Gamma-Secretase Represents a Therapeutic Target for the Treatment of Invasive Glioma Mediated by the p75 Neurotrophin Receptor

LiMei Wang 1,2,3, Jennifer J. Rahn 1,2,3, XueQing Lun 1,3, Beichen Sun 1,3, John J. P. Kelly 4,5, Samuel Weiss 4,5, Stephen M. Robbins 1,2,3, Peter A. Forsyth 1,2,3,6*, Donna L. Senger 1,3,6*

1 Department of Oncology, University of Calgary, Calgary, Canada, and Tom Baker Cancer Centre, Calgary, Canada, 2 Department of Biochemistry and Molecular Biology, University of Calgary, Calgary, Canada, 3 Clark H. Smith Brain Tumor Center and Southern Alberta Cancer Research Institute, Calgary, Canada, 4 Department of Cell Biology and Anatomy, University of Calgary, Calgary, Canada, 5 Hotchkiss Brain Institute, Calgary, Canada, 6 Department of Clinical Neurosciences, University of Calgary, Calgary, Canada


citation: Wang L, Rahn JJ, Lun X, Sun B, Kelly JJP, et al. (2008) Gamma-secretase represents a therapeutic target for the treatment of invasive glioma mediated by the p75 neurotrophin receptor. PLoS Biol 6(11): e289. doi:10.1371/journal.pbio.0060289

Introduction

Human malignant glioma (MG) is one of the most common primary central nervous system tumors in adults. These tumors are diffuse, highly invasive, with dismal prognosis, and long-term survivors are rare [1,2]. MG extend tendrils of tumor several centimeters away from the main tumor mass. These, as well as the recently identified brain tumor-derived stem-like cells [3–6], herein called brain tumor-initiating cells (BTICs), act as “disease reservoirs,” rendering these tumors refractory to available treatments such as surgery or radiotherapy [7,8]. The highly invasive nature of these tumors is the result of genotypic and phenotypic changes that result in the activation of a number of coordinate cellular programs, including those necessary for migration (e.g., motility) and invasion (e.g., extracellular matrix [ECM] degradation) [9] and changes in pathway signaling that impart resistance to conventional treatments by reducing proliferation and increasing resistance to apoptosis [8,10,11]. A detailed understanding of the mechanisms underlying this invasive behavior is essential for the development of effective therapies.

Several genes, including those that encode uPA/uPAR, ephrinB3/EphB2, matrix metalloproteinases (MMPs), a disintegrin and metalloproteases (ADAMs), cathepsins, and integrins, have previously been implicated in glioma invasion [12]. More recently, gene expression profiling identified several subclasses of gliomas that separate tumors into good and poor prognosis groups of which diffuse infiltrative gliomas are divided into four such subclasses [13]. One of these four subclasses, designated hierarchical cluster 2B (HC2B), was found to include several genes with specific roles in cell migration and invasion, and membership in this group was found to strongly correlate with poor patient survival. Our understanding of the proteins that initiate, and the pathways that regulate, glioma invasion is continually expanding, such as the recent discovery that CD95 via the activation of the PI3K/Akt/glycogen synthetase kinase (GSK3β) pathway regulates glioma invasion [14]. However, despite recent advances and efforts to target these specific molecules or pathways, no clinically relevant agents have been identified as yet. Using a discovery-based approach and a series of functional, biochemical, and clinical studies, we have identified the p75 neurotrophin receptor (p75NTR) as a critical regulator of glioma invasion [15]. We found that p75NTR, through a neurotrophin-dependent mechanism, dramatically enhanced migration and invasion of genetically distinct glioma and that robust expression of p75NTR was detected in the highly invasive tumor cell population from primary central nervous system tumors in adults. These tumors are diffuse, highly invasive, with dismal prognosis, and long-term survivors are rare [1,2]. MG extend tendrils of tumor several centimeters away from the main tumor mass. These, as well as the recently identified brain tumor-derived stem-like cells [3–6], herein called brain tumor-initiating cells (BTICs), act as “disease reservoirs,” rendering these tumors refractory to available treatments such as surgery or radiotherapy [7,8]. The highly invasive nature of these tumors is the result of genotypic and phenotypic changes that result in the activation of a number of coordinate cellular programs, including those necessary for migration (e.g., motility) and invasion (e.g., extracellular matrix [ECM] degradation) [9] and changes in pathway signaling that impart resistance to conventional treatments by reducing proliferation and increasing resistance to apoptosis [8,10,11]. A detailed understanding of the mechanisms underlying this invasive behavior is essential for the development of effective therapies.

Several genes, including those that encode uPA/uPAR, ephrinB3/EphB2, matrix metalloproteinases (MMPs), a disintegrin and metalloproteases (ADAMs), cathepsins, and integrins, have previously been implicated in glioma invasion [12]. More recently, gene expression profiling identified several subclasses of gliomas that separate tumors into good and poor prognosis groups of which diffuse infiltrative gliomas are divided into four such subclasses [13]. One of these four subclasses, designated hierarchical cluster 2B (HC2B), was found to include several genes with specific roles in cell migration and invasion, and membership in this group was found to strongly correlate with poor patient survival. Our understanding of the proteins that initiate, and the pathways that regulate, glioma invasion is continually expanding, such as the recent discovery that CD95 via the activation of the PI3K/Akt/glycogen synthetase kinase (GSK3β) pathway regulates glioma invasion [14]. However, despite recent advances and efforts to target these specific molecules or pathways, no clinically relevant agents have been identified as yet. Using a discovery-based approach and a series of functional, biochemical, and clinical studies, we have identified the p75 neurotrophin receptor (p75NTR) as a critical regulator of glioma invasion [15]. We found that p75NTR, through a neurotrophin-dependent mechanism, dramatically enhanced migration and invasion of genetically distinct glioma and that robust expression of p75NTR was detected in the highly invasive tumor cell population from
**Author Summary**

Despite technical advances, clinical prognosis of patients with malignant glioma, with an average survival of less than one year, has not changed. The highly invasive nature of these tumors, together with the recently identified brain tumor-initiating cells, provide disease reservoirs that render these tumors incurable by conventional therapies. Here, we present the first evidence to our knowledge that regulated intramembrane proteolysis of the neurotrophin receptor p75NTR is a critical regulator of glioma invasion. Inhibition of this process by clinically relevant γ-secretase inhibitors dramatically impairs the highly invasive nature of genetically distinct glioblastomas and brain tumor-initiating cells and prolongs survival. These data highlight regulated intramembrane proteolysis as a therapeutic target of malignant glioma and implicate the application of γ-secretase inhibitors in the treatment of these devastating tumors.

p75NTR-positive glioblastoma patient specimens. In this current study, we investigated the mechanism by which p75NTR imparts this highly invasive behavior to malignant glioma, and assessed the use of a clinically applicable agent in abrogating this invasive behavior.

p75NTR elicits a large array of diverse biological responses that are regulated by a complex layer of mechanisms. These intricate layers of control have been proposed to explain the variety of cellular effects triggered by p75NTR activation. Key p75NTR signaling pathways already identified include Ras homolog gene family, member A (RhoA), Jun N-terminal kinase (JNK), mitogen-activated protein kinase (MAPK), and nuclear factor κ B (NFκB) [16]. These pathways are believed to be activated by upstream proteins that directly associate with various regions of the p75NTR intracellular domain (ICD).

These proteins include guanine nucleotide dissociation inhibitor (RhoGDI), ribosome-inactivating protein-2 (RIP-2), and p75NTR-associated cell death executor (NADE) [17–20], which associate with a region referred to as the “death domain”; Schwann cell factor-1 (SC-1); neurotrophin receptor-interacting MAGE homolog (NRAGE); tumor necrosis factor (TNF) receptor-associated factor (TRAF), and neurotrophin receptor interacting factor (NRIF) [21–23], which associate with the juxtamembrane region of p75NTR, and a PDZ-containing protein Fas-associated phosphatase-1 (FAP-1), which associates with the C-terminal Ser-Pro-Val (SPV) [24]. What proteins or biological processes are activated by p75NTR, however, is highly cell context specific. In addition to associating with other signaling molecules, p75NTR, similar to amyloid precursor protein (APP) and Notch, has been shown to undergo regulated α-secretase and γ-secretase cleavage, referred to as regulated intramembrane proteolysis (RIP). Cleavage of several type-1 transmembrane receptors has been implicated and shown to be necessary in eliciting some downstream biological responses [25–28]. α-Secretase cleavage of full-length p75NTR by a sheddase liberates the extracellular domain (ECD), leaving an unstable membrane-bound C-terminal fragment (CTF) that is cleaved by the γ-secretase complex to release an ICD with potential signaling capability [26,29]. Here, we show for the first time to our knowledge that regulated intramembrane proteolysis of p75NTR is a requirement for the highly invasive behavior of p75NTR-positive malignant glioma, and designate RIP as a clinical target for the treatment of invasive malignant glioma.

**Results**

p75NTR Proteolytic Processing Occurs in Patient Specimens Endogenously Expressing p75NTR

In a previous study, our laboratory identified p75NTR as a potent mediator of invasion in human glioma using a novel invasive glioma mouse model generated by serial in vivo selection [15]. In that study, we found that p75NTR was expressed in 22% mid-grade astrocytomas (two of nine) and 85% of glioblastoma multiforme (GBM) specimens (17 of 20), and that the p75NTR-positive glioma cells in the patient tumor cell population were more migratory than the p75NTR-negative glioma cells. Here, we investigate the mechanism underlying this p75NTR-induced invasion. In neurons, p75NTR is a substrate for sequential α- and γ-secretase-mediated intramembrane proteolysis generating 24 kDa CTF and 19 kDa ICD fragments, respectively, and the generation of these fragments are required for some of its biological functions [26,28,30–34]. We therefore sought to determine whether intramembrane proteolysis of p75NTR occurred in malignant glioma patient specimens. To do this, we assessed whether the generation of the 24-kDa CTF and the 19-kDa ICD occurred in a panel of surgically resected human glioma specimens and normal human brain. Tumor and normal tissue taken at the time of surgery were immediately snap frozen in liquid nitrogen and stored at −80 °C. Frozen tissue was digested in lysis buffer and analyzed by western blots using a p75NTR cytoplasmic-specific antibody that not only detected the full-length p75NTR protein, but also detected p75NTR-positive fragments migrating at 24 and 19 kDa, respectively, in the p75NTR-positive specimens (eight of nine GBMs and two of five Grade III glioma) (Figure 1A). Hence, p75NTR processing occurs in human glioma tumors, and this suggested the possibility that p75NTR processing is required for glioma invasion.

p75NTR Is Proteolytically Processed in Invasive Glioma Cells

To address the possible role(s) of p75NTR proteolytic processing in glioma cells, we assessed whether the appearance of the p75NTR-positive fragments at 24 and 19 kDa was the result of proteolytic processing of the full-length p75NTR in invading glioma cells. We have previously established the highly invasive glioma cell lines U87R and U251R for which p75NTR accounts for their invasive behavior [15]. The U87R and U251R invasive glioma cells were grown in the absence or presence of the proteasome inhibitor epoxomicin, a compound used to inhibit rapid degradation of proteins often associated with RIP-mediated proteins [26,35]. Western blot analysis showed that in addition to the 75-kDa full-length p75NTR protein, 24-kDa and 19-kDa fragments (Figure 1B, lane 1 and 5) were present and stabilized in the presence of 1 μM epoxomicin (Figure 1B, lane 2 and 6). These results are in agreement with the model that the full-length p75NTR protein is cleaved, releasing the ECD, CTF, and intracellular fragments. Next, we verified that the appearance of the 24-kDa and 19-kDa fragments was the result of sequential cleavage of p75NTR by an α-secretase and then a γ-secretase. First, we determined whether treatment of p75NTR glioma cells (U87p75NTR) using the TNF-α protease inhibitor (TAPI)-2, known to inhibit metalloproteases and ADAMs such as tumor necrosis factor-α converting enzyme (TACE) [30–38] and previously shown to inhibit the proteolytic processing of...
Figure 1. p75NTR is Proteolytically Processed in Patient Specimens and Invasive Glioma Cells

(A) Western blot analysis detected p75NTR-positive fragments migrating at 24 and 19 kDa in patient specimens expressing p75NTR (eight of nine GBMs, two of five Grade III glioma) but was undetectable in tissue from normal human brain. U87 cells transfected with p75NTR and grown in the presence of the proteasome inhibitor epoxomicin (1 μM) were used as a positive control (C). Western blots probed for beta-actin were used as a loading control. Molecular weight markers are indicated on the left.

(B) The highly invasive glioma cell lines U87R and U251R isolated by serial in vivo selection were treated with the proteasome inhibitor epoxomicin (Epo, 1 μM) and/or the γ-secretase inhibitor, Compound X (CompX, 2 μM) for 4 h. Western blots for p75NTR were probed with an antibody specific to the cytoplasmic domain of p75NTR which detects full-length (75 kDa), CTF (indicated by the less than symbol [<]; 25 kDa), and ICD (indicated by the asterisk [*]; 19 kDa) peptides. The ICD is derived from the cleavage of the CTF, which is shown to visibly accumulate in the presence of a γ-secretase inhibitor. Molecular weight markers are indicated on the left.

(C) p75NTR proteolytic processing is a global event in human glioma cells. pcDNA3.1 encoding human p75NTR or the empty pcDNA3.1 vector were stably transfected into U87, U251, U118, and U343. Cells expressing p75NTR (U87p75, U251p75, U343p75, and U118p75) were treated as described above. Western blot analysis showed that all glioma cell lines tested cleaved the full-length p75NTR to generate first the 24-kDa CTF and then the 19-kDa ICD.

doi:10.1371/journal.pbio.0060289.g001
p75NTR in neurons [26,32,33,39], could inhibit p75NTR processing in glioma cells. TAPI-2 inhibited the proteolytic processing of p75NTR as indicated by the lack of CTF and ICD, and abrogated p75NTR-mediated invasion (Figure S1). Since TAPI-2 has broad specificity, and glioblastomas are known to produce high levels of many proteases, including members of the MMP, ADAM, and ADAMTS families, leaving the exact identity of the α-secretase unclear, we focused our efforts on the second cleavage event. To determine whether the generation of the 19-kDa fragment was the result of cleavage of p75NTR by a γ-secretase, U87R and U251R cells were treated with 2 μM Compound X (Calbiochem), a specific inhibitor of γ-secretase, for 4 h in the absence or presence of epoxomicin. Western blot analysis of p75NTR revealed that in the presence of the γ-secretase inhibitor, an accumulation of the 24-kDa fragment occurred without subsequent cleavage to the 19-kDa ICD, consistent with the release of the ICD of p75NTR by γ-secretase (Figure 1B, lanes 3, 4, 7, and 8). The role of processing of p75NTR was not limited to a single glioma cell line and was a general mechanism observed in glioma cells established from genetically distinct individuals (U87p75, U251p75, U343p75, and U118p75). We found that in all p75NTR-positive glioma cell lines, full-length p75NTR was cleaved to generate two fragments of 19 and 24 kDa: ICD and CTF, respectively (Figure 1C). These results demonstrate that regulated intramembrane proteolysis of p75NTR is a global event occurring in highly invasive p75NTR-positive human glioma cells.

γ-Secretase Inhibition Significantly Abrogated p75NTR-Induced Glioma Migration and Invasion In Vitro

In neurons, ectodomain shedding of p75NTR by α-secretase and then γ-secretase cleavage to generate an ICD fragment can result in the activation of downstream events [26–28,30–34]. To test whether the processing of p75NTR resulting in the release of the ICD fragment has a functional role in glioma invasion, we analyzed in vitro migration and invasion of U87R, U251R, U87p75NTR, and U251p75NTR glioma cell lines using circular monolayer migration assays (Figure 2A and 2B) and 3D-collagen invasion assays (Figure 2C and 2D) in the absence and presence of the γ-secretase inhibitor, Compound X. p75NTR-mediated glioma migration and invasion were significantly inhibited in the presence of Compound X. In contrast, when the proteasome inhibitor epoxomicin was used to stabilize p75NTR-ICD, a significant increase in migration and invasion was seen (unpublished data), consistent with increased invasion observed when a cDNA construct mimicking the ICD fragment was ectopically expressed in U87 glioma cells (Figure 3Aa and 3B). To determine whether γ-secretase inhibition was confined to glioma invasion or had effects on other biological processes, we assessed the effect of γ-secretase inhibition on survival and proliferation of p75NTR-positive glioma cells. No significant change was observed on either survival or proliferation in vitro (Figure S2).

Biochemical Characterization of U87 and U251 Glioma Cells Expressing p75NTR Wild-Type or Cleavage-Resistant Chimeric p75NTR Alleles

It is well known that γ-secretase has many substrates [40,41]. To directly test the role of p75NTR processing in glioma invasion, we constructed cleavage-resistant chimeric proteins of p75NTR by replacing either the transmembrane (p75FasTM) or the extracellular stalk domain of p75NTR (p75FasS) with equivalent domains from the Fas receptor [39] (Figure 3C). Both p75NTR and Fas receptors are members of the TNF receptor superfamily, and although they each contain similar domains, Fas, unlike p75NTR, does not undergo RIP. Since ectopic expression of p75NTR in the human glioma cell lines U87 and U251 was sufficient to mediate glioma invasion [15], these cell lines were used as a model system to assess the p75NTR chimeric mutants. U87 and U251 were therefore stably transfected with the cleavage-resistant p75NTR constructs (p75FasS and p75FasTM). To ensure proper function of all p75NTR protein constructs, we assessed their location, topography, and ability to bind neurotrophin in both U87 and U251 glioma cell lines. Receptor orientation and localization at the plasma membrane was confirmed by flow cytometric analysis using a monoclonal antibody specific to the ECD domain of p75NTR (Figure S3A). As expected, all p75NTR constructs were expressed at the plasma membrane with the correct topography. Next, we assessed whether the chimeric constructs could still bind neurotrophin. Previously, we demonstrated that in the absence of p75NTR, glioma cells secrete high levels of brain-derived neurotrophic factor (BDNF) protein into the culture medium in vitro. When these same cells express p75NTR, the majority of the BDNF is found to be cell associated, presumably bound to p75NTR [15]. To confirm that the p75NTR cleavage-resistant chimeric forms retained the ability to bind neurotrophin, ELISA assays were performed to detect BDNF expression in the conditioned medium and total cell lysates of U87 and U251 cells expressing p75FasL, p75FasTM, p75FasS, and p75CRD130 (Figure S3B). p75CRD130 is a neurotrophin-biding mutant created by inserting four amino acids after amino acid residue 130 [15,42–47]. Expression of the chimeric p75NTR proteins (p75FasTM and p75FasS), just like the p75 wild type, resulted in a shift in BDNF localization from the conditioned medium to the cell lysate. This was in contrast to the cells expressing the neurotrophin-binding mutant (p75CRD130) or the empty vector (pcDNA) where the bulk of BDNF was detected in the culture medium. These data demonstrate that p75NTR cleavage-resistant chimeric constructs p75FasTM and p75FasS retained their ability to bind neurotrophin (Figure S3C). Once we confirmed the correct expression and binding of the various p75NTR constructs, western blots using a p75NTR cytoplasmic domain-specific antibody were performed to evaluate proteolytic processing of the various p75NTR receptors (Figure 3D). In cells expressing p75FasS, only the full-length protein was detected, consistent with inhibition of the α-secretase cleavage, whereas the full-length 75 kDa and the 24-kDa fragment were detected in cells expressing the p75FasTM construct corresponding to the ectodomain shedding of p75NTR by α-secretase but with inhibition of the γ-secretase cleavage. Moreover, in the presence of epoxomicin, no additional p75NTR fragments were observed (Figure S4). These results demonstrate the cleavage-resistant chimeric p75NTR alleles were expressed with correct biochemical characteristics in U87 and U251 glioma cells. In addition, and consistent with the hypothesis that proteolytic processing of p75NTR is required for glioma invasion, only the full-length 75 kDa band was detected in lysates from U87 cells expressing p75CRD130, a p75NTR construct that was unable to induce glioma invasion [15].
Cleavage-Resistant Chimeric Forms of p75NTR Do Not Induce Glioma Migration and Invasion In Vitro or In Vivo

Since we have shown that neurotrophin binding is required for p75NTR-mediated glioma invasion [15], and the neurotrophin-binding mutant p75CRD130 does not undergo RIP, it would appear that RIP of p75NTR is required for glioma invasion. To determine whether this is in fact true, U87 and U251 cells expressing the p75NTR cleavage-resistant constructs were assessed for their invasive ability using 3D-collagen invasion assays. We found that expression of cleavage-resistant forms of p75NTR (p75FasS, p75FasTM, and p75CRD130), which prevented receptor proteolysis, blocked p75NTR-mediated glioma invasion (Figure 4A and 4B), providing evidence to support a role for γ-secretase–dependent release of p75NTR ICD in mediating glioma invasion.

To determine whether p75NTR processing was required for glioma invasion in vivo, U87 glioma cell lines ectopically expressing p75FasS and p75FasTM were implanted into the brains of immunocompromised (SCID) mice. U87 glioma cells expressing full-length p75NTR (U87p75) or control vector (U87pcDNA) were used for comparison. Twenty-eight days after implantation, the mice were sacrificed, and frozen brain sections were stained with antibodies against human nuclei, to visualize all glioma cells (Figure 4C, upper panel) or with anti-human p75NTR (Figure 4C, bottom panel). Implantation of U87 glioma cells stably transfected with the control pcDNA vector led to the formation of well-circumscribed tumors, while U87 glioma cells ectopically expressing p75NTR formed tumors with highly infiltrative edges. In sharp contrast to the p75NTR-expressing U87 tumors, tumors expressing either p75FasTM or p75FasS formed well-circumscribed tumors similar to the p75NTR-negative tumors (U87pcDNA).

Figure 2. γ-Secretase Inhibitor Significantly Inhibited p75NTR-Induced Migration and Invasion in Glioma Cells

(A and B) Monolayer circular migration assays of U87R and U251R cells, which endogenously express p75NTR, and U87p75 and U251p75 cells, which ectopically express p75NTR, were performed. Briefly, cells were seeded in monolayer wells through a cell sedimentation manifold. p75NTR-negative U87T, U87pcDNA, U251T, and U251pcDNA cells were used for comparison. Once the sedimentation manifolds were removed, cells were given complete medium containing 2 μM Compound X (CompX). Digital images of the cells were taken before migration at 0 h and then again at 72 h. Best-fit circles were drawn around the area covered by the cells at the 0 h and 72 h time points, and the actual cell area was determined using Axiovision 4.2 imaging software. Quantitative migration scores were calculated as the increase in the area covered by the cells beyond the initial area of the cells. γ-Secretase inhibitor Compound X significantly inhibited p75NTR-induced migration. Values shown are the mean ± s.e.m. from three independent experiments; triple asterisks (***), indicate p < 0.001 as compared to control (one-way analysis of variance [ANOVA] with the Neuman-Keuls post-test).

(C and D) 3D-collagen invasion assays of U87R, U87p75, U251R, and U251p75 cells were performed by mixing cells with 3D-collagen matrix (collagen, fibronectin, and laminin) and then seeding them into 8.0-μm pore size transwell chambers in the presence or absence of 2 μM Compound X for 6 h. Cells were fixed and stained, and invasive cells were counted. U87T, U87pcDNA, U251T, and U251pcDNA cells were used for comparison. Inhibition of γ-secretase by Compound X significantly inhibited p75NTR-induced glioma invasion. Values shown are the mean ± s.e.m. from three independent experiments; triple asterisks (***), indicate p < 0.001 as compared to control (one-way ANOVA with the Neuman-Keuls post-test).

doi:10.1371/journal.pbio.0060289.g002

Cleavage-Resistant Chimeric Forms of p75NTR Do Not Induce Glioma Migration and Invasion In Vitro or In Vivo

Since we have shown that neurotrophin binding is required for p75NTR-mediated glioma invasion [15], and the neurotrophin-binding mutant p75CRD130 does not undergo RIP, it would appear that RIP of p75NTR is required for glioma invasion. To determine whether this is in fact true, U87 and U251 cells expressing the p75NTR cleavage-resistant constructs were assessed for their invasive ability using 3D-collagen invasion assays. We found that expression of cleavage-resistant forms of p75NTR (p75FasS, p75FasTM, and p75CRD130), which prevented receptor proteolysis, blocked p75NTR-mediated glioma invasion (Figure 4A and 4B), providing evidence to support a role for γ-secretase–dependent release of p75NTR ICD in mediating glioma invasion.

To determine whether p75NTR processing was required for
parable results were seen in three independent experiments. In conjunction with the in vitro data, these data suggest that proteolytic processing of p75NTR is required for glioma invasion in vivo (Figure 4C).

It is well known that the microenvironment of tumors can change the biochemical characterization and function of cells. We have demonstrated in vitro that glioma cells expressing p75NTR undergo proteolytic processing to generate first the 24-kDa CTF and then the 19-kDa ICD. Other p75NTR constructs were generated, including the neurotrophin-binding mutant in which the ligand-binding site was mutated by inserting four amino acids after amino acid residue 130, designated p75CRD130 (CRD130), and the p75NTR intracellular domain construct consisting of amino acids 238 to 399 (p75-ICD).

Processing by western blot. The 24- and 19-kDa fragments were found in the highly invasive glioma cells, U87R, U251R, and U87p75NTR. In contrast, neither the 24-kDa nor the 19-kDa fragment was seen in cells expressing p75FasS, and as expected, only the 24-kDa fragment was detected in cells expressing the p75FasTM, consistent with their in vitro characterization (Figure 4D). These data further support a role for RIP of p75NTR in glioma invasion.

**p75NTR Proteolytic Processing Occurs in BTICs from Patient Specimens That Endogenously Express p75NTR**

Our data demonstrated that 85% of GBM specimens (17/20) express p75NTR, that the p75NTR-positive glioma cells in...
the original patient tumor cell population were more migratory [15], and that 24- and 19-kDa p75\textsuperscript{NTR}-positive fragments are present in p75\textsuperscript{NTR}-positive primary Grade III and GBM patient specimens (Figure 1). We therefore wanted to determine whether the appearance of these fragments in the malignant glioma patient specimens was the result of intramembrane proteolysis of p75\textsuperscript{NTR}. To do this, we established primary cultures from human glioma patient tumors. The recent discovery that human stem cell-like tumor cells, termed BTICs, retain characteristics that closely recapitulate the original patient tumor [3–6,48,49] prompted us to establish the primary patient tumor cultures under neural stem cell–promoting conditions. BTICs share characteristics with neural stem cells (NSCs) such as continuous self-renewal, extensive brain parenchymal migration and infiltration, and potential for full or partial differentiation, properties not found in established glioma cell lines [50,51]. Operative samples of human GBM were obtained at the time of surgery, and brain tumor sphere cultures were established in NS-A basal medium plus epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) (EF medium). Immunocytochemical analysis of BTICs established from five

Figure 4. Cleavage-Resistant Chimeric p75\textsuperscript{NTR} Proteins Do Not Induce Migration and Invasion In Vitro or In Vivo

(A and B) The invasive ability of U87 (A) and U251 (B) stably transfected with p75\textsuperscript{NTR} or the p75\textsuperscript{NTR} cleavage-resistant chimeric constructs (p75FasS and p75FasTM) were assessed using 3D-invasion assays. Expression of p75\textsuperscript{NTR} significantly increased invasion in the genetically distinct glioma cell lines U87 and U251, whereas neither p75Fas nor p75FasTM stimulated glioma invasion as compared to the p75\textsuperscript{NTR}-negative control cells (pcDNA). Values shown are the mean ± s.e.m. from three independent experiments; (the less than symbol [<] indicates CTF; the asterisk [*] indicates ICD; triple asterisks [**+] indicate p < 0.001 vs. p75\textsuperscript{NTR} wild type, one-way ANOVA with Neuman-Keuls post-test).

(C) U87 cells stably expressing p75\textsuperscript{NTR} or the cleavage-resistant p75FasS and p75FasTM were implanted into the brains of SCID mice and allowed to grow for 28 d. The mice were sacrificed, and frozen brain sections were stained with antibodies against human nuclei (top row, brown) and human p75\textsuperscript{NTR} (bottom row, brown). Sections were counterstained with toluidine blue (blue). Scale bars in (C) represent 100 µm. Implantation of U87 glioma cells stably transfected with the empty pcDNA vector or p75\textsuperscript{NTR} cleavage-resistant chimeras (p75FasTM and p75FasS), led to the formation of well-circumscribed tumors. In marked contrast, animals implanted with U87 glioma cells ectopically expressing p75\textsuperscript{NTR} (U87p75) developed tumors with highly infiltrative edges. Similar results were seen in three independent experiments with three animals in each group.

(D) p75\textsuperscript{NTR} cleavage-resistant chimeras do not generate ICD in vivo. Brain cryosections from SCID mice implanted with U87 glioma cells expressing p75\textsuperscript{NTR}, p75FasTM, or p75FasS were lysed in 2× loading buffer. Proteins were resolved on 10% SDS-PAGE gels and probed with a p75 cytoplasmic-specific antibody. U87R, U251R, and U87p75, which express high levels of p75\textsuperscript{NTR} and exhibit increased invasive activity, process full-length p75\textsuperscript{NTR} to generate both CTF and ICD in vivo. In contrast, and consistent with the in vitro data, the p75\textsuperscript{NTR} ICD peptide was not detected in tumors from animals expressing p75FasS or p75FasTM. Cell lysates from U87 cells expressing p75\textsuperscript{NTR} and grown in the presence of epoxomicin (p75\textsuperscript{NTR}–Epo) or expressing pcDNA (pcDNA) were used as positive and negative controls, respectively. doi:10.1371/journal.pbio.0060289.g004
glioma patients expressed the early neural cell progenitor proteins Nestin, Musashi, hSox2, and CD133 (J. J. P. Kelly, S. Weiss, P. A. Forsyth, and D. L. Senger; unpublished data). In addition, four out of five glioma tumor progenitor cells in vitro expressed high levels of p75NTR as detected by immunocytochemistry (A) and western blot (B). U87 cells stably expressing pcDNA and p75NTR were used as negative and positive controls, respectively. Cultures were counterstained with DAPI (blue) to visualize the cell nucleus. (B) p75NTR expressed on the BTICs undergoes RIP. BTICs were grown in the absence and presence of 2 μM γ-secretase and/or 1 μM epoxomicin. Western blot analysis detected full-length p75NTR, CTF, and ICD with the accumulation of the 24-kDa CTF in the presence of the γ-secretase inhibitor (CompX).

(C) Brain tumor-initiating cells (BTIC) express p75NTR in vivo. BTIC cells were implanted into the brains of SCID mice and allowed to establish for 4–8 wk. Animals were sacrificed, and frozen sections were stained with anti-p75NTR (brown). Sections were counterstained with toluidine blue (blue). Tumors established from BTICs express high levels of p75NTR and form highly infiltrative tumors. M1 and M2 are individual mice.

doi:10.1371/journal.pbio.0060289.g005

Figure 5. Regulated Intramembrane Proteolysis Occurs in Brain Tumor-Derived Stem-Like Cells (BTICs) from Glioma Patient Specimens

(A and B) Primary cultures from human glioma patient tumor grown under brain tumor stem cell-promoting conditions (BTIC) express high levels of p75NTR (red) in vitro as detected by immunocytochemistry (A) and western blot (B). U87 cells stably expressing pcDNA and p75NTR were used as negative and positive controls, respectively. Cultures were counterstained with DAPI (blue) to visualize the cell nucleus. (B) p75NTR expressed on the BTICs undergoes RIP. BTICs were grown in the absence and presence of 2 μM γ-secretase and/or 1 μM epoxomicin. Western blot analysis detected full-length p75NTR, CTF, and ICD with the accumulation of the 24-kDa CTF in the presence of the γ-secretase inhibitor (CompX).

(C) Brain tumor-initiating cells (BTIC) express p75NTR in vivo. BTIC cells were implanted into the brains of SCID mice and allowed to establish for 4–8 wk. Animals were sacrificed, and frozen sections were stained with anti-p75NTR (brown). Sections were counterstained with toluidine blue (blue). Tumors established from BTICs express high levels of p75NTR and form highly infiltrative tumors. M1 and M2 are individual mice.

doi:10.1371/journal.pbio.0060289.g005

p75NTR Processing and Glioma Invasion

p75NTR as a substrate for γ-secretase [26,33,39] adds to a growing list of proteins shown to be substrates for γ-secretase cleavage, including APP [52–54], Notch [55,56], and Notch ligands Delta1 and Jagged2 [57], ErbB4 [58], CD44 [59,60], and E-cadherin [61,62]. Our in vitro data and the recent application of γ-secretase inhibitors (e.g., LY-450139 and LY-411575) in advanced clinical trials for Alzheimer disease [63–65] prompted us to investigate the use of γ-secretase inhibitors to treat highly invasive gliomas. Using an intracranial glioma model, we assessed the therapeutic potential of γ-secretase inhibitors. Parallel experiments were performed using the genetically distinct U87p75NTR and U251p75NTR glioma cell lines and the p75NTR-positive BTICs established from a patient GBM. Cells were implanted intracerebrally
Figure 6. γ-Secretase Inhibitor Significantly Blocked p75NTR Induced Glioma Migration and Invasion In Vivo

(A–C) U87p75NTR (A), U251p75NTR (B), or p75NTR-positive BTICs established from a patient GBM specimen (C) were implanted intracerebrally into SCID mice (m1–m3 and m7–m9 represent individual mice). Three days (U87p75NTR and U251p75NTR) or 5 d (BTIC) later, mice were administered s.c. 10 mg/kg γ-secretase inhibitor DAPT (γ-SI) or vehicle (corn oil) alone, once/day for 2–3 wk (three to five mice/group). The mice were sacrificed, and frozen brain sections were stained with an antibody against human p75NTR (brown). Sections were counterstained with toluidine blue to visualize the cell nucleus (blue). Control animals given vehicle alone (corn oil; middle row) developed tumors with highly infiltrative edges (top panels). In marked contrast,
animals that received daily injections of the γ-secretase inhibitor DAPT (bottom panels) developed localized tumors with demarcated edges. Similar results were seen in two independent experiments with three to five animals in each group. (D) γ-secretase inhibitor blocks p75NTR processing in vivo. Brain cryosections from three individual SCID mice implanted with U87p75NTR, U251p75NTR, or p75NTR-positive BTICs and administered s.c. 10 mg/kg γ-secretase inhibitor DAPT or vehicle (corn oil) alone were lysed in 2× loading buffer. Proteins were resolved on 10% SDS-PAGE gels and probed with a p75 cytoplasmic-specific antibody. In animals given vehicle alone, western blot analysis detected p75NTR-positive fragments migrating at 75, 24, and 19 kDa, whereas in cryosections from animals given the γ-secretase inhibitor DAPT, only the full-length p75NTR and 25-kDa CTF were detected. U87 cells expressing p75NTR and grown in the presence of epoxomicin (control; C) were used as a positive control. [The less than symbol (<) indicates CTF; the asterisk (*) indicates ICD.] (E) γ-secretase inhibitor increases survival of animals bearing U87p75NTR xenografts. Kaplan-Meier survival curves of SCID mice harboring U87p75NTR intracranial tumors given s.c. injections of 10 mg/kg γ-secretase inhibitor DAPT or vehicle (corn oil) alone, once/day beginning on day 3. Animals given the γ-secretase inhibitor (GSI) survived significantly longer than control animals (p < 0.0001).

doi:10.1371/journal.pbio.0060289.g006

Discussion

The p75NTR signaling cascade is a complex signaling axis that depends on numerous factors, including cellular context and specific protein interactions that influence biological outcomes to regulated intramembranous proteolysis of p75NTR. For example, a recent report showed that in primary cultures of cerebellar neurons, p75NTR ectodomain shedding and subsequent γ-cleavage is necessary for the growth inhibitory signal of neurotrophin dependent [15] and that this neurotrophin-induced invasion is dependent on RIP of p75NTR.

To assess the role of RIP in p75NTR-mediated glioma invasion, we used a pharmacological and molecular approach both in vitro and in vivo to demonstrate that (1) p75NTR proteolytic process occurs in glioma cell lines, surgically resected tumor specimens, and BTICs isolated from patient specimens; (2) cleavage-resistant alleles of p75NTR are insufficient to mediate glioma invasion; and (3) pharmacological inhibition with a clinically applicable γ-secretase inhibitor results in a dramatic decrease of glioma invasion both in vitro and in vivo and significantly prolonged survival of animals bearing p75NTR-positive intracranial tumors. Together, these data highlight the potential of using pharmacological inhibition to interfere with RIP as a therapeutic intervention for highly infiltrative p75NTR-positive gliomas.

One of the initial steps in regulating RIP is the shedding of the ECD by an α-secretase. This shedding event is required for subsequent cleavage of the CTF to generate an ICD. In order to show that both of these proteolytic events were important in the processing of p75NTR, we made a series of chimeric molecules with the Fas receptor, a related family member that does not generate CTF and ICD fragments [39,67]. The means by which the ECD is shed from the full-length p75NTR protein and what its biological role is are not yet understood [68–70]. Glioma cells are known to express many proteases, including serine, cysteine, and metalloproteases that are involved in invasion and tumor progression. The ADAM metalloproteinase disintegrins, including ADAM17 and ADAM10, have been described as prominent sheddases for p75NTR as well as other transmembrane type-1 receptors such as APP [71–73], with recent in vivo evidence establishing a correlation with glioma invasion and an increase in ADAM17 under hypoxic stress [74,75]. The use of inhibitors targeting these proteases may thus result in preventing RIP of p75NTR. Here, we have shown that the broad-range metalloproteinase inhibitor TAPI-2 was able to prevent both proteolytic processing of p75NTR in glioma and p75NTR-mediated invasion. Although a possible therapeutic
strategy for highly invasive p75NTR-positive tumors, previous clinical attempts to inhibit the protease-rich environment of tumors using broad-spectrum MMP inhibitors have so far proven to be ineffective as anti-cancer agents, with phase II and III trials failing to show efficacy or survival benefit [76,77]. The reason for this lack of efficacy may result in part from the fact that glioblastomas produce high levels of proteases, many of which have been suggested to help facilitate tumor cell survival and invasiveness [74,78–83]. Attempts, therefore, to inhibit the ectodomain shedding of p75NTR in a clinical setting may prove difficult.

The second proteolytic event with possible direct therapeutic importance is mediated by the γ-secretase complex, which is composed of several proteins including presenilin, nicastrin, APH-1, and presenilin enhancer 2 (PEN-2) [84]. This protein complex is known to be essential in the normal processing of amyloid β-peptides from β-APP. Abnormal accumulation of amyloid β-peptides with the formation of plaques is believed to be the pathogenesis of Alzheimer disease. Given the connection between Alzheimer disease and γ-secretase, there has been great interest in developing compounds that can inhibit this protein complex with some of these compounds already in phase II/III clinical trials [85]. The exact molecular mechanism(s) by which the ICD fragment of p75NTR exerts the invasive behavior of glioma cells is unknown. As is the case with the Notch signaling pathway [41,86,87], there have been recent studies to suggest that the ICD fragment can translocate to the nucleus, but whether it acts as part of a transcriptional complex is unclear [28,90]. In addition, myelin-associated glycoprotein binding to cerebellar neurons induces α- and γ-secretase proteolytic cleavage of p75NTR, and the resulting ICD fragment is necessary for both the activation of the small molecular weight GTPase, RhoA, and inhibition of neurite outgrowth [33]. Whether these processes or others are regulated by the p75NTR ICD fragment in glioma cells remains to be determined.

In our present study, we show that neurotrophin-induced p75NTR proteolytic processing is required for p75NTR-mediated glioma invasion in vitro and in vivo. Furthermore, daily administration of the γ-secretase inhibitor DAPT to animals bearing p75NTR-positive intracranial tumors significantly prolonged survival. These results are intriguing and support the possible clinical application of γ-secretase inhibitors for the treatment of these deadly tumors. We cannot, however, exclude the possibility that we are inhibiting the processing of other proteins that may be involved in glioma invasion since γ-secretase is known to mediate the proteolytic processing of several transmembrane proteins [52,53,55,57–61]. The biochemical evidence presented here, however, supports the hypothesis that the anti-invasive effect of γ-secretase inhibition is at least in part the result of inhibition of p75NTR RIP. Moreover, the fact that we did not observe any significant effects on proliferation or survival of the human glioma cells in vivo suggests that the dominant mechanism of activity is the inhibition of p75NTR-mediated glioma invasion.

Excitingly, we also found that a large percentage (four out of five) BTICs from primary glioma patient tissue express high levels of p75NTR. This rare population of cells with stem-like properties and the ability to repopulate the tumor [3–6] have been shown to be resistant to our current therapies (radiation and temozolomide) and thus may represent a “disease reservoir” for these devastating tumors [88,89]. Unlike the U87 parental cells, these cells are highly invasive in vivo, and treatment with a γ-secretase inhibitor dramatically blocked their invasive nature (Figure 6). Several recent studies have demonstrated strong similarities between BTICs and neural stem and progenitor cells [4,90,91]. However, whether human glioblastoma stem cells arise from mutated neural stem cells or a more mature cell type that acquires self-renewal capacity remains to be determined. Interestingly, a small population of cells (0.3%) within the stem cell niche of the adult rat subventricular zone has neurosphere-forming capacity, express p75NTR [92], and appear to be maintained from birth through adulthood [93–95]. In addition, the more migratory p75NTR glioma cell population in clinical glioblastoma patient specimens also represents a small percentage of the main tumor mass [15]. It is intriguing to note that glioma cells that express high levels of p75NTR seem to possess many characteristics of BTICs, including self-renewal, extensive brain parenchymal migration, and potential for differentiation (J. J. P. Kelly and S. Weiss, unpublished data). Whether p75NTR is an early brain tumor stem cell marker, at least for some GBMs, remains to be determined.

In a previous study, we postulated that p75NTR itself may be a valid target for the treatment of glioma, and now we propose that abrogation of the cellular processing of p75NTR represents an additional therapeutic target. Although these inhibitors may have application in malignant glioma, they may have an even broader application for cancer, as p75NTR has also been implicated in other cancers, including melanoma, specifically the more aggressive melanomas that metastasize to the brain [96–98]. Thereby, therapies that target the processing of p75NTR may also be beneficial for other metastatic cancers.

Materials and Methods

Cell culture. The human glioma cell lines U87, U118, and U343 were obtained from the American Type Culture Collection. The human glioma cell line U251N was a kind gift from V. W. Yong (University of Calgary, Calgary, Alberta, Canada). All cells were maintained in complete media (Dulbecco’s modified eagle’s medium [DMEM] F12, 50% fetal bovine serum [FBS], 0.1 mM nonessential amino acids, 2 mM l-glutamine, and 1 mM sodium pyruvate [Gibco BRL, http://www.invitrogen.com]) at 37 °C in a humidified 5% CO2 incubator. Cells were passaged by harvesting with trypsin (Gibco BRL) at 80%–90% confluence. Stable transfectants of U87, U251, U118, and U343 cells were maintained in identical media with the exception of the addition of 400 µg/ml of G418 (Invitrogen, http://www.invitrogen.com).

Construction of p75NTR cleavage-resistant plasmids. The human p75NTR expression vector was constructed as described previously [98]. The expression plasmids containing the p75NTR mutants were constructed by subcloning of PCR fragments containing the desired p75NTR sequences. Chimeric proteins were created by replacing either the transmembrane (p75FaTM) or the extracellular stalk domain of p75NTR (p75FaS) with equivalent domains from the Fas receptor [Figure 3C] as described by Domeniconi et al. 2905 [33]. The neurotrophin-binding mutant that was mutated at a four-amino acid (ARRA) insertion after amino acid residue 130 was termed p75CRD130 [15,42,43,47]. The p75NTR intracellular domain construct was created using amino acids 236–399 of the wild-type receptor plus a methionine at the amino terminus (p7-ICD). The original p75NTR template were from B. Hempstead (p73WT; Weill Medical College of Cornell University, New York, New York) and M. Chao (pT3/T7-p75; New York University School of Medicine, New York, New York). All constructs were inserted into pcDNA 3.1 expression vectors (Invitrogen). The sequences of all the mutant expression plasmids were confirmed prior to stable transfection.

Transfection of glioma cell lines. Transfection of glioma cell lines was performed as described previously [15]. Briefly, cells to be transfected were seeded at 2 × 104 cells/well in six-well plates, and
incubated at 37 °C overnight. Vector DNA was introduced to the cells using FuGENE 6 transfection reagent (Roche Diagnostic, http://www.roche.com) according to the manufacturer’s instructions. The following day, the medium was changed to fresh complete medium containing the antibiotic G418 (concentration determined by toxicity curve for each cell line) to select for those cells that had taken up the vector. Cells were grown under antibiotic selection until the desired cells were at confluence. For U87p75NTR, U251p75NTR, U118p75NTR, U87p75CRD130, U251p75FasTM, and U251p75FasS transfected cells were identified by flow cytometry and western blot analysis.

Western blotting. The desired cells were washed in ice-cold PBS and lysed by gentle rocking in lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 10 mM NaF, 0.09% NaN₃, 0.5% sodium deoxycholate, 0.1% SDS, 1% Nonidet P-40, 1% Triton X-100, 1 mM EDTA, 60 mM β-ocytol glucoside, 25 μg/ml aprotinin, 10 μg/ml leupeptin, 3 mM sodium orthovanadate, 1 mM PMSF) at 4 °C by gentle rocking for 20 min and then centrifuged at 3,000 x g for 10 min. The supernatants were removed, and protein quantification was performed using the bicinchoninic acid (BCA) assay (Pierce Biotechnology, http://www.piercenet.com). Proteins were resolved on 12% SDS-PAGE gels, and western blot analyses were performed using the following primary antibodies: rabbit polyclonal anti-human p75NTR (Promega), mouse monoclonal anti-human p75NTR ECD (Upstate Biotechnology), mouse monoclonal anti-β-tubulin (Sigma-Aldrich, http://www.sigmaaldrich.com). The antibodies were detected using a horseradish peroxidase-conjugated secondary antibody (Pierce Biotechnology) and were visualized using enhanced chemiluminescence (Amersham Biosciences).

Flow cytometric analysis of p75NTR cleavage. A total of 1 × 10⁶ cells stably transfected with p75NTR wild type or p75NTR cleavage-resistant constructs p75FasTM and p75FasS were collected using a Puck’s EDTA at 37 °C and then washed in PBS containing 1 mM EDTA (PBS/EDTA). Cells were exposed to the monoclonal anti-p75NTR, clone ME20-4 (which recognizes the extracellular domain) at 37 °C for 30 min on ice. Cells transfected with pcDNA vector alone were used as negative controls. After washing with PBS/EDTA, cells were incubated with Alexa-488 conjugated goat anti-mouse IgG (Invitrogen/Molecular Probes, http://www.probes.invitrogen.com). A total of 1 × 10⁵ cells were suspended in 10 μl of 10% FBS complete medium with the following extracellular matrix (ECM) proteins: 5 μg/ml laminin, 5 μg/ml fibronectin, 5 μg/ml chondroitin sulfate proteoglycan, Chemicon. A total of 1 × 10⁵ cells were suspended in 350 μl of collagen gel solution, and 70 μl of the collagen/cell mixture was pipetted into the Transwell chamber (five chambers for each cell line). Chambers were immediately transferred to a 37 °C incubator for 60 min to allow the matrix to polymerize. Once polymerized, 100 μl of serum-free DMEM was added to the upper chamber and 1.0 ml of 10% FBS complete medium with the γ-secretase inhibitor Compound X (2 μM) was added to the lower chamber. Transwell chambers were kept at 37 °C for 6 h, at which time the chamber was washed with PBS, fixed with 80% ethanol, and stained with hematoxylin. Any cells remaining in the top chamber were removed, and membranes were mounted on glass slides. Four different fields were counted for each membrane.

ELISA. U87 and U251 glioma cells stably transfected with p75NTR wild type or the cleavage-resistant constructs were allowed to condition culture medium for 5 d. The conditioned medium was then collected, centrifuged, and filtered through a 0.2-μm syringe filter (VWR International, http://www.vwr.com). The remaining cells were washed with ice-cold PBS, and total cellular lysates were extracted as described. Western blotting was performed using the BCA assay (Pierce Biotechnology), and BDNF, nerve growth factor (NGF), or neurotrophic factor 3 (NT-3) ELISA (R&D Systems, http://www.rndsystems.com) was performed as per the company protocol. Briefly, MaxiSorp ELISA plates (Nalgene International, http://www.nalgene.com) were coated with monoclonal anti-human BDNF, NGF, or NT-3 (R&D Systems), nonspecific binding was blocked, and then the standards of serial dilutions of recombinant human BDNF, NGF, or NT-3 (Sigma-Aldrich) and equal volumes of conditioned medium or equal volumes of control buffer were added. The plates were incubated for 2 h, washed, and nonspecific binding was blocked with the corresponding biotinylated antibody, streptavidin HRP, and a tetramethylbenzidine substrate (R&D Systems). Absorbance was measured at 450 nm.

In vitro p75NTR proteolysis. For in vitro p75NTR cleavage assessment, the desired cells were treated with 4 h at 37 °C and 5% CO₂ with the proteasome inhibitor epoxomicin (1 μM) (Calbiochem, http://www.emd-biosciences.com) and/or γ-secretase inhibitor Compound X (2 μM) (Calbiochem). DMSO was used as the vehicle control. Cells were then washed one time with cold PBS on ice, lysed in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, 1% Triton X-100, 1 mM EDTA, 60 mM β-ocytol glucoside, 25 μg/ml aprotinin, 10 μg/ml leupeptin, 3 mM sodium orthovanadate, 1 mM PMSF) at 4 °C with protease inhibitors and centrifuged for 5 min at 14,000g supernatants were identified by BCA assay and/or Western blot analysis. The conditioned medium was harvested by trypsinization, sonication, and clarified by centrifugation. The conditioned medium was analyzed using the BCA assay (Pierce Biotechnology) and/or Western blot analysis.

Circular Monolayer Migration Assay. Migration assays were performed using a microliter-scale radial monolayer migration assay as described previously [15]. Briefly, ten-well Teflon-masked microscope slides were coated with 20 μg/ml laminin, followed by the addition of 50 μl of medium to each well. Sedimentation conditions were at 37 °C and 75% humidity. The appropriate control was cell migration through the central lumen of the cell sedimentation cylinder at 2,000 cells/well for 6 h. The remaining cells were collected using Puck’s EDTA (Promega).}

**3D-invasion assay.** To test the invasive ability of the p75NTR cleavage-resistant constructs, actively growing glioma cells U87 and U251 stably transfected with p75NTR and p75NTR cleavage-resistant constructs were harvested by trypsinization, washed, and resuspended in steroid PBS/137 mM NaCl, 8.1 mM Na₂HPO₄, 2.68 mM KCl, and 1.47 mM KH₂PO₄ (pH 7.5). These cells were implanted intracerebrally into the right putamen of SCID mice (1 × 10⁵ cells/mouse) at a depth of 3 mm through a scalp incision and a 0.5-mm burr hole made 1.5–2 mm right of the midline and 0.5–1 mm posterior to the coronal suture. Animals were housed in groups of three to five and maintained on a 12-h light/dark schedule with a temperature of 22 ± 1 °C and a relative humidity of 50% ± 5%. Food and water were available ad libitum. All protocols were reviewed and approved by the University of Calgary Animal Care Committee.

**In vivo studies using an intracranial glioma model.** Actively growing glioma cells stably transfected with p75NTR and p75NTR cleavage-resistant constructs were harvested by trypsinization, washed, and resuspended in sterile PBS/137 mM NaCl, 8.1 mM Na₂HPO₄, 2.68 mM KCl, and 1.47 mM KH₂PO₄ (pH 7.5). These cells were implanted intracerebrally into the right putamen of SCID mice (1 × 10⁵ cells/mouse) at a depth of 3 mm through a scalp incision and a 0.5-mm burr hole made 1.5–2 mm right of the midline and 0.5–1 mm posterior to the coronal suture. Animals were housed in groups of three to five and maintained on a 12-h light/dark schedule with a temperature of 22 ± 1 °C and a relative humidity of 50% ± 5%. Food and water were available ad libitum. All protocols were reviewed and approved by the University of Calgary Animal Care Committee.

An intraperitoneal administration of ketamine (85 mg/kg) plus xylazine (15 mg/kg) (MTC Pharmaceuticals). The stereotactic injection used a 5-μl syringe (Hamilton Co., www.hamiltoncompany.com) with a 30-g needle mounted on a Kopf stereotactic apparatus (Kopf Instruments). After withdrawal of the needle, the incision was sutured, and animals were sacrificed at specific time points (generally weekly, from 2–6 wk postinjection) or when they lost 20% of their body weight or had difficulty ambulating, feeding, or grooming. For some experiments, BrdU was given by intraperitoneal injection 24 h prior to sacrifice. For sacrifice, mice were intracardially perfused with PBS or 4% paraformaldehyde/O.C.T. compound (Electron Microscopy Sciences, http://www.emdsciduum.com), and cryo-sectioned into 7–9-μm sections for examination by immunohistochemistry and in vivo p75NTR proteolysis assessment.

**Immunohistochemistry.** Frozen sections were air-dried at room temperature, fixed with cold acetone, and then rinsed with PBS. Endogenous peroxidases in the sections were inactivated with 0.075% H₂O₂/methanol, and nonspecific binding was blocked with 10%...
normal goat serum in PBS. The sections were incubated with rabbit polyclonal anti-human p75NTR ICD antibody (Promega) or mouse monoclonal anti-human nuclei (Chemicon) in blocking buffer overnight at 4 °C. Following washing with PBS, the appropriate biotinylated secondary antibody (Vector Laboratories, http://www.vectorlabs.com) was applied. The secondary antibodies were then detected using the VECTASTAIN Elite ABC Kit (Vector Laboratories) and detected by addition of SIGMAFAST DAB (3,3'-diaminobenzidine tetrahydrochloride) (Sigma-Aldrich), which was converted to a brown reaction product by the peroxidase. Toluidine blue (for frozen sections) was used as an nuclear counterstain. Sections were then dehydrated in an ethanol/xylene series and mounted with Entellan (Electron Microscopy Sciences).

In vivo p75NTR proteolysis. For detection of p75NTR proteolytic processing in vivo, the desired cells were implanted intracerebrally into SCID mice as described previously. Mice were sacrificed 3–4 wk later. Following sacrifice, the brains were removed, frozen in Tissue-Tek O.C.T. compound, and cryosectioned into 7–9-μm sections, and alternating sections were stained with toluidine blue. Based on the size of tumor, cryosections were lysed in 2% lysis buffer (0.1 M Tris-HCl [pH 6.8], 1% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.02% bromophenol blue). Proteins were resolved on 12% SDS-PAGE gels, and western blots were performed using an anti-p75NTR cytoplasmic specific antibody (Promega).

Detection of p75NTR proteolysis in patient specimens. Tumor and normal tissue samples were obtained from the Foothills Hospital, Calgary, Alberta. Operative samples of human gliomas were obtained during brain tumor surgery and transported to the laboratory in serum-free DMEM-F12. Primary culture of brain tumor-initiating cells (BTICs). Briefly, necrotic and ICD (indicated by an asterisk [*]; 19 kDa) peptides. In cell lysates

Survival of p75NTR-Expressing U87 Glioma Cells. Figure S2. -Secretase Inhibition Does Not Effect Proliferation or Survival of p75NTR-Expressing U87 Glioma Cells. Inhibition of -secretase does not effect proliferation or survival of the highly invasive human glioma cell lines U87R isolated by serial in vivo selection or the U87p75 cells, which ectopically express p75NTR.

In vivo selection or the U87p75 cells, which ectopically express p75NTR. The results show that 2 μM -secretase inhibitor Compound X (CompX), and survival and proliferation were generated using the Kaplan-Meier method. The log-rank test was used to compare the distributions of survival times. A p-value of less than 0.05 was considered statistically significant.

Supporting Information

Figure S1. TNF-α Protease Inhibitor (TAPI)-2 Prevents the Proteolytic Processing of p75NTR and Also Decreases the Migration of U87-p75NTR Cells

(A) A total of 7.5 × 104 U87p75 or U87pcDNA cells were plated in collagen III-coated six-well plates and treated with either normal growth medium or medium supplemented with TAPI-2 for 20 μM overnight. Western blots for p75NTR were probed with an antibody specific to the cytoplasmic domain of p75NTR, which detects full-length (75 kDa), CTF (indicated by the less than symbol [\(<\)]: 25 kDa) and ICD (indicated by an asterisk [*]; 19 kDa) peptides. In cell lysates from U87p75 glioma cells, peptides corresponding to the full-length, CTF, and ICD fragments were detected, whereas only the full-length p75NTR receptor was detected in cells treated with TAPI-2.

(B) A total of 5 × 104 U87p75 or U87pcDNA cells were plated into the upper chambers of Transwell plates coated with brain-like matrix collagen I (C, 5 μg/cm²), which was coated with plasminogen-depleted, chondroitin sulfate proteoglycans, and laminins added as minor components). The cells were treated with either normal growth medium, or medium supplemented with 20 μM TAPI-2 for 4 h. Cells were fixed/stained with 1% crystal violet in ethanol, and the cells on the under side of the membrane (migrated cells) were counted by light microscopy (n = 7; double asterisks [**] indicate p = 0.019). Found at doi:10.1371/journal.pbio.0060289.sg001 (258 KB PPT).

Figure S2. γ-Secretase Inhibition Does Not Effect Proliferation or Survival of p75NTR-Expressing U87 Glioma Cells.

Inhibition of γ-secretase does not effect proliferation or survival of the highly invasive human glioma cell lines U87R isolated by serial in vivo selection or the U87p75 cells, which ectopically express p75NTR. U87R and U87p75 were treated with and without 2 μM γ-secretase inhibitor Compound X (CompX), and survival and proliferation were assessed at 72 h by MTT assay (A) and crystal violet proliferation assay (B and C). The results show that 2 μM γ-secretase inhibitor CompX does not effect survival (A) or proliferation (B and C) of U87R and U87p75 (p > 0.05).

Found at doi:10.1371/journal.pbio.0060289.sg002 (365 KB PPT).

Figure S3. Biochemical Characterization of U87 and U251 Glioma Cells Expressing p75NTR Wild Type or Cleavage-Resistant Chimeric Alleles

(A) Expression and topography of the full-length, chimeric constructs (p75FasTM and p75FasS) and the neurotrophin-binding mutant p75CD130 at the plasma membrane were confirmed by flow
The cell lysate, consistent with the binding of BDNF to p75NTR. In produced a shift in BDNF localization from the conditioned medium to the cell lysate, consistent with the binding of BDNF to p75NTR. In contrast, cells expressing the ligand-binding mutant p75CRD130 did not produce a shift in BDNF localization in accordance with the inability to bind ligand. Values shown are the mean (s.e.m.) for a single experiment. Similar results were seen in three independent experiments; triple asterisks (***) indicate p < 0.001 as compared to pcDNA control for each cell line (one-way ANOVA with the Neuman-Keuls post-test).

Figure S4. Proteolytic Processing of p75NTR Mutant Chimeras

U87 (left panel) and U251 (right panel) stably transfected with p75NTR wild type, the cleavage-resistant chimeras (FasS and FatTM), or the neurotrophin-binding mutant CRD130 were treated with the protease-inhibitor epoxomicin (Epo, 2 μM) and the specific inhibitor of γ-secretase, Compound X (CompX, 2 μM) for 4 h. Western blots for p75NTR were probed with an antibody specific to the cytoplasmic domain of p75NTR which detects full-length (75 kDa), CTF (indicated by the less than symbol <; 25 kDa), and ICD (indicated by the asterisk [*]; 19 kDa) peptides. In cell lysates from glioma cells expressing p75FasTM chimera, only the 24-kDa fragment was detected, whereas the p75FatS chimera and the p75CRD130 ligand binding site mutant did not display any cleaved products. Similar results were seen in three independent experiments.

References

1. Scott JN, Winchester NB, Brasher PM, Fulton D, MacKinnon JA, et al. (1999) Which glioblastoma multiforme patient will become a long-term survivor? A population-based study. Ann Neurol 46: 183–188.
2. Senger D, Cairncross JS, Forsyth PA (2003) Long-term survivors of glioblastoma: stabilization or important unrecognized molecular subtype? Cancer J 9: 214–221.
3. Galli R, Binda E, Orfanelli U, Cipelletti B, Gritti A, et al. (2004) Isolation and characterization of tumorigenic, stem-like neural precursors from human glioblastoma: statistical aberration or important unrecognized molecular subtype? Cancer J 9: 214–221.
4. Hemmati HD, Nakano I, Lazarreff A, Masterman-Smith M, Geschwind DH, et al. (2003) Cancerous stem cells can arise from pediatric brain tumors. Proc Natl Acad Sci U S A 100: 15178–15183.
5. Singh SK, Clarke ID, Terasaki M, Bonn VE, Hawkins C, et al. (2003) Identification of a cancer stem cell in human brain tumors. Cancer Res 63: 5821–5828.
6. Singh SK, Hawkins C, Clarke ID, Squire JA, Bayani J, et al. (2004) Identification of human brain tumor initiating cells. Nature 432: 396–401.
7. Giese A, Loo MA, Tran N, Haskett D, Coons SW, et al. (1996) Dichotomy of glioma cell strands. Proc Natl Acad Sci U S A 93: 5854–5858.
8. Giese A, Bjerkvig R, Berens ME, Westphal M (2003) Molecular mechanisms of glioma cell migration and invasion. J Neurooncol 64: 458–478.
9. Khursigara G, Bertin J, Yano H, Moffett H, DiStefano PS, et al. (2001) A novel MAGE protein, interacts with the p75 neurotrophin receptor and modulate Rho activity and axonal outgrowth. Neuron 24: 235–248.
10. Joy AM, Beaudry CE, Tran NL, Ponce FA, Holz DB, et al. (2003) Migrating glioma cells activate the PI-3 K-pathway and display decreased susceptibility to apoptosis. J Cell Sci 116: 4409–4417.
11. Mariani L, Beaudry C, McDonough WS, Hoelzinger DB, Demuth T, et al. (2003) A population-based study. Ann Neurol 46: 183–188.
12. Mariani L, Beaudry C, McDonough WS, Hoelzinger DB, Demuth T, et al. (2003) Identification of a cancer stem cell in human brain tumors. Cancer Res 63: 5821–5828.
13. Mariani L, Beaudry C, McDonough WS, Hoelzinger DB, Demuth T, et al. (2003) Identification of human brain tumor initiating cells. Nature 432: 396–401.
14. Kleber S, Sancho-Martinez I, Wiestler B, Beisel A, Gieffers C, et al. (2008) Yes and PI3K bind CD95 to signal invasion of glioblastoma. Cancer Cell 13: 235–248.
15. Johnston AL, Lue N, Rahn J, Lincioni A, Wang L, et al. (2007) The p75 neurotrophin receptor central regulator of glioma invasion. PLoS Biol 5: e212. doi:10.1371/journal.pbio.0050212.
16. Reichardt LF (2006) Neurotrophin-regulated signalling pathways. Philos Trans R Soc Lond B Biol Sci 361: 1543–1564.
17. Yamashita T, Tolyama M (2003) The p75 receptor acts as a displacement factor for the colony-stimulating factor 1 receptor that releases Rho effectors Rho-GEF. Nat Neurosci 6: 461–467.
18. Yamashita T, Tucker KL, Barde YA (1999) Neurotrophin binding to the p75 receptor modulates Rho activity and axonal outgrowth. Neuron 24: 585–593.
19. Khursigara G, Bertin J, Yano H, Moffett H, DiStefano PS, et al. (2001) A prosurvival function for the p75 receptor death domain mediated via the caspase recruitment domain receptor-interacting protein 2. J Neurosci 21: 1624–1636.
20. Mukai J, Shoji S, Kinumura MT, Okubo S, Sano H, et al. (2002) Structure-function analysis of NAD: identification of regions that mediate nerve growth factor-induced apoptosis. J Biol Chem 277: 13973–13982.
21. Chittka A, Chao MV (1999) Identification of a zinc finger protein whose subcellular distribution is regulated by serum and nerve growth factor. Proc Natl Acad Sci U S A 96: 10705–10710.
22. Salehi AH, Roux PP, Kubu CJ, Zeindler C, Bhakar A, et al. (2000) NRAGE, a novel MAGE protein, interacts with the p75 neurotrophin receptor and modulate apoptosis induction. J Biol Chem 275: 30202–30208.
23. Xie Y, Mehlen P, Rahiizadeh S, VanArsdale T, Zhang H, et al. (1999) TRAF family proteins interact with the common neurotrophin receptor and modulate apoptosis induction. J Biol Chem 274: 30202–30208.
24. Irie S, Hachiya T, Rabizadeh S, Maruyama W, Mukai J, et al. (1999) Functional interaction of Fas-associated phosphatase-1 (FAP-1) with p75(NTR) and their effect on NF-kappaB activation. FEBs Lett 460: 191–198.
25. Parolis-Piquard R, Petit A, Kawarai T, Sunycha C, Alves da Costa C, et al. (2005) Presenilin-dependent transcriptional control of the Abeta-degrading enzyme neuraminidase by intracellular domains of betaAPP and APLP. Neuron 46: 541–554.

Acknowledgments

We thank Dr. Barbara Hempstead (Cornell University, New York) and Dr. Moses Chao (New York University, New York) for providing cDNAs. We would also like to thank Laurie Robertson for her technical assistance with the flow cytometry, and the Brain Tumor Stem Cell Core that is funded by funds from the Hotchkiss Brain Institute at the University of Calgary and the Stem Cell National Centre of Excellence.

Author contributions. JJR, XL, SMR, PAF, and DLS conceived and designed the experiments. LW, JJR, XL, BS, and JJPK performed the experiments. LW, SMR, PAF, and DLS analyzed the data. JJPK and SW contributed reagents/materials/analysis tools. LW, SMR, and DLS wrote the paper.

Funding. LW was supported by a fellowship from the Alberta Heritage Foundation for Medical Research (AHFMR). JJR was supported by a fellowship from the Translational Research Training Program from funds from the Alberta Cancer Board (ACB) and the Canadian Institutes of Health Research (CIHR), and more recently by a fellowship from the Jocle Syverson American Brain Tumor Association Fellowship. JJPK was funded by a Clinical AHFMR fellowship, SW and SMR are scientists of the AHFMR, and SMR currently holds a Canada Research Chair in Cancer Biology. DLS is supported by funds from the ACB. This work was supported in part by a generous donation from the Clark H. Smith Family and by grants from the CIHR (PAF and DLS) and ACB (SMR).

Competing interests. The authors have declared that no competing interests exist.
26. Kanning KC, Hudson M, Amieux PS, Wiley JC, Bothwell M, et al. (2003) Proteolytic processing of the p57 neurotrophin receptor and two homologs generates C-terminal fragments with signaling capability. J Neurosci 23: 5425–5436.

27. Lachance C, Glarner A, Aloyz R, Zelinger S, et al. (1997) Transgenic mice expressing the intracellular domain of the p75 neurotrophin receptor undergo neuronal apoptosis. J Neurosci 17: 6988–6998.

28. Poddlepy S, Kichev A, Pedrazza C, Saurat J, Encinas M, et al. (2006) Pro-NGF from Alzheimer’s disease and normal human brain displays distinctive abilities to induce shedding and nuclear translocation of intracellular domain of p75NTR and apoptosis. Am J Pathol 169: 119–131.

29. Jung KM, Tan S, Landman N, Petrova K, Murray S, et al. (2003) Regulated intramembrane proteolysis of the p75 neurotrophin receptor modulates its association with the TRA receptor. J Biol Chem 278: 42161–42169.

30. Frade JM (2005) Nuclear translocation of the p75 neurotrophin receptor cytoplasmic domain in response to neurotrophin binding. J Neurosci 25: 1407–1417.

31. Pascual CV, Ferre E, Dickson KM, Barker PA (2004) A pro-apoptotic fragment of the p75 neurotrophin receptor is expressed in p75NTRExonIV null mice. J Neurosci 24: 1917–1923.

32. Ahmed Z, Mazibrada G, Seabright RJ, Dent RG, Berry M, et al. (2006) TACE-induced cleavage of NgR and p75NTR in dorsal root ganglion cultures disinhbits outgrowth and promotes branching of neurites in the presence of inhibitory CNS myelin. FASEB J 20: 1939–1941.

33. Domeniconi M, Zampieri N, Spencer T, Hilaire M, Mellado W, et al. (2005) MAG induces regulated intramembrane proteolysis of the p75 neurotrophin receptor to inhibit neurite outgrowth. Neuron 46: 849–855.

34. Kenchappa RS, Zampieri N, Chao MV, Barker PA, Teng HK, et al. (2006) Ligand-dependent cleavage of the p75 neurotrophin receptor is necessary for NRP1 nuclear translocation and apoptosis in sympathetic neurons. Neuron 50: 219–232.

35. Ober G, Li J, Paulus K, Wolf E, Gurney M, et al. (2001) The Notch intracellular domain is ubiquitinated and negatively regulated by the mammalian Sel-10 homolog. J Biol Chem 276: 35847–35853.

36. Black RA, Rauch CT, Kozlowski CJ, Peschon J, Stack JB, et al. (1997) A metalloproteinase disintegrin that releases tumour-necrosis-factor-alpha from cells. Nature 385: 729–733.

37. Franzke CW, Tasan K, Schacke H, Zhou Z, Tryggvason K, et al. (2002) Transmembrane collagen XVII, an epithelial adhesion protein, is shed from the cell surface by ADAMs. EMBO J 21: 5026–5035.

38. Moss ML, Rasmussen FH (2007) Fluorescent substrates for the proteinases ADAM17, ADAM10, ADAM2, and ADAM12 useful for high-throughput proteinase screening. Anal Biochem 366: 144–148.

39. Zampieri N, Xu CF, Neubert TA, Chao MV (2005) Cleavage of P75 neurotrophin receptor by alpha-secretase and gamma-secretase requires specific receptor domains. J Biol Chem 280: 14563–14571.

40. Kopan R, Ilagan MX (2004) Gamma-secretase: proteasome of the membrane? Nat Rev Mol Cell Biol 5: 499–504.

41. Mumms JS, Kopan R (2000) Notch signaling from the outside in. Dev Biol 228: 151–165.

42. Yan H, Chao MV (1991) Disruption of cysteine-rich repeats of the p75 neurotrophin receptor leads to loss of high affinity nerve growth factor receptor. J Biol Chem 266: 12999–12124.

43. Baldwin AN, Butler CM, Wilcher AA, Shooter EM (1993) Studies on the structure and binding properties of the cysteine-rich domain of rat low affinity nerve growth factor receptor (p75NFGFR). J Biol Chem 267: 8352–8358.

44. Wilcher AA, Butler CM, Radeke MJ, Shooter EM (1991) Nerve growth factor binding domain of the nerve growth factor receptor. Proc Natl Acad Sci U S A 98: 159–163.

45. Johnson D, Lanahan A, Buck CR, Sehgal A, Morgan C, et al. (1986) Expression and structure of the human NGF receptor. Cell 47: 543–554.

46. Hempstead BL, Partal N, Thiel B, Chao MV (1999) Deletion of cytoplasmic sequences of the nerve growth factor receptor leads to loss of high affinity ligand binding. J Biol Chem 264: 12999–12104.

47. Baldwin AN, Butler CM, Wilcher AA, Shooter EM (1992) Studies on the structure and binding properties of the cysteine-rich domain of rat low affinity nerve growth factor receptor (p75NFGFR). J Biol Chem 267: 8352–8358.

48. Wilcher AA, Butler CM, Radeke MJ, Shooter EM (1991) Nerve growth factor binding domain of the nerve growth factor receptor. Proc Natl Acad Sci U S A 98: 159–163.

49. De Strooper B, Safigt P, Craebersaa K, Vanderstichele H, Ghede G, et al. (1998) Deficiency of presenilin-1 inhibits the normal cleavage of amyloid precursor protein. Nature 391: 387–390.

50. Tanaka H, Yano K, Tanaka K, Otsuka T, Harada M, et al. (2000) Pro-NGF from Alzheimer’s disease and normal human brain displays distinctive abilities to induce shedding and nuclear translocation of intracellular domain of p75NTR and apoptosis. Am J Pathol 169: 119–131.

51. Jung KM, Tan S, Landman N, Petrova K, Murray S, et al. (2003) Regulated intramembrane proteolysis of the p75 neurotrophin receptor modulates its association with the TRA receptor. J Biol Chem 278: 42161–42169.

52. Frade JM (2005) Nuclear translocation of the p75 neurotrophin receptor cytoplasmic domain in response to neurotrophin binding. J Neurosci 25: 1407–1417.

53. Pascual CV, Ferre E, Dickson KM, Barker PA (2004) A pro-apoptotic fragment of the p75 neurotrophin receptor is expressed in p75NTRExonIV null mice. J Neurosci 24: 1917–1923.
profiling proteases and inhibitors in human cancer cells. Mol Cancer Res 1: 333–345.

82. Rao JS (2003) Molecular mechanisms of glioma invasiveness: the role of proteases. Nat Rev Cancer 3: 489–501.

83. Wildeboer D, Naus S, Amy Sang QX, Bartsch JW, Pagenstecher A (2006) Metalloproteinase disintegrins ADAM8 and ADAM19 are highly regulated in human primary brain tumors and their expression levels and activities are associated with invasiveness. J Neuropathol Exp Neurol 65: 516–527.

84. Kimberly WT, LaVoie MJ, Ostazewski BL, Ye W, Wolfe MS, et al. (2003) Gamma-secretase is a membrane protein complex comprised of presenilin, nicastrin, Aplh-1, and Pen-2. Proc Natl Acad Sci U S A 100: 6382–6387.

85. Wildeboer D, Naus S, Amy Sang QX, Bartsch JW, Pagenstecher A (2006) Metalloproteinase disintegrins ADAM8 and ADAM19 are highly regulated in human primary brain tumors and their expression levels and activities are associated with invasiveness. J Neuropathol Exp Neurol 65: 516–527.

86. Kimberly WT, LaVoie MJ, Ostazewski BL, Ye W, Wolfe MS, et al. (2003) Gamma-secretase is a membrane protein complex comprised of presenilin, nicastrin, Aplh-1, and Pen-2. Proc Natl Acad Sci U S A 100: 6382–6387.

87. DeAngelo DJ, Stone RM, Heaney ML, Nimer SD, Paquette RL, et al. (2006) Phase I clinical results with tandutinib (MLN518), a novel FLT3 antagonist, in patients with acute myelogenous leukemia or high-risk myelodysplastic syndrome: safety, pharmacokinetics, and pharmacodynamics. Blood 108: 3674–3681.

88. Bao S, Wu Q, Sathornsumetee S, Hao Y, Li Z, et al. (2006) Stem cell-like glioma cells promote tumor angiogenesis through vascular endothelial growth factor. Cancer Res 66: 7843–7848.

89. Clement V, Sanchez P, de Tribolet N, Radovanovic I, Ruiz i Altaha A (2007) HEDGEHOG-GLI1 signaling regulates human glioma growth, cancer stem cell self-renewal, and tumorigenicity. Curr Biol 17: 165–172.

90. Lee J, Kotlarova S, Kotlarov Y, Li A, Su Q, et al. (2006) Tumor stem cells derived from glioblastomas cultured in bFGF and EGF more closely mirror the phenotype and genotype of primary tumors than do serum-cultured cell lines. Cancer Cell 9: 391–403.

91. Phillips TM, McBride WH, Pajonk F (2006) The response of CD24(-)/low/CD44+ breast cancer-initiating cells to radiation. J Natl Cancer Inst 98: 1777–1785.

92. Young KM, Merson TD, Sotthibundhu A, Coulson EJ, Bartlett PF (2007) p75 neurotrophin receptor expression defines a population of BDNF-responsive neurogenic precursor cells. J Neurosci 27: 5446–5455.

93. Yan Q, Johnson EM Jr (1989) Immunohistochemical localization and biochemical characterization of nerve growth factor receptor in adult rat brain. J Comp Neurol 290: 585–598.

94. Calza L, Giardino L, Pozza M, Betti C, Micera A, et al. (1998) Proliferation and phenotype regulation in the subventricular zone during experimental allergic encephalomyelitis: in vivo evidence of a role for nerve growth factor. Proc Natl Acad Sci U S A 95: 3209–3214.

95. Giuliani A, D’Intino G, Paradini M, Giardino L, Calza L (2004) p75(NTR)-immunoreactivity in the subventricular zone of adult male rats: expression by cycling cells. J Mol Histol 35: 749–758.

96. Herrmann JL, Menter DG, Hamada J, Marchetti D, Nakajima M, et al. (1993) Mediation of NGF-stimulated extracellular matrix invasion by the human melanoma low-affinity p75 neurotrophin receptor: melanoma p75 functions independently of trkA. Mol Biol Cell 4: 1205–1216.

97. Menter DG, Herrmann JL, Marchetti D, Nicolson GL (1994) Involvement of neurotrophins and growth factors in brain metastasis formation. Invasion Metastasis 14: 372–384.

98. Shonukan O, Bagayogo I, McCrea P, Chao M, Hempstead B (2003) Neurotrophin-induced melanoma cell migration is mediated through the actin-bundling protein fascin. Oncogene 22: 3616–3623.