A Novel Role for Extracellular Signal-Regulated Kinase 5 and Myocyte Enhancer Factor 2 in Medulloblastoma Cell Death

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Abstract

Expression of the neurotrophin-3 receptor, tyrosine kinase C (TrkC), is associated with favorable prognosis in medulloblastoma patients. This may be due to increased tumor apoptosis induced by TrkC activation. Neurotrophin-3/TrkC–induced apoptosis is inhibited by the mitogen-activated protein (MAP) kinase (MAPK) pharmacologic antagonists SB203580 and PD98059. In addition to extracellular signal-regulated kinase (ERK)-1/2, PD98059 also inhibits the more recently identified neurotrophin-responsive MAPK, ERK5 (big MAPK 1). In the present study, we investigate the contribution of ERK5 and its target myocyte enhancer factor 2 (MEF2) to neurotrophin-3/TrkC–induced medulloblastoma cell death. Neurotrophin-3 not only enhanced ERK5 phosphorylation but also significantly enhanced the transcriptional activity of MEF2, a specific target of ERK5. Overexpression of both ERK5 and MEF2 induced a statistically significant increase in cell death of neurotrophin-3–responsive and nonresponsive medulloblastoma cell lines (Daoy-trkC and Daoy) and primary cultures of patched heterozygous mouse medulloblastomas. Only those cells expressing MAP/ERK kinase 5 (MEK5) plus ERK5 or MEF2 constructs underwent apoptosis, indicating that overexpression of either is sufficient to induce medulloblastoma cell death. Expression of a dominant-negative MEF2 or small interfering RNA for the ERK5 activator, MEK5, significantly inhibited neurotrophin-3–induced cell death. The dominant-negative MEF2 construct also blocked MEK5/ERK5–induced cell death, supporting a role for MEF2 downstream of ERK5. Coimmunoprecipitation studies revealed direct interaction of phosphorylated ERK5 with MEF2 in response to neurotrophin-3. Our investigation of the mechanism of neurotrophin-3/TrkC–induced apoptosis has identified a novel role for both MEK5/ERK5 and MEF2 in cell death, suggesting that these molecules can be exploited to induce apoptosis in both TrkC-expressing and nonexpressing medulloblastoma cells. (Cancer Res 2005; 65(13): 5683-9)

Introduction

Elevated expression of the neurotrophin-3 receptor, tyrosine kinase C (TrkC), by medulloblastoma is associated with a favorable clinical outcome (1, 2). It is thought that this may in part be due to increased levels of apoptosis within the tumor, induced by activation of the TrkC receptor (3, 4). We showed in earlier work that activation of TrkC by neurotrophin-3 induces apoptosis in primary cultures of medulloblastoma and a TrkC-expressing medulloblastoma cell line. Although activation of multiple signaling pathways was shown, apoptosis was blocked by treatment with the p38 mitogen-activated protein (MAP) kinase (MAPK) inhibitor, SB203580, and the classic extracellular signal-regulated kinase (ERK)/MAPK inhibitor, PD98059.

Our initial findings suggested a key role for ERK1 and 2 (3), and since these studies were published it has been discovered that neurotrophins also activate ERK5. Some of the functions previously attributed to ERK1 and 2 may in fact be mediated by this more recently discovered MAPK (5, 6). Like the classic MAPKs, ERK1 and 2, it is inhibited by PD98059, putting it among the candidate molecules involved in neurotrophin-3 signaling. ERK5 is expressed developmentally and has been shown to selectively mediate neurotrophin-mediated survival of developing but not mature neurons (5, 7). We therefore tested ERK5 as a potential mediator of neurotrophin-3–induced cell death and looked at its downstream targets. Our previous work showed neurotrophin-3–induced apoptosis to occur over 48 to 96 hours, and to be inhibited by the general translation inhibitor cyclohexamide (3). These data are consistent with a mechanism dependent on new gene transcription and translation. A common nuclear target of both p38 and ERK5 is the myocyte enhancer factor 2 (MEF2) family of MADS-box transcription factors. ERK5 is a potent enhancer of MEF2 transcriptional activity (8–10).

All four members of the MEF2 family are expressed during embryogenesis, but MEF2A and MEF2D have been shown to play a specific role in cerebellar development (11). Moreover, both MEF2A and MEF2D have been shown to promote differentiation and survival of cerebellar granule cells (8, 12, 13), the cells from which medulloblastomas are most often derived (14).

Our results show a unique role for ERK5 and MEF2 in neurotrophin-induced medulloblastoma cell death. To date, both ERK5 and MEF2 have been associated with neuronal cell survival following activation of neurotrophin receptors. ERK5, in particular, is emerging as a prosurvival molecule in multiple tumor types (15–17). In defining a role for ERK5 in tumor cell death, our results illustrate that biological effects of signaling molecules are context dependent.

Materials and Methods

Cell culture and transfection. The medulloblastoma cell line Daoy was obtained from the American Tissue Culture Collection (Manassas, VA) and maintained as previously described (3). The Daoy-trkC cell line, Daoy cells stably transfected with full-length TrkC, was produced and maintained as previously described (3). Medulloblastoma cell lines D458 and D384 were donated by Dr. Darrell Bigner. Mouse primary tumor cultures were obtained from patched heterozygous mice (18). Human primary medulloblastoma cultures were obtained from
tumors taken from patients at diagnosis and placed in ice-cold Hanks (Invitrogen GIBCO, Carlsbad, CA) at time of harvest. Tumors were finely minced and grown as suspension cultures in neurobasal media supplemented with 0.1 mmol/L nonessential amino acids, 1 mmol/L sodium pyruvate, N2 supplement (1:100 Invitrogen GIBCO), and 10% FCS. Cells were seeded into Primaria 24-well plates (Falcon, BD Biosciences, Palo Alto, CA) and transfected following a 24-hour recovery period.

For transfection, Daoy and Daoy-trkC cells were seeded on poly-L-lysine–coated coverslips (2). Cells were transfected with DNA/Clonfectin (Clontech, BD Biosciences, Palo Alto, CA) at a ratio of 1:2, with a total of 1 μg DNA per well. Mouse primary cultures were transfected using FuGENE 6 (according to the supplied protocol, Roche Applied Science, Indianapolis, IN). For assays involving ERK5 and MAPK/ERK kinase 5 (MEK5), constructs were used at a ratio of 1:1. Cotransfection studies with green fluorescent protein (GFP) used ratios of 1 GFP:2 ERK5:2 MEK5 and 1 GFP:2 MEF2.

**DNA constructs and antibodies.** All MEF2 and ERK5 constructs were obtained from Dr. Rosalind Segal and Dr. Michael Greenberg. Details of constructs were previously published (6, 12). Antibodies were raised against a bacterial expressed His6-tagged full-length human MEF2C as previously described (19).

**Luciferase assay.** Cells were transfected with the constitutively active Renilla luciferase construct, the MEF2 response element-firefly luciferase construct, and the appropriate combination of the constructs described above at a ratio of 0.1:0.4:0.5. Approximately 24 hours following transfection, cells were serum deprived for 1 hour, at which time, if appropriate, SB203580 or PD98059 was added to the media. Cells were treated with neurotrophin-3 (50 ng/mL; Amgen, Thousand Oaks, CA) and harvested between 2 and 8 hours later into passive lysis buffer (Promega, Madison, WI). Luciferase levels were assayed using the Promega dual luciferase assay system (according to the supplied protocol).

**Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay.** Fixed monolayer cultures were assessed for DNA degradation by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) using digoxigenin-labeled nucleotide incorporation and a rhodamine- or fluorescein-labeled digoxigenin antibody according to the protocol of the manufacturer (Apoptag; Intergen, Gaithersburg, MD). Cells were counterstained with bis-benzimide (Hoechst 33342, 1 μmol/L), which was also used to indicate condensed nuclei associated with apoptosis. Cell images were captured using a Nikon video microscopy system (10× objective) and SPOT imaging software. For cells cotransfected with GFP, a mouse anti-GFP antibody (1:500; Roche) was incorporated into the protocol following the stop wash, and a goat anti-rabbit antibody conjugated to Cy2 (1:300; Calbiochem, San Diego, CA) was added to the TUNEL antibody incubation step. Cells were imaged using a Zeiss LSM510 META confocal microscope using an Argon/2 488-nm laser line as the sole excitation wavelength and a goat anti-rabbit antibody conjugated to Cy2 (1:300; Calbiochem) detected by enhanced chemiluminescence (Hyperfilm ECLplus, Amersham Biosciences, Piscataway, NJ). Specificity of the anti–phospho–ERK5 antibody was confirmed by the absence of the observed protein band in the presence of a blocking peptide (Calbiochem) at a concentration 10-fold greater than that of the antibody. A nonphosphorylated blocking peptide (Calbiochem) was used as a control.

**Mitogen-activated protein/extracellular signal-regulated kinase 5 small interfering RNA.** Monolayer Daoy-trkC cultures were transfected with small interfering RNA (siRNA; MEG5 or negative control; Ambion, Austin, TX) at a final concentration of 0.1 μmol/L along with the nontargeting cotransfection marker, siGLO RISC-free siRNA (Dharmacon, Lafayette, CO), using siPORT Lipid (Ambion) according to the instructions of the manufacturer for 6 hours at 37°C. Transfections were halted using serum-containing media and recovered for 12 hours. After washing with PBS, cultures were fed serum-free media, some containing neurotrophin-3 (50 ng/mL) for 48 hours. Cultures were either harvested for isolation of total cellular RNA and protein or for fixation in 4% paraformaldehyde and Hoechst 33342 staining. RNA was used as template for quantitative reverse transcriptase-PCR (RT-PCR) of MEF5 RNA levels using SYBR-green and the following primers: sense, 5'-GGCTTACACCTCGTCAGCAG-3'; antisense, 5'-CCTGGAGGCTCCATTCTCA-3'. Hoechst-stained cells were microscopically examined for evidence of nuclear condensation or fragmentation and for fluorescently labeled siGLO to normalize results for transfection efficiency. Mock-transfected and siGLO-only controls were also evaluated.

**Coimmunoprecipitation of extracellular signal-regulated kinase 5 and myocyte enhancer factor 2.** Daoy and Daoy-trkC cells were treated with neurotrophin-3 (50 ng/mL) for 20 and 30 minutes. Protein lysates (200 μg) were incubated with anti-MEF2 antibody (1:500) for 2 hours at detergent-compatible protein assay (Bio-Rad Laboratories, Hercules, CA). Each protein sample (20 μg) was size fractionated by SDS-polyacrylamide (10%) gel electrophoresis. The accuracy of protein estimation and loading was confirmed by Coomassie blue staining.

Proteins were transferred onto nitrocellulose membrane (Bio-Rad Laboratories) and blocked with a 5% blocking solution (Bio-Rad). Membranes were incubated with rabbit polyclonal phospho-specific anti-ERK5 antibody (1:700, Biosource International, Camarillo, CA), total anti-MEF2A (1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA), anti-MEF2C (1:500), and anti-MEF2D (1:1,000, Santa Cruz Biotechnology) overnight at 4°C. Membranes were incubated for 1 hour in horseradish peroxidase–conjugated goat anti-rabbit antibody (1:10,000, Calbiochem), detected by enhanced chemiluminescence (Hyperfilm ECLplus, Amersham Biosciences, Piscataway, NJ). Specificity of the anti–phospho–ERK5 antibody was confirmed by the absence of the observed protein band in the presence of a blocking peptide (Calbiochem) at a concentration 10-fold greater than that of the antibody. A nonphosphorylated blocking peptide (Calbiochem) was used as a control.

**Western blotting.** Neurotrophin-3 (50 ng/mL) was added 1 hour following serum deprivation. Cells were harvested into lysis buffer at 10, 20, 30, and 45 minutes, 1, 3, 6, 24, and 48 hours following neurotrophin-3 stimulation. Protein concentration was estimated using the Bio-Rad detergent-compatible protein assay (Bio-Rad Laboratories, Hercules, CA). Each protein sample (20 μg) was size fractionated by SDS-polyacrylamide (10%) gel electrophoresis. The accuracy of protein estimation and loading was confirmed by Coomassie blue staining.
4°C. Protein A-agarose beads (25 μL; 50% beads in lysis buffer; Sigma, St. Louis, MO) were added to the lysates for 1 hour at 4°C. Protein was eluted by boiling in SDS-loading buffer and subsequent Western blots were probed with anti–phospho-ERK5 antibody as previously described.

Results

Neurotrophin-3–induced cell death is inhibited by both the p38 inhibitor SB203580 and the extracellular signal-regulated kinase inhibitor PD98059. Neurotrophin-3 (50 ng/mL) induced a 3- to 6-fold increase in apoptosis of the TrkC-expressing Daoy cells, as shown by Hoechst staining and TUNEL, respectively (Daoy-trkC; Fig. 1). This was significantly blocked by pretreatment with either the MAPK inhibitor PD98059 or the p38 inhibitor SB203580 (P < 0.0001, Fig. 1), suggesting that both the p38 and the classic MAPK (ERK1/2) cascades mediate induction of cell death by neurotrophin-3.

Daoy-trkC cells and a primary human medulloblastoma culture express extracellular signal-regulated kinase 5, which is phosphorylated in response to neurotrophin-3. Although it was previously shown that neurotrophin-3 stimulates phosphorylation and subsequent activation of ERK1 and 2, it is becoming increasingly evident that some of the functions previously attributed to these two molecules may be a consequence of ERK5 activation. We examined the effect of neurotrophin-3 on the phosphorylation status of ERK5 in medulloblastoma cell lines and a primary human medulloblastoma culture (Fig. 2A). Neurotrophin-3 induced phosphorylation of ERK5 in the TrkC-expressing Daoy cells and the human primary tumor culture N5138. An increase in phosphorylated ERK5 was not induced by exposure to neurotrophin-3 in Daoy cells, which do not express TrkC. Specificity of the ERK5 antibody was confirmed using ERK5 blocking peptides. The band corresponding to phosphorylated-ERK5 was eliminated by preincubation with a phosphorylated ERK5 blocking peptide but not by a nonphosphorylated ERK5 blocking peptide.

Daoy and Daoy-trkC cells express myocyte enhancer factors 2A, C, and D, of which the activity is enhanced by extracellular signal-regulated kinase 5. We also considered specific downstream targets of both p38 and ERK5, focusing on the MEF2 family of transcription factors, which have been found to play a role in cerebellar granule cell survival (11, 12). Expression of three of the four MEF2 family members was detected by Western blotting of Daoy-trkC and Daoy protein extracts (Fig. 2B). Antibodies raised...
against MEF2A, C, and D detected multiple bands in the Daoy, Daoy-trkC, D458, and D341 cell lines, and a primary human medulloblastoma culture (N5138). Higher molecular weight bands corresponding to MEF2A and MEF2D (62 kDa) and smaller molecular weight bands pertaining to MEF2C (54 kDa) were detected (Fig. 2B).

This was confirmed at the mRNA level in the Daoy-trkC cell line by RT-PCR. Primers to the 3′ UTR of MEF2A, C, and D generated cDNA products of the expected sizes: 603, 584, and 402 bp, respectively (results not shown). We were unable to detect MEF2B RNA in the Daoy-trkC cells. This was not unexpected because it has not been detected in cerebellum to date. Previous studies have shown the expression of MEF2B to be limited to the developing cortex and muscle (20).

**Neurotrophin-3 activates endogenous myocyte enhancer factor 2 in Daoy-trkC cells, an effect mediated by mitogen-activated protein kinase.** Because MEF2A, C, and D are highly expressed in the medulloblastoma cell lines, we examined the effect of neurotrophin-3 on the transcriptional activity of endogenous MEF2. Using a luciferase reporter gene driven by three MEF2 response elements, we showed a 5-fold increase in endogenous MEF2 transcriptional activity with neurotrophin-3 treatment (\(P = 0.019\); Fig. 3). This induction of endogenous MEF2 activity by neurotrophin-3 was significantly blocked by the classic MAPK inhibitor PD98059, but not by the p38 inhibitor SB203580 (Fig. 3; \(P = 1.0\) and \(P < 0.0001\) when compared with the untreated control). This suggests that in medulloblastoma cells, it is ERK and not p38 that mediates neurotrophin-3 activation of MEF2. This conclusion was supported by overexpression of a constitutively active MAPK kinase-6 and wild-type p38 in the luciferase assay, where no significant activation of endogenous MEF2 was seen (Fig. 3). As a positive control, these constructs were examined in the TUNEL assay where they were shown to induce medulloblastoma cell death (results not shown). VP16-MEF2 was used as a constitutively active positive control demonstrating the validity of the luciferase assay.

**Figure 3. Neurotrophin-3 activates MEF2-responsive transcription in medulloblastoma cell lines.** Cells were grown in 24-well plates for 24 hours before transfection with a luciferase reporter construct incorporating three MEF2 response elements. Cells were exposed to neurotrophin-3 for 8 hours following a 24-hour recovery period. Cells were lysed and luciferase activity measured. Columns, fold change in activity when compared with an untreated control; bars, SE. Statistical significance relative to an untreated control was determined using an unpaired \(t\) test (\(n = 9\)) with Bonferroni correction for multiple comparisons.

Daoy-trkC cells overexpressing MEK5 and ERK5 also showed a significant increase in endogenous MEF2 transcriptional activity (\(P < 0.0001\) when compared with an untreated control; Fig. 3). This data supports a role for MAPK in neurotrophin-3–induced MEF2 activation, and confirms previous studies showing ERK5 to enhance the transcriptional activity of MEF2 (8, 10).

**The overexpression of extracellular signal-regulated kinase 5 and myocyte enhancer factor 2 is sufficient to induce apoptosis in Daoy and Daoy-trkC cells.** Because our experiments indicated that neurotrophin-3 activates both ERK5 and MEF2, we examined the effect of both of these molecules on survival of medulloblastoma cell lines. Coexpression of a constitutively active MEK5 and wild-type ERK5 induced a 4-fold increase in apoptosis of Daoy and Daoy-trkC cells (\(P = 0.0021\) and \(P < 0.0001\), respectively; Fig. 4A).

Daoy and Daoy-trkC cells transfected with VP16-MEF2 showed up to a 3-fold increase in apoptosis over that of the mock-transfected control (\(P = 0.0117\) and 0.0004, respectively; Fig. 4A). Wild-type MEF2A also induced a 3-fold increase in apoptosis of both Daoy and Daoy-trkC cells (\(P < 0.0001\) and \(P = 0.0007\), respectively).

Cells were also cotransfected with GFP and either MEF2 or MEK5 plus ERK5 construct to show the percentage of apoptosis in transfected cells only. Up to 58% of Daoy-trkC cells expressing MEK5 and ERK5 were also TUNEL positive and therefore apoptotic (Fig. 4C). This was significantly higher than that observed in the pcDNA-transfected control cells (\(P < 0.0001\)) and, in contrast to neurotrophin-3–induced cell death, was not significantly affected by the presence of serum (\(P = 0.62\). Transfection with the MEF2 expression plasmid also increased the level of apoptosis to as much as 70% (\(P < 0.0001\)). These results equate to a 5-fold increase in cell death compared with controls. This is similar to the result previously shown by the total cell count and supports our hypothesis that only the ERK5- and MEF2-transfected cells are apoptotic.

Expression of a dominant-negative MEF2 construct significantly blocked neurotrophin-3–induced apoptosis of Daoy-trkC cells (\(P = 0.70\) when compared with the untreated control). Neurotrophin-3–induced Daoy-trkC cell death was also inhibited by siRNA-mediated knockdown of MEK5 (Fig. 4B). MEK5 siRNA transfection of Daoy-trkC cells resulted in a significant decrease in MEK5 mRNA to 12 ± 0.2% SE (\(P < 0.0005\)) of control levels. All values were normalized to parallel control transfections with siRNA for the housekeeping gene GAPDH or a nonhomologous negative control sequence (Ambion). MEK5 siRNA also resulted in a reproducible decrease in neurotrophin-3/TrkC–induced apoptosis down to 67 ± 3.0% SE (\(P < 0.003\)) compared with negative control siRNA-treated cells (Fig. 4). Western blot analysis with a MEK5 monoclonal antibody showed a significant decrease of MEK5 protein to 71 ± 3.7% SE (\(P < 0.004\)) in the MEK5 siRNA–treated cultures compared with negative control.

Apoptosis induction by MEK5 and ERK5 was blocked by transfection with a dominant-negative MEF2 construct to levels not significantly different from the mock-transfected control (\(P = 0.94\) and \(P = 0.99\), respectively; Fig. 4A). This suggests that the induction of cell death by ERK5 is mediated by MEF2. The dominant-negative MEF2 construct used in this study is mutated in the ERK5 phosphorylation site, further supporting a combined role for ERK5 and MEF2 in medulloblastoma cell death. We used a coimmunoprecipitation method to look for a direct interaction between ERK5 and MEF2 in response to neurotrophin-3. MEF2C was immunoprecipitated from control and neurotrophin-3–stimulated Daoy-trkC cell lysates. Immunoblotting with the phosphorylated ERK5–specific...
antibody showed that in response to neurotrophin-3, phosphorylated ERK5 coimmunoprecipitates with MEF2C, suggesting a direct interaction between the two molecules (Fig. 5). Control cells under serum-free conditions also had a low basal level of active ERK5 associated with MEF2.

Extracellular signal-regulated kinase 5 and myocyte enhancer factor 2 overexpression induces apoptosis in primary cultures of cerebellar tumors from the patched heterozygous mouse model of medulloblastoma. Our ultimate goal was to validate our findings in primary human tumor cultures but, due to the resistance of human medulloblastoma cells to transfection and the scarcity of tumor tissue for culture, we were unable to do this. As an alternative, we validated our findings in cerebellar tumors obtained from the patched heterozygous mouse model of Gorlin’s syndrome. These mice spontaneously develop cerebellar tumors comparable to desmoplastic medulloblastoma by gene expression analysis (4) and their differential response to neurotrophin-3.

Both neurotrophin-3–responsive (line A) and nonresponsive (line B) mouse cerebellar tumors exhibited an increase in apoptosis in response to the overexpression of MEF2C and ERK5 (Fig. 6; \( P = 0.01 \) and \( P = 0.013 \), respectively). MEF2C induces a 4-fold increase in cell death as compared with an untreated mock-transfected control. Similarly constitutively active MEK5 and wild-type ERK5 induced up to a 4-fold increase in cell death.
MEF2 has not previously been associated with cell death. To the contrary, there is an emerging view that these factors promote proliferation and survival (16). We have now shown that the pathways responsible for induction of medulloblastoma cell death are the same as those involved in survival of normal cerebellar granule cells, the cell type from which these tumors are thought to be derived. Elucidation of signaling downstream of TrkC showed activation of multiple signaling pathways, including phosphatidylinositol 3-kinase, ERK1/2, p38, and c-jun-NH₂-kinase, the majority of which have prosurvival functions during cerebellar development. We subsequently found neurotrophin-3 to activate ERK5 and enhance endogenous MEF2 activity. ERK5 and MEF2 have not previously been associated with cell death. To the contrary, there is an emerging view that these factors promote proliferation and survival of various tumor types (15–17). Our results show that the biological actions previously attributed to ERK1/2 may in fact be mediated by this more recently discovered MAPK (5, 6). It has been shown in normal cerebellar granule cells that ERK5 is responsible for mediating neurotrophin-induced cell survival (21). We have now shown that the pathways responsible for induction of medulloblastoma cell death are the same as those involved in survival of normal cerebellar granule cells, the cell type from which these tumors are thought to be derived. Elucidation of signaling downstream of TrkC showed activation of multiple signaling pathways, including phosphatidylinositol 3-kinase, ERK1/2, p38, and c-jun-NH₂-kinase, the majority of which have prosurvival functions during cerebellar development. We subsequently found neurotrophin-3 to activate ERK5 and enhance endogenous MEF2 activity. ERK5 and MEF2 have not previously been associated with cell death. To the contrary, there is an emerging view that these factors promote proliferation and survival of various tumor types (15–17). Our results show that the biological actions of these signaling molecules are highly context dependent, both with respect to their actions in normal cells as well as to different types of cancer.

Having identified MEF2 as a downstream target of ERK5, we looked at expression of the four family members in medulloblastoma cell lines. MEF2A, C, and D were found to be present in all of the cell lines tested, which has not previously been shown. Given the role of these factors in the developing brain and granule cell survival, it is not surprising that we see expression in tumors derived from granule cells. We showed MEF2 to be not only activated by neurotrophin-3 but also necessary for neurotrophin-3–induced apoptosis of medulloblastoma cell lines. Overexpression of MEF2 alone was also shown to be sufficient to induce apoptosis of these cells. MEF2 has not previously been associated with cell death in neuronal cell populations or tumors, although there is some evidence for the involvement of MEF2D and its transcriptional target Nur77 in apoptosis of immature T cells following T-cell receptor activation (22). In our system we have not been able to study MEF2D, but a recent study in normal granule cells shows this MEF to also have a prosurvival role (13, 23).

The precise transcription targets of activated MEF2 involved in promotion of neuronal survival and maturation are not currently known. However, it has been reported that MEF2 activity regulates c-jun expression (24) and that brain-derived neurotrophic factor induces the MEF2-dependent transcription of neurotrophin-3 (25). We considered whether or not the overexpression of MEF2 or MEK5/ERK5 in medulloblastoma cell lines was indirectly influencing apoptosis by enhancing the expression of neurotrophin-3 with consequent paracrine or autocrine stimulation of apoptosis. This is not the case in our system because similar levels of apoptosis were observed following overexpression of MEF2 and ERK5 in both the TrkC-expressing Daoy-trkC cells and the TrkC-deficient Daoy cells.

As yet the role of p38 in neurotrophin-3–induced medulloblastoma cell death remains unresolved. Neurotrophin-3 induced phosphorylation of p38 whereas overexpression of p38 and constitutively active MAPK kinase-6, a specific upstream activator of p38, induced a significant increase in apoptosis. However, we did not find p38 to induce a significant increase in endogenous MEF2 activity, suggesting that in medulloblastoma cells MEF2 is not downstream of p38. The p38 inhibitor SB203580, however, did slightly reduce neurotrophin-3 enhancement of MEF2 activity. Although MAPK kinase-6 and p38 expression is sufficient to induce medulloblastoma cell death, these data suggest at most a supportive role for p38 in neurotrophin-3–induced activation of MEF2. Further work is required to elucidate the interaction between ERK5 and p38 pathways in neurotrophin-3–induced apoptosis.

Our study suggests that neurotrophin-3 actually stimulates signaling through pathways found normally in cerebellar granule cells to support cell survival. Why then do these same pathways induce death in medulloblastoma cells? There are many possible explanations for this apparent paradoxical response. The most likely being that these tumor cells are very divergent from their natural environment.
proposed cell of origin and it is possible that these signaling pathways are in some way disrupted (4). As a consequence, the tumor cells cannot complete the pathway, with the default being apoptosis. A similar phenomenon was previously described in a medulloblastoma cell line with regard to its response to fibroblast growth factor (FGF; ref. 26). This study showed that FGF, normally involved in cerebellar neuron maturation, triggers neuronal differentiation in a medulloblastoma cell line immediately followed by apoptosis. We did not look at markers of differentiation in neurotrophin-3–treated cells but we did note an increase in the expression of p27KIP1 (data not shown). This cell cycle regulatory molecule is highly expressed in granule cells where it is thought to be involved in their exit from the cell cycle before differentiation (27). Medulloblastoma gene expression data also showed very definite differences between normal cerebellum and tumors, suggesting that there may be key differences in the targets of signaling pathways (14). If multiple genes are dysregulated, then the outcome of a pathway could be completely altered. This is not a new phenomenon; for example, when oncogenes such as MYC are dysregulated, and hence all of their targets, treating cells with a differentiation or survival factor often results in death (28–30).

An important conclusion of this study is the demonstration of medulloblastoma cell death dependent of neurotrophin-3 and TrkC. We have shown both ERK5 and MEFD2 to play an important role in medulloblastoma cell death and, due to the equally important roles of these factors in survival of normal cerebellar granule cells, exploitation of this signaling pathway identifies a potentially very useful therapeutic target.

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