RNA extraction and reverse transcriptase–polymerase chain reaction (RT-PCR)
Total RNA was extracted from primary tumor samples with Trizol (Invitrogen, Carlsbad, CA92008) and Qiagen RNEasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Generally, 2 µg of total RNA were reverse transcribed into cDNA with PowerScript Moloney Murine Leukemia Virus Reverse Transcriptase (Becton, Dickson, Heidelberg, Germany). Nested-PCR was performed in a final volume of 50 µL containing 1 unit of Taq gold polymerase, 200 µmol/L dNTP, and 0.4 µmol/L of each primer in a buffer consisting of 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3), 2 mmol/L $MgCl_2$. Primers were designed to obtain whole sequences from the 2nd Ig like domain to the kinase domain. Primers for TRKA were #112 (5′-CAACGCTCTGGAGTCTCTC-3′) and #182 (5′-GGCGAATTAGATACTTCGAGATC-3′) for the first round, #134 (5′- GTGTTCACCACATCAAGCGCC-3′) and #135 (5′-CTAGCCCAGGACATCCAGGTAGAC-3′), #130 (5′-TCCCGGCCAGTGTGCAGCTG-3′) and #133 (5′-CTTGACAGCCACCAGCATCTTGTC-3′) for the second round of PCR. Primers for TRKB were #152 (5′-CTAGCCTAGAATGTCCAGGTAGACC- 3′) and #153 (5′-CTGTCAACCTCACTGTGCATTTTGCAC-3′) for the first round, #151 (5′-TTGTTCAGCACATCAAGCGACATAAC-3′) and #170 (5′-CAAGTTCTGAAGGAGGGTATGGATGCC-3′), #171 (5′-GAATCTCCAACCTCAGACCACCACTG- 3′) and #205 (5′-CATCCTTCAGGGTCTTCACTGCC-3′) for the second round of PCR. Primers for TRKC were #149 (5′-AGTGTTGCCCTCACTGTCTACTATCC-3′) and #165 (5′-ATTCATGACCACCAGCCA-3′) for the first round, #147 (5′-ATGTGCAGCACATTAAGAGGAGAG- 3′) and #148 (5′-CTAGCCAAGAATGTCCAGGTAGATTG-3′), #169 (5′-TGAGCCTGGAGGAGCCTGAGCTG-3′) and #150 (5′-CTTCACAGCCACAAGCATCTTGTCC-3′) for the second round. Primers for detection of fusion transcript TPM3/TRK were #172 (5′- ATGGCTGGGATCACCACCATCGAGG-3′) and #135 (5′-CTAGCCCAGGACATCCAGGTAGAC-3′) for the first round, #136 (5′-CTGAAGTGTCTGAGTGCTG-3′) and #133 for the second round of PCR. PCR fragments were directly sequenced on a capillary sequencer (Beckman Coulter, Krefeld, Germany).

Cytogenetic and molecular genetic analysis
Pretreatment samples from all patients were studied centrally by G-banding and fluorescence Rbanding analysis and fluorescence *in situ* hybridization (FISH). Cytogenetic studies were performed using standard techniques, and chromosomal abnormalities were described according to the International System for Human Cytogenetic Nomenclature. All specimens were also analyzed by FISH using a comprehensive DNA probe set allowing detection of the most relevant AMLassociated genomic aberrations. In addition, diagnostic samples from patients with AML were analyzed for mutations in FLT3-ITD.

Retroviral transductions, *in vivo* tumorigenesis assays, and tumor phenotyping
Murine hematopoietic cells were retrovirally transduced with ecotropic supernatants, using conditions that led to efficient gene marking with low provirus copies (<3) per cell.[1] Polyclonal cultures of retrovirally engineered 32D cells (a non-leukemogenic myeloid, interleukin-3 (IL-3) dependent murine cell line) were used for *in vivo* tumorigenesis experiments. 10^7 cells per recipient were inoculated by intravenous injection into sublethally irradiated (2.5 Gy) C3H/Hej mice.[2] Hematopoietic stem/progenitor cells (HSC/HPC) enriched lineage negative (Lin^-) bone

marrow (BM) cells were isolated from C57Bl/6J.Ly5.2 mice and transduced as previously described.[1] Genemodified cells were transplanted by tail vein injection into lethally irradiated syngeneic recipients (aged 8–16 weeks). All animals were obtained from and kept in the animal laboratories of Hannover Medical School. Animal experiments were approved by the local ethical committee and performed according to their guidelines. Diagnoses of nonlymphoid hematopoietic neoplasms were made according to the Bethesda proposals.[2,3] Mice that did not develop leukemia were analyzed after a maximal observation period of up to 37 weeks according to the same protocol. Moribund mice were humanely killed for necropsy, or analyzed when found dead before onset of autolysis. Mice were macroscopically examined for pathological abnormalities during dissection. Enlarged organs were weighed. BM, spleen, liver, kidney, lung, and thymus were fixed in a buffered 4% formalin solution and embedded in Paraplast plus (Kendall, Mansfield, MA, USA). Sections (2 μm or 2.5 μm thick) were routinely stained with hematoxylin and eosin. For histologic examinations a light microscope (Axioplan2, Zeiss, Oberkochen, Germany) with 10×, 20×, 63×, and 100× Plan-NEOFLUAR objective lenses was used. The MRGrab (Version 1.0, Zeiss, Oberkochen, Germany) with a digital camera (AxioCam MRc) was utilized for image processing. Blood cell counts were determined by microscopic counting or by using an automatic analyzer (ABC Counter, Scil, Viernheim, Germany). Cells from infiltrated organs and peripheral blood were subjected to flow cytometry. Leukocyte morphology was also evaluated in Pappenheim stained blood smears and cytospins of BM, spleen, thymus, or liver cells. After red blood cell lysis, cells were stained with lineage-specific antibodies against Gr1, CD11b (myeloid cells), CD19 (B-cells), Ter119 (erythroid cells), CD3, CD4, and CD8 (T-cells) (Pharmingen, Hamburg, Germany). Antibodies were usually directly linked to PE or APC fluorochromes. Dead cells were excluded by propidium iodide staining.

BDNF-Elisa

For the quantification of BDNF in cell culture supernatants the BDNF E_{max} ImmunoAsssay System (Promega) was used.[4] The supernatants were passed through a 0.45 μM filter, stored at −80°C or directly analysed following the manufacturer's instructions. Samples were measured in triplicate with and without acid treatment.

Small interfering RNA (siRNA)-mediated knockdown

Two different TRKB shRNA constructs targeted 2 different regions on the human TRKB cDNA were tested. For shRNA67-8, an effective down regulation of TRKB in mice was already shown.[5] shRNAs used in that approach were mutated to fit the human TRKB sequence exactly. The sequences for the different shRNAs were the following: shRNA67-8: 5′-GAT CCCCTTGTGGATTCT GGATTAAACTCGAGTTTAATCCAGAATCCACAATTTTTA-3′; control shRNA67-11: 5′-GATCCC CGATTCGATTATGGTTAGATCTCGAGATCTAACCATAATCGAATCTTTTTA-3′; shRNA67-10: 5′-GATCCCCGATTTCTCATGGTTTGGATCTCGAGATCCAAACCATGAGAAATCTTTTTA-3′; control shRNA67-13: 5′-GATCCCCGCGTTTCTGGATAGTATTTCTCGAGAAATACTATCCAGAAACGC TTTTTA-3′. Double stranded shRNA-oligos were subcloned into the pSuper Plasmid (OligoEngine) via HindIII/BglII and from there a HincII/SmaI fragment containing the H1-Promotor and the shRNAoligo were transferred to ΔU3 region of a lentiviral vector expressing DsRed under the control of the spleen focus forming virus promoter (SFFV). Vectors were pseudotyped with the

envelope glycoprotein of vesicular stomatitis virus. #681 cells co-expressing human TRKB and BDNF were transduced with an MOI of up to 100 by spin inoculation (transduction efficiency >60%). During and after transduction #681 cells were cultivated in the presence of mIL-3 to avoid potential loss of cells with TRKB knocked-down. To analyze clonal growth, transduced cells were plated in M3234 media (StemCell Technologies, Vancouver, Canada) with or without supplementation of IL-3. The assays were plated as duplicates, and colonies were counted on day 6. Colony plating efficiency was iv calculated as the colony numbers without IL-3 over the numbers in the presence of IL-3. To check the effect of knocking-down TRKB in leukemic cells in liquid culture, 6 days after transduction #681 cells were also cultured at cell density of 10^5/ml in the presence or absence of IL-3. Cell viability was checked in different time points.

Statistical Analysis

Length of follow-up was the time from entry into the prospective study to date of death (failure) or alive at last contact (censored). The endpoints of interest were overall survival (OS) or in the case of event free survival (EFS), measured from the date of documented CR, relapse (failure) or death in CR (failure). OS and EFS data were determined by G-rho family of test (log-rank) and an estimate of a survival curve for censored data was visualized using Kaplan-Meier curves. To correlate OS with expression of TRKs and evaluate the power of potential clinical predictors, univariate and multivariate analysis using Cox proportional hazard models were computed. To provide quantitative information on the relevance of results, 95% confidence intervals of hazard ratios were calculated. Comparisons between pairwise datasets were performed by two sample Student's t-Test for continuous variables and by Pearson's chi-squared contingency table and goodness-of-fit tests in terms of categorical variables. All computations were performed with the R statistical software using the Graphics, Splines, Stats, and Survival libraries. A p-value <0.05 was considered significant.

REFERENCES

1. Li Z, Schwieger M, Lange C, et al. Predictable and efficient retroviral gene transfer into murine bone marrow repopulating cells using a defined vector dose. *Exp Hematol*. 2003;31:1206-1214.
2. Meyer J, Rhein M, Schiedlmeier B, et al. Remarkable leukemogenic potency and quality of a constitutively active neurotrophin receptor, deltaTrkA. *Leukemia*. 2007;21:2171-2180.
3. Kogan SC, Ward JM, Anver MR, et al. Bethesda proposals for classification of nonlymphoid hematopoietic neoplasms in mice. *Blood*. 2002;100:238-245.
4. Pearse RN, Swendeman SL, Li Y, Rafii D, Hempstead BL. A neurotrophin axis in myeloma: TrkB and BDNF promote tumor-cell survival. *Blood*. 2005;105:4429- 4436.
5. Bartkowska K, Paquin A, Gauthier AS, Kaplan DR, Miller FD. Trk signaling regulates neural precursor cell proliferation and differentiation during cortical development. *Development*. 2007;134:4369-4380.
6. Duchemin AM, Ren Q, Mo L, Neff NH, Hadjiconstantinou M. GM1 ganglioside induces phosphorylation and activation of Trk and Erk in brain. *J Neurochem*. 2002;81:696-707.