snider, W. D. (2003) Neuron 38, 403–416
7. Markus, A., Patel, T. D., and Snider, W. D. (2002) Curr. Opin. Neurobiol. 12, 523–531
8. Moqrich, A., Earley, T. J., Watson, J., Andahazy, M., Backus, C., Martinez-Zanca, D., Wright, D. E., Reichardt, L. F., and Patapoutian, A. (2004) Nat. Neurosci. 7, 812–818
9. Huang, E. I., Zang, K., Schmidt, A., Saulys, A., Xiang, M., and Reichardt, L. F. (1999) Development 126, 2869–2882
10. Ma, L., Merenemies, I., and Parada, L. F. (2000) Development 127, 3777–3788
11. Lei, L., Ma, L., Nef, S., Thai, T., and Parada, L. F. (2001) Development 128, 1147–1158
12. Ma, L., Lei, L., Eng, S. R., Turner, E., and Parada, L. F. (2003) Development 130, 3525–3534
13. Lei, L., Laub, F., Lush, M., Romero, M., Zhou, J., Luikart, B., Klesse, L., Ramirez, F., and Parada, L. F. (2005) Genes Dev. 19, 1354–1364
14. Okuda, T., van Deursen, J., Hibbert, S. W., Grosveld, G., and Downing, J. (1996) Cell 84, 321–330
15. Komori, T., Yagi, H., Nomura, S., Yamaguchi, A., Sasaki, K., Deguchi, K., Shimizu, Y., Bronson, R. T., Gao, Y. H., Inada, M., Sato, M., Okamoto, R., Kitamura, Y., Yoshiki, S., and Kishimoto, T. (1997) Cell 89, 755–764
16. Blyth, K., Cameron, E. R., and Neil, J. C. (2005) Nat. Rev. Cancer 5, 376–387
17. Li, Q. L., Ito, K., Sakakura, C., Fukamachi, H., Inoue, K., Chi, X. Z., Lee, K. Y., Nomura, S., Lee, C. W., Han, S. B., Kim, H. M., Kim, W. J., Yamamoto, H., Yamashita, N., Yano, T., Ikeda, T., Itohara, S., Inazawa, J., Abe, T., Hagiwara, A., Yamagishi, H., Ooe, A., Kaneda, A., Sugimura, T., Ushijima, T., Bae, S. C., and Ito, Y. (2002) Cell 109, 113–124
18. Inoue, K., Ozaki, S., Shiga, T., Ito, K., Masuda, T., Okado, N., Iseda, T., Kawaguchi, S., Ogawa, M., Bae, S. C., Yamashita, N., Itohara, S., Kudo, N.,
Transcriptional Repression of TrkB by Runx3

and Ito, Y. (2002) Nat. Neurosci. 5, 946–954
19. Levanon, D., Bettouin, D., Harris-Cerruti, C., Woolf, E., Negreanu, V., Elaim, R., Bernstein, Y., Goldbergden, D., Xiao, C., Fliegau, M., Kremer, E., Otto, F., Brenner, O., Lev-Tov, A., and Groner, Y. (2002) EMBO J. 21, 3454–3463
20. Kramer, I., Sigrist, M., de Nooij, J. C., Tanuchii, I., Jessell, T. M., and Arber, S. (2006) Neuron 49, 379–393
21. Marmigere, F., Montelius, A., Wegner, M., Groner, Y., Reichardt, L. F., and Enners, P. (2006) Nat. Neurosci. 9, 180–187
22. Chen, C. L., Broom, D. C., Liu, Y., de Nooij, J. C., Li, Z., Cen, S., Samad, O. A., Jessell, T. M., Woolf, C. J., and Ma, Q. (2006) Neuron 49, 365–377
23. Hanai, J., Chen, F. K., Kanno, T., Ohtani-Fujita, N., Kim, W. Y., Gwo, W. H., Imamura, T., Ishidou, Y., Fukuhi, M., Shi, M. J., Stavnezer, J., Kawabata, M., Miyazono, K., and Ito, Y. (1999) J. Biol. Chem. 274, 31577–31582
24. Michaud, I., Wu, F., Osato, M., Callot, G. M., Yanagida, M., Asou, N., Shigesada, K., Ito, Y., Benso, K. F., Raskind, W. H., Rossier, C., Antonarakis, S. E., Israels, S., McNicol, A., Weiss, H., Horwitz, M., and Scott, H. S. (2002) Blood 99, 1364–1372
25. Yoshida, T., Kanegane, H., Osato, M., Yanagida, M., Miyawaki, T., Ito, Y., and Shigesada, K. (2002) Am. J. Hum. Genet. 71, 724–738
26. Birney, E., Andrews, T. D., Bevan, P., Cacccamo, M., Chen, Y., Clarke, L., Coates, G., Cuff, J., Curwen, V., Cutts, T., Down, T., Eyras, E., Fernandez-Suarez, X. M., Gane, P., Gibbins, B., Gilbert, J., Hammond, M., Hotz, H. R., Iyer, V., Jekosch, K., Kahari, A., Kasprzyk, A., Keefe, D., Keenan, S., Lehvaslaiho, H., McCVicker, H., Melsopp, C., Meidll, P., Morgen, E., Pettett, R., Potter, S., Proctor, G., Rae, M., Searle, S., Slater, G., Smedley, D., Smith, J., Spooner, W., Stabenu, A., Stalker, J., Storey, R., Uret-Vidal, A., Woodward, K. C., Cameron, G., Durbin, R., Cox, A., Hubbard, T., and Clamp, M. (2004) Genome Res. 14, 925–928
27. Gruber, C. A., Bree, J. M., Gliberbern, A., and Turner, E. E. (1997) Mol. Cell. Biol. 17, 2391–2400
28. Kaplan, D. R., Matsumoto, K., Lucarelli, E., and Thiele, C. J. (1993) Neuron 11, 321–331
29. Lucarelli, E., Kaplan, D. R., and Thiele, C. J. (1995) J. Biol. Chem. 270, 24725–24731
30. Ito, Y., and Miyazono, K. (2003) Curr. Opin. Genet. Dev. 13, 43–47
31. Zhang, D., Mehler, M. F., Song, Q., and Kessler, J. A. (1998) J. Neurosci. 18, 3314–3326
32. Chalazonitis, A., D’Autreppeaux, F., Guha, U., Pham, T. D., Faure, C., Chen, J. J., Roman, D., Kan, L., Rothman, T. P., Kessler, J. A., and Gershon, M. D. (2004) J. Neurosci. 24, 4266–4282
33. Brderlau, A., Faigle, R., Elmi, M., Zerebski, A., Sjoberg, S., Fuji, M., Miyazono, K., and Funa, K. (2004) Mol. Biol. Cell 15, 3863–3875
34. Hallikas, O., Palin, K., Sinjihunna, N., Rautiainen, R., Partanen, J., Ukkonen, E., and Taiapile, J. (2006) Cell 124, 47–59
35. Prabhakar, S., Poulin, F., Shoukry, M., Afzal, V., Rubin, E. M., Couronne, O., and Pennacchio, L. A. (2006) Genome Res. 16, 855–863
36. O’Neill, L. P., VerMilyea, M. D., and Turner, B. M. (2006) Nat. Genet. 38, 835–841
37. Rhoades, K. L., Singh, N., Simon, L., Glidden, B., Cedar, H., and Chess, A. (2000) Curr. Biol. 10, 789–792
38. Farinas, I., Wilkinson, G. A., Backus, C., Reichardt, L. F., and Patapoutian, A. (1998) Neuron 21, 325–334
39. Rifkin, J. T., Todd, V. J., Anderson, L. W., and Lefcort, F. (2000) Dev. Biol. 227, 465–480
40. White, F. A., Silos-Santiago, I., Moller, D. C., Nishimura, M., Phillips, H., Barbaric, M., and Snider, W. D. (1996) J. Neurosci. 16, 4662–4672
41. Lin, J., Saito, T., Anderson, D. J., Lance-Jones, C., Jessell, T. M., and Arber, S. (1998) Cell 95, 393–407
42. Farinas, I., Yoshida, C. K., Backus, C., and Reichardt, L. F. (1996) Neuron 17, 1065–1078
43. Elshamy, W. M., and Ernfors, P. (1996) Neuron 16, 963–972
44. Elshamy, W. M., Fridvall, L. K., and Ernfors, P. (1998) Neuron 21, 1003–1015
45. Friedel, R. H., Schnurh, H., Stubbusch, J., and Barde, Y. A. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 12670–12675
46. Liebl, D. J., Klesse, L. J., Tessarollo, L., Wohlman, T., and Parada, L. F. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 2297–2302
47. Muhr, J., Andersson, E., Persson, M., Jessell, T. M., and Ericson, J. (2001) Cell 104, 861–873
48. O’Connor, R., and Tressier-Lavigne, M. (1999) Neuron 24, 165–178
49. Durst, K. L., and Hiebert, S. W. (2004) Oncogene 23, 4220–4224
50. Yarmus, M., Woolf, E., Bernstein, Y., Fainaru, O., Negreanu, V., Levanon, D., and Groner, Y. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 7384–7389
51. Zou, Y. R., Sunshine, M. J., Taniuchi, I., Hatam, F., Killeen, N., and Littman, D. R. (2001) Nat. Genet. 29, 332–336
52. Taniuchi, I., Osato, M., Egawa, T., Sunshine, M. J., Bae, S. C., Komori, T., Ito, Y., and Littman, D. R. (2002) Cell 111, 621–633
53. Bae, S. C., and Choi, J. K. (2004) Oncogene 23, 4336–4340
54. Nakagawara, A., Azar, C. G., Scavarda, N. J., and Brodeur, G. M. (1994) Mol. Cell. Biol. 14, 759–767
55. Sato, H., Mimata, H., Nomura, Y., Ito, Y., and Nomura, Y. (2001) Int. J. Urol. 8, S28–S34
56. Eggert, A., Grotzer, M. A., Ikekagi, N., Zhao, H., Cnaan, A., Brodeur, G. M., and Evans, A. E. (2001) J. Clin. Oncol. 19, 689–696
57. Schabas, G. M., Fujikoa, S., Schmidt, C., Li, Z., Frederick, W. A., Yang, W., Yokoi, K., Evans, D. B., Abbruzzese, J. J., Hess, K. R., Zhang, W., Fidler, I. J., and Chiao, P. J. (2005) Clin. Cancer Res. 11, 440–449
58. Pearse, G. R., Swendsen, S. L., Li, Y., Rafii, D., and Hemphstead, B. L. (2005) Blood 105, 4429–4436
59. Douma, S., Van Laar, T., Zevenhoven, J., Meuwissen, R., Van Garderen, E., and Peper, D. S. (2004) Nature 430, 1034–1039