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ORIGINAL ARTICLE

Glioma invasion mediated by the p75 neurotrophin receptor (p75^{NTR}/CD271) requires regulated interaction with PDLIM1

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The invasive nature of glioblastoma renders them incurable by current therapeutic interventions. Using a novel invasive human glioma model, we previously identified the neurotrophin receptor p75^{NTR} (aka CD271) as a mediator of glioma invasion. Herein, we provide evidence that preventing phosphorylation of p75^{NTR} on S303 by pharmacological inhibition of PKA, or by a mutational strategy (S303G), cripples p75^{NTR}-mediated glioma invasion resulting in serine phosphorylation within the C-terminal PDZ-binding motif (SPV) of p75^{NTR}. Consistent with this, deletion (ΔSPV) or mutation (SPM) of the PDZ motif results in abrogation of p75^{NTR}-mediated invasion. Using a peptide-based strategy, we identified PDLIM1 as a novel signaling adaptor for p75^{NTR} and provide the first evidence for a regulated interaction via S425 phosphorylation. Importantly, PDLIM1 was shown to interact with p75^{NTR} in highly invasive patient-derived glioma stem cells/tumor-initiating cells and shRNA knockdown of PDLIM1 *in vitro* and *in vivo* results in complete ablation of p75^{NTR}-mediated invasion. Collectively, these data demonstrate a requirement for a regulated interaction of p75^{NTR} with PDLIM1 and suggest that targeting either the PDZ domain interactions and/or the phosphorylation of p75^{NTR} by PKA could provide therapeutic strategies for patients with glioblastoma.

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INTRODUCTION

Human malignant glioma is one of the most common adult primary central nervous system tumors with a median survival of only 14.6 months after diagnosis.[1,2] A major barrier to effective treatment of glioblastoma is their highly invasive nature; they extend tendrils several centimeters away from the main tumor mass rendering them incurable by localized therapy including surgery or radiotherapy.[3,4] Invading malignant glioma cells comprise a cell population that are genotypically and phenotypically distinct from their noninvasive counterparts, activating a number of coordinate cellular programs including those necessary for migration, invasion and survival.[4–12] Many individual genes have been implicated in glioma invasion and recently studies identified a subclass of glioma-expressing genes involved in cell migration and invasion that strongly correlate with poor patient survival.[13–17]

We previously found that the neurotrophin receptor, p75^{NTR}, was upregulated in invasive glioma cells[18,19] and established p75^{NTR} as a major contributor to their invasive nature.[18,19] p75^{NTR} is a transmembrane glycosylated receptor expressed by neurons, neural stem cells, astrocytes, oligodendrocytes precursors and Schwann cells[20] where it functions through interactions with several ligands and co-receptors[21–23] to mediate cell death, survival, migration and axonal growth inhibition (reviewed in Reichardt[23] and Kraemer *et al.*[24]). Using an unbiased approach, we found that p75^{NTR} through a neurotrophin-dependent mechanism regulates the invasive behavior of glioma cells,[18,19] a function also observed in Schwann cells, neural crest cells, melanocytes and melanoma.[25–31]

Although the precise elucidation of p75^{NTR} downstream signaling remains unknown, a number of effector pathways have been uncovered including RhoA,[32] JNK,[33] MAPK,[34] NFκB,[26] ceramide[35] and HIF.[36] Although the exact biological implications of these signaling events are largely undefined, cellular context, localization and proteolytic processing are major determinants for p75^{NTR}-mediated events.[24,37,38] p75^{NTR} itself does not contain intrinsic kinase activity and instead requires association with other proteins,[24] some of which require the phosphorylation of the receptor by kinases such as the cAMP-dependent protein kinase PKA, where in cerebellar neuron phosphorylation by PKA regulates the translocation of p75^{NTR} to lipid rafts.[39] p75^{NTR} also contains protein interaction domains including a PDZ-binding motif at the extreme C-terminus of the receptor shown to interact with the Fas-associated phosphatase (FAP-1) in 293T cells[40] and Par3 during Schwann cell myelination.[41] PDZ domains have been found in a number of intracellular signaling proteins[42,43] and, in the context of a LIM domain, can interact with the actin cytoskeleton.[44–48] PDLIM1 is a member of the ALP subfamily of PDZ/LIM proteins, which are postulated to have a role in actin cytoskeleton architecture.[46,49–54]

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There is mounting evidence that the neurotrophin receptor family and their respective ligands are rationale therapeutic targets. For example, p75^{NTR} (CD271) has been shown to be a crucial molecule driving melanoma initiation, self-renewal, invasion and metastasis,[30,55,56] features also associated with glioma.[18,19,57–60] We have previously provided evidence for the targeting of p75^{NTR} using clinically applicable γ-secretase inhibitors that prevent intramembrane proteolysis of p75^{NTR}, a prerequisite for glioma invasion.[18,19] Use of γ-secretase inhibitors have, however, generated considerable debate owing to the numerous cellular targets and unforeseen clinical complications.[61,62] Thereby, the identification of pathways utilized by p75^{NTR} to drive cancer invasion and tumorigenesis would provide alternate avenues for therapeutic targeting of this signaling axis. Thus, as an alternative approach, we undertook defining the molecular signaling downstream of p75^{NTR} that are required to mediate glioma invasion. Herein, we provide evidence that phosphorylation of p75^{NTR} by PKA modulates the phosphorylation of specific amino acid residues within the cytoplasmic domain of p75^{NTR} resulting in a regulated interaction with PDLIM1, a novel signaling adaptor required for p75^{NTR}-mediated glioma invasion.

RESULTS

PKA inhibition abrogates p75^{NTR}-induced glioma invasion

PKA has been shown to induce serine phosphorylation of the cytoplasmic tail of p75^{NTR}, an event required for lipid-raft localization and downstream signaling in cerebellar neurons.[39] Consistent with a role for compartmentalized signaling cholesterol depletion by cholesterol oxidase or methyl-β-cyclodextrin abolished the ability to mediate glioma invasion *in vitro* (Supplementary Figure S1) suggesting a role for lipid rafts and perhaps PKA for p75^{NTR}-mediated glioma invasion. To determine whether PKA activity is important for p75^{NTR}-regulated activities in glioma, we checked whether PKA activation could promote glioma invasion. Using a PKA-selective pharmacological inhibitor KT5720, invasive U87 human glioma cells stably expressing p75^{NTR} (CD271) (U87p75^{NTR}) were assessed for their ability to invade collagen. Treatment of U87p75^{NTR} cells with KT5720 resulted in a dose-dependent inhibition of invasion as compared with noninvasive U87pcDNA cells (Figure 1a). This inhibitory effect was also observed in a highly invasive glioma cell line established by serial *in vivo* selection (U87R) in which p75^{NTR} also regulates its invasive behavior[18] (Figure 1b), and importantly, in three independent p75^{NTR} expressing patient-derived primary cultures, herein referred to as brain tumor-initiating cells ((BT042, BT134, BT147) Figures 1c and d). This decrease in invasion was also observed following treatment with the adenylyl cyclase inhibitor 2′, 5′-dideoxyadenosine which inhibits the production of cAMP (Figure 1e). Moreover, glioma cells expressing p75^{NTR} had significantly higher levels of cAMP as assessed using a transcriptional CRE-reporter assay, and transcriptional activity was further augmented by the adenylyl cyclase activator forskolin (1 μM) (Figure 1f). Taken together, these data suggest that cAMP/PKA-induced p75^{NTR} phosphorylation is required for glioma invasion.

Mutation of the PKA phosphorylation site (S303G) abrogates p75^{NTR}-mediated invasion

In mouse cerebellar neurons, PKA phosphorylates the cytoplasmic tail of p75^{NTR} on serine 304 (human equivalent S303).[39] To assess the importance of S303 phosphorylation by PKA and its potential role in p75^{NTR}-mediated glioma invasion, U87 cells stably expressing a mutant construct of p75^{NTR}/CD271 with a single point mutation at S303 were generated (p75S303G; Figure 2a) and assessed for receptor localization, topology and ability to bind neurotrophin. Previously, we demonstrated that glioma cells secrete high levels of the p75^{NTR} ligand BDNF and upon p75^{NTR}

expression the ligand becomes cell associated, presumably bound to p75^{NTR}.[18] Flow cytometry analysis using a monoclonal antibody specific to the extracellular domain of p75^{NTR} confirmed plasma membrane expression and correct topography of p75S303G (Supplementary Figure S2). In addition, glioma-derived BDNF was localized to the cell membrane, similar to the wild-type receptor, suggesting that BDNF binding was not compromised in the mutant (Supplementary Figure S3).

The p75S303G cells were then assessed for their invasive ability. Mutation of p75^{NTR} at S303 resulted in a significant decrease in invasion compared with the wild-type p75^{NTR} despite these constructs being expressed at comparable levels *in vitro* (Figures 2b and c). To confirm whether the loss of invasive ability *in vitro* was maintained *in vivo*, p75S303G cells were implanted into the brains of immunocompromised (SCID) mice. U87p75^{NTR} or U87pcDNA were used for comparison. Twenty-eight days after implantation, mice were killed and formalin-fixed paraffin-embedded brain sections were stained with Hematoxylin & Eosin (Figure 2d, left panel) or antibodies against human nucleolin or the extracellular matrix protein tenascin C[63] to visualize the tumor (Figure 2d, center and right panel). Implantation of U87pcDNA glioma cells formed well-circumscribed tumors, while U87p75^{NTR} glioma cells formed tumors with highly infiltrative edges. In striking contrast to the invasive U87p75^{NTR} and consistent with the *in vitro* data, p75S303G-expressing tumors were well circumscribed, similar to the U87pcDNA. Comparable results were seen in three independent experiments. Taken together, these data suggest that phosphorylation of p75^{NTR} at S303 is required for p75^{NTR} mediated-glioma invasion.

As we have demonstrated that p75^{NTR} undergoes γ-secretase-mediated processing in human glioma, and this was required for p75^{NTR}-induced invasion,[19] the possibility existed that mutation of the PKA phosphorylation site would affect regulated intramembrane proteolysis of p75^{NTR}. However, as observed in Figure 2b, both the C-terminal fragment (α-secretase generated) and intracellular domain fragment (γ-secretase generated) were detected in p75S303G-expressing cells *in vitro*. These data suggest that in addition to intramembrane proteolysis of p75^{NTR}, other elements are required for the invasive behavior, such as posttranslational modifications and/or specific subcellular localization. In addition, although no difference was observed in the overall growth or viability in the presence of the PKA inhibitor KT5720 or in the PKA-defective p75^{NTR}-expressing glioma cells *in vitro,* a reproducible reduction in bromodeoxyuridine incorporation was observed *in vivo* (Figure 2e) suggesting that the brain microenvironment has an impact on the growth rate or the cellular viability of these cells.

PKA regulates the phosphorylation of Serine425 in the p75^{NTR}-PDZ-binding motif

To confirm that S303 on p75^{NTR} is phosphorylated in human glioma, we assessed endogenous phosphorylation by nano-liquid chromatography–tandem mass spectrometry. Phosphorylation of S303 was detected on the wild-type receptor but no phosphorylation was detected at amino acid 303 in p75S303G cells (Figure 3a). Phosphorylation was, however, detected on seven other serine residues (287, 303, 305, 308, 311, 313, 325 and 425) including a previously unidentified phosphorylation event on S425 of p75^{NTR} (Figure 3b). This newly identified phosphorylation site is located within the extreme C-terminus of the protein and is part of a PDZ-binding motif.[40,64] Phosphorylation of S425 was also observed in cells treated with the PKA inhibitor KT5720 suggesting a regulatory role for this specific phosphorylation event (Figure 3b).
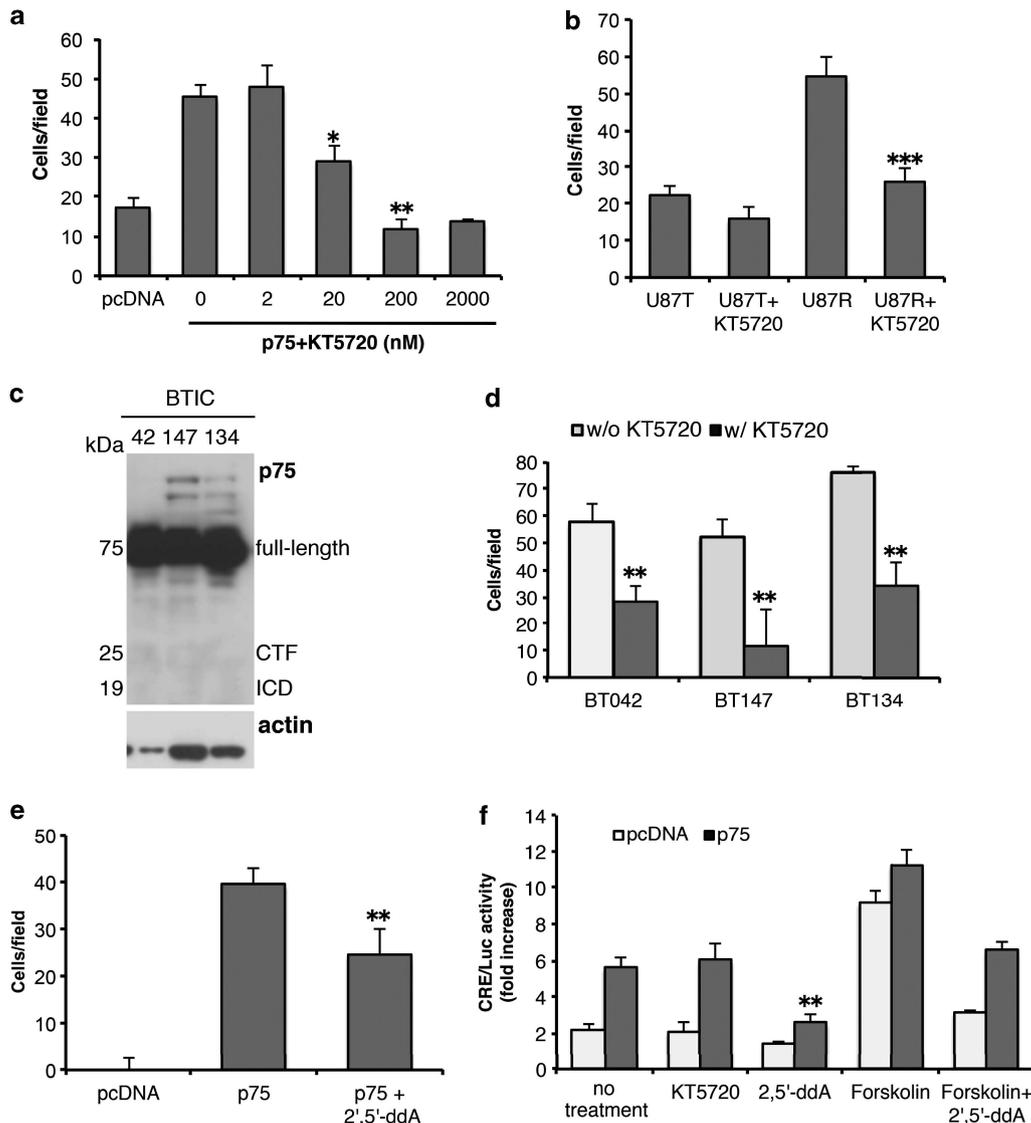
Figure 1. PKA inhibition significantly abrogated p75[NTR]-induced glioma invasion. (**a**) Treatment with increasing concentrations of the PKA inhibitor KT5720 (KT) inhibited invasion of U87 cells expressing full-length p75[NTR] (p75). Following pre-treatment with KT5720 for 1 h, the invasive ability of the U87 cells expressing full-length p75[NTR] (p75) or empty vector (pcDNA; control) were determined using collagen-coated transwells. Similar results were observed in two independent experiments. Asterisk (*) indicate $P < 0.05$ and double asterisks (**) indicate $P < 0.01$ versus non-treated U87p75 cells (one-way ANOVA with the Newman-Keuls post-test). (**b**) Highly invasive U87R and noninvasive U87T cells were isolated by serial *in vivo* selection and assessed for their invasive ability in the absence or presence of KT5720 (200 nM). Asterisks (***) indicate $P < 0.001$ as compared with non-treated cells. (**c**) Western blot shows the expression of p75[NTR] in patient-derived glioma cells BT042, BT147 and BT134. (**d**) Histogram shows that the invasion of the patient-derived glioma cells (BTIC) in the presence of fetal bovine serum was suppressed by KT5720 (200 nM). Asterisks (**) indicate $P < 0.01$ as compared with the corresponding non-treated BTIC. (**e**) p75[NTR]-mediated glioma invasion is dependent on cAMP. U87pcDNA or p75 cells were treated for 1 h with an adenylyl cyclase inhibitor (2′, 5′-dideoxyadenosine(2′, 5′-ddA)) and invasion assays (described above) were performed. Asterisk (**) indicate $P < 0.01$ versus non-treated U87p75 cells. (**f**) U87p75[NTR] treated with the PKA inhibitor KT5720 (1 h, 200 nM), 2′, 5′-ddA (1 h, 30 μM) or the adenylyl cyclase activator forskolin (1 μM) in the presence or absence of 2′, 5′-ddA were assessed for cAMP activity using a CRE-luciferase reporter construct. Histogram shows the fold increases in luciferase activity as compared with vehicle-treated cells. Asterisk (**) indicate $P < 0.01$ versus non-treated U87p75 cells. Data represent the mean ± s.e.m. of triplicates, representative of at least three independent experiments.

Mutation of the p75[NTR]-PDZ-binding motif reduces glioma invasion

To assess the physiological relevance of phosphorylation at S425 and the importance of the C-terminal PDZ-binding domain of p75[NTR], we generated two specific p75[NTR] mutants, both predicted to compromise p75[NTR] binding to PDZ-containing effector proteins.[40,64] The first construct was generated by deleting the entire PDZ-binding motif (SPV) at the extreme C-terminal of p75[NTR] (ΔSPV; Figure 4a). In the second the C-terminal valine was

substituted with methionine (SPM; Figure 4a). U87 glioma cells were stably transfected with ΔSPV or SPM constructs and receptor expression (Figure 4b; Supplementary Figure S4) and BDNF binding (Supplementary Figure S5) was confirmed. Disruption of the PDZ-binding domain resulted in a significant decrease in p75[NTR]-mediated invasion *in vitro* (Figure 4c). To confirm this *in vivo*, these cells were implanted into the brains of SCID mice and allowed to grow for 28 days. The mice were killed, and paraffin-embedded brain sections were stained with antibodies against human nuclei (Figure 4d; brown,
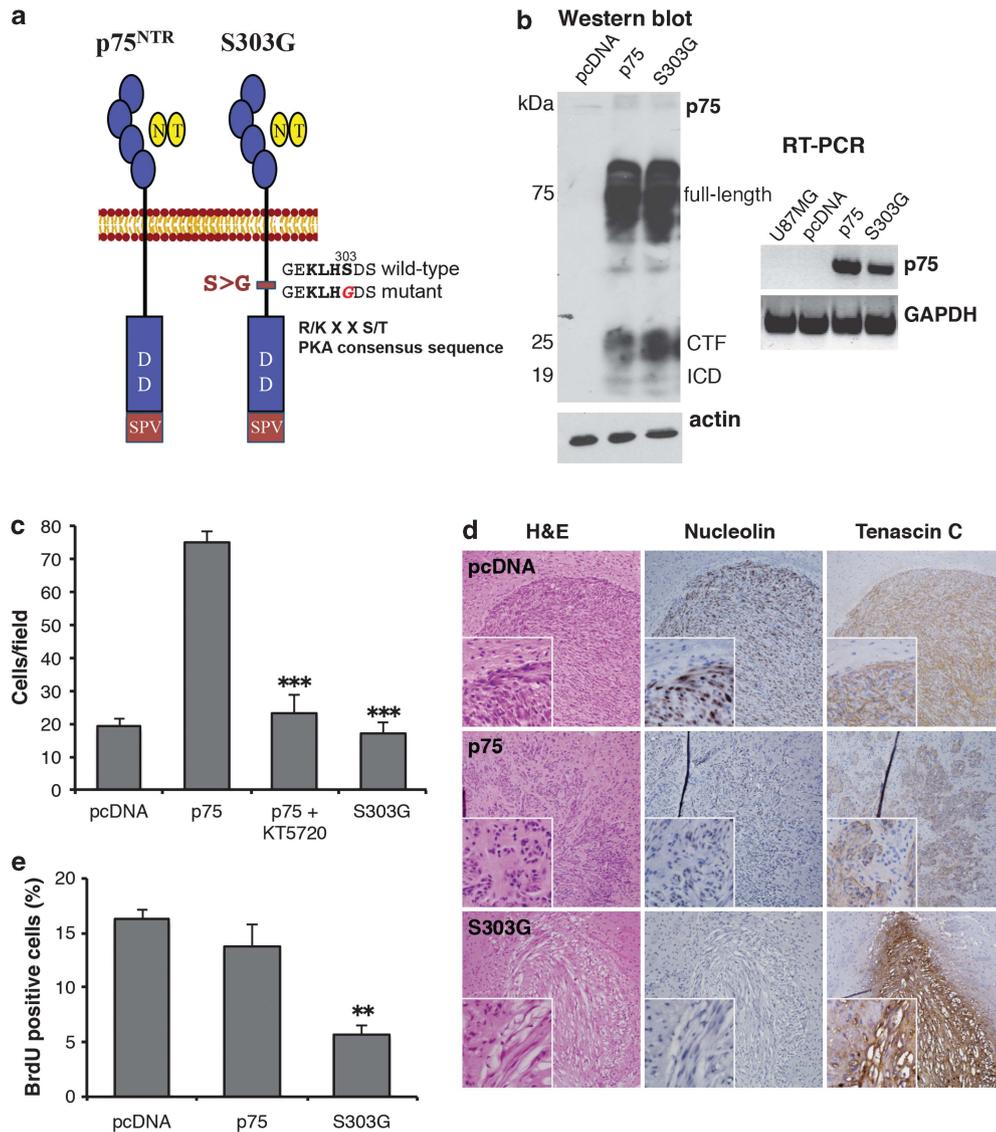
Figure 2. Mutation of the PKA phosphorylation site (S303G) abrogates p75^{NTR}-mediated invasion *in vitro* and *in vivo*. (**a**) Schematic diagram of wild-type and mutant p75^{NTR} constructs. p75^{NTR} has one putative PKA recognition consensus site (R/K X X S/T; *KLHS*) within the intracellular domain (ICD) of the protein. A PKA phosphorylation-defective mutant was generated by substitution of amino acid S303 by glycine (S303G). (**b**) U87MG glioma cells were stably transfected with pcDNA3.1 (pcDNA), pcDNA 3.1 containing wild-type p75^{NTR} (p75) or pcDNA 3.1 containing the S303G mutant of p75^{NTR}. RT–PCR and western blot confirm the expression of the wild-type and mutant receptors. RT–PCR analysis of GAPDH and western blot analysis of actin were included as loading controls. Western blot analysis detected the full-length p75^{NTR}, the 25-kDa C-terminal fragment (CTF) and the 19-kDa ICD of p75^{NTR}. (**c**) Invasion of S303G cells in collagen-coated transwell chambers was significantly decreased compared with wild-type p75^{NTR}-expressing cells (p75). Noninvasive U87pcDNA were used for comparison. Histogram shows the mean ± s.e.m. from three independent experiments; asterisks (***) indicate $P < 0.001$ as compared with non-treated U87p75 (one-way ANOVA with the Neuman-Keuls post-test). (**d**) pcDNA, p75 and S303G cells (5×10^4 cells) were implanted intracerebrally into SCID mice and allowed to grow for 28 days. The mice were killed, and paraffin brain sections were stained with Hematoxylin & Eosin (H&E) or antibodies against human nucleolin or tenascin-C (brown) to visualize the tumors. Sections were counterstained with hematoxylin (blue). (**e**) *In vivo* tumor proliferation was determined by injecting bromodeoxyuridine (BrdU) into tumor-bearing mice 24 h prior to killing of the mice. Frozen brain sections were stained with an antibody against BrdU and counterstained with toluidine blue to visualize the cell nucleus. Cells that had divided during the 24 h prior to killing of the mice stained positively for BrdU, and the percentage of BrdU-positive cells was counted. Histogram represents the percentage of BrdU-positive cells in five consecutive fields. Values shown are the mean ± s.e.m. from five independent mice; asterisks (**) indicate $P < 0.01$ as compared with control U87pcDNA (one-way ANOVA with the Neuman-Keuls post-test).

center panel) and p75^{NTR} (Figure 4d; brown, right panel). Disruption of the PDZ-binding motif of p75^{NTR} resulted in tumors with distinctly demarcated edges. Moreover, tumors generated from glioma cells expressing either SPM or ΔSPV p75^{NTR} mutants were smaller and showed significant reduction of bromodeoxyuridine incorporation *in vivo* (Figure 4e), a result analogous to tumors generated using the PKA phosphorylation-defective U87

cells. These data suggest that the PDZ-binding domain of p75^{NTR} is required for p75^{NTR}-mediated glioma invasion.

PDLIM1 directly interacts with the PDZ-binding motif of p75^{NTR} an interaction that is regulated by phosphorylation of S425
As the PDZ-binding domain was important for p75^{NTR}-mediated glioma invasion, we sought to identify the PDZ domain-containing
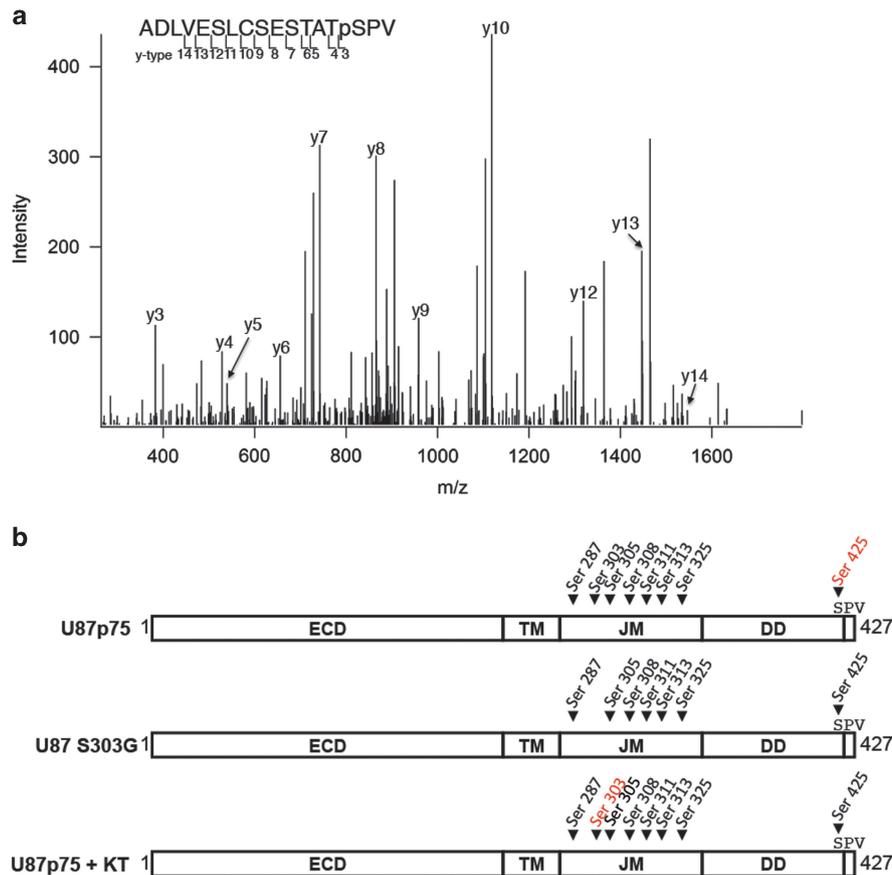
Figure 3. PKA regulates the phosphorylation of S425 within the p75^NTR-PDZ binding motif. p75^NTR (U87p75), mutant S303G p75^NTR or cells expressing p75^NTR that were treated with the PKA inhibitor KT5720 (200 nM) were lysed, immunoprecipitated with anti-p75^NTR, run on 10% sodium dodecyl sufate–polyacrylamide gel electrophoresis and proteins were visualized by silver stain. Protein bands representing p75^NTR were removed from the gel and in-gel trypsin digestion was performed. Digested peptides and phosphorylated serine residues were identified using nano-liquid chromatography–tandem mass spectrometry (LC/MS/MS) on a LTQ Orbitrap velos (Thermo Scientific; Southern Alberta Mass Spectrometry Center). (a) Modification and peptide search were performed with Mascot software v2.3. The MS/MS spectrum for the phospho-peptide ADLVESLCSESTAT(pS)PV, with annotated b and y ions, is shown. Phosphorylation at serine -1 is indicated by presence of y3 ion (+80Da for the mass of the phosphate group). (b) Shown is a schematic representation of p75^NTR indicating serine phosphorylated residues (black text) within the intracellular domain of p75^NTR isolated from U87 cells expressing wild-type p75^NTR (U87p75), mutant S303G p75^NTR or cells expressing p75^NTR that were treated with the PKA inhibitor KT5720 (200 nM). Red text indicates unphosphorylated serine residues.

protein that binds p75^NTR to regulate invasion. Using biotinylated peptides corresponding to the C-terminus of either p75^NTR (p75WT peptide, Biotin-N-LCSESTATSPV-COOH, residues 417-427), or a valine deletion mutant of p75^NTR (p75^MT peptide, Biotin-N-LCSESTATSP-COOH, residues 417–426) as affinity reagents, we identified PDLIM1 as a potential p75^NTR-interacting protein using nano-liquid chromatography–tandem mass spectrometry (Supplementary Figure S6). PDLIM1 (aka CLP-36, elfin, hCLIM1) was selected for further investigation based on the presence of an N-terminal PDZ domain and a C-terminal LIM domain that has been shown to associate with α-actinin-1 and α-actinin-4[46,65] and implicated in cell motility, endocytosis and cancer invasion.[66–71] To assess the specificity of the PDLIM1-p75^NTR interaction in glioma cells, we first demonstrated that PDLIM1 bound the wild-type p75^NTR peptide but not the PDZ-binding motif mutant (Figure 5a). Next, based on the identification of a PKA-regulated phosphorylation on S425 (Figure 3), we determined whether the interaction of PDLIM1 with p75^NTR was regulated by phosphorylation of S425. Therefore, p75^NTR C-terminal peptides containing an unphosphorylated S425 (SPV, Biotin-N-LCSESTATSPV-COOH, residues 417–427), a phosphorylated S425 (^Phospho SPV, Biotin-N-LCSESTAT^Phospho SPV-COOH, residues 417–427) or deleted for the

PDZ-binding motif (ΔSPV, Biotin-N-VESLCSESTAT-COOH, residues 414–424) were assessed for their ability to interact with PDLIM1. As shown in Figure 5b, both the ^Phospho SPV and ΔSPV peptide were compromised for their ability to bind PDLIM1 suggesting that PDLIM1 interacts with the C-terminal cytoplasmic domain of p75^NTR when S425 is in an unphosphorylated state.

To determine whether there is a direct interaction between PDLIM1 and p75^NTR, in vitro pull-down experiments were performed using His-tagged PDLIM1 and a biotinylated-p75^NTR C-terminal peptide (Biotin-N-LCSESTATSPV-COOH, residues 417–427) (Figure 5c). As predicted, His-tagged PDLIM1 was found to directly bind the p75^NTR peptide containing an unphosphorylated serine at position 425 (SPV), an interaction that was dramatically reduced when S425 was phosphorylated (^Phospho SPV), when the C-terminal valine was either substituted by methionine (SPM, Biotin-N-LCSESTATSPM-COOH, residues 417–427) or deleted (ΔV, Biotin-N-SLCSESTATSP-COOH, residues 416-426) or when the entire PDZ-motif (ΔSPV) was removed. No change in binding was observed when proline within the PDZ-binding domain was substituted with aspartate (SDV, Biotin-N-LCSESTATSDV-COOH, residues 417-427). Furthermore, and consistent with these data, immunoprecipitation of lysates
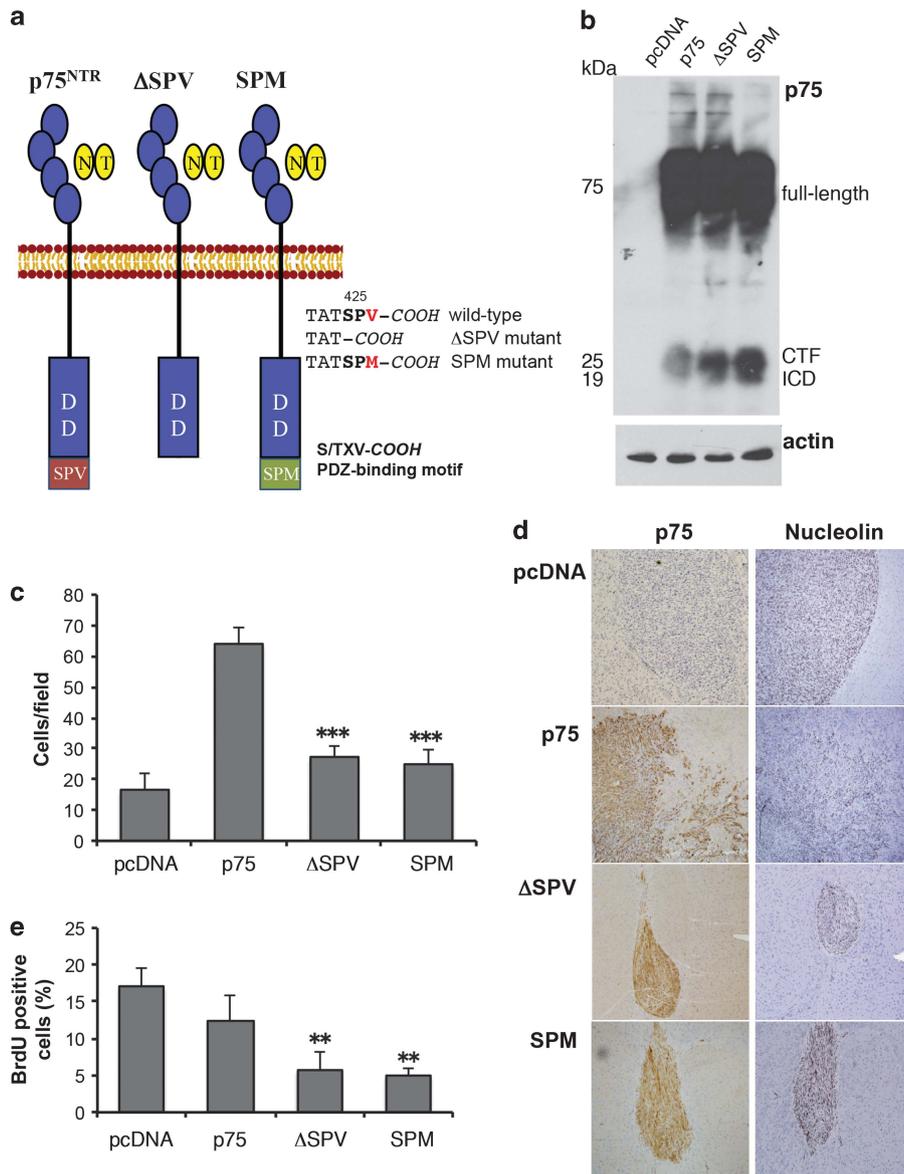
Figure 4. p75^{NTR} C-terminal PDZ-binding motif is required for glioma invasion *in vitro* and *in vivo*. (**a**) Schematic diagram of the p75^{NTR} PDZ-binding motif mutants (ΔSPV; SPM). (**b**) Western blot analysis using a C-terminal domain antibody to p75^{NTR} confirms the expression of the wild-type (p75) and mutant (ΔSPV; SPM) p75^{NTR} constructs. Western blot for actin was used as a loading control. (**c**) Histogram shows the number of U87 p75^{NTR} wild-type and PDZ-binding mutants (ΔSPV; SPM) that migrated through a collagen-coated transwell. Values are the mean ± s.e.m. from three independent experiments; asterisks (***) indicate $P < 0.001$ as compared with p75^{NTR} (one-way ANOVA with the Neuman-Keuls post-test). (**d**) U87pcDNA, p75^{NTR}, ΔSPV and SPM glioma cells (5×10^4 cells) were implanted intracerebrally into SCID mice and allowed to grow for 28 days. The mice were killed, and paraffin brain sections were stained with human nucleolin and p75^{NTR} (brown) to visualize the tumors. Sections were counterstained with hematoxylin (blue). (**e**) Bromodeoxyuridine (BrdU) was injected into the tumor-bearing mice 24 h prior to sacrifice. Frozen brain sections were stained with an antibody against BrdU and counterstained with toluidine blue to visualize the cell nucleus. Histogram represents the percentage of BrdU-positive cells in five consecutive fields. Values shown are the mean ± s.e.m. from five independent mice; asterisks (**) indicate $P < 0.01$ as compared with U87pcDNA (one-way ANOVA with the Neuman-Keuls post-test).

from p75^{NTR}-expressing glioma cells with either p75^{NTR} or PDLIM1 antibodies resulted in the co-precipitation of PDLIM1 and p75^{NTR}, respectively (Figures 5d and e, Supplementary Figure S7). Similar results were observed using lysates from U87 cells expressing only the intracellular domain of p75^{NTR} or other glioma cell lines (U251, U118) expressing the p75^{NTR} (Supplementary Figures S7 and S8). In contrast, the ability to co-precipitate PDLIM1 with the mutant alleles of p75^{NTR} (S303G, ΔSPV and SPM) or p75^{NTR} cells treated with the PKA inhibitor KT5720 was severely compromised (Figure 5d). Most importantly, the interaction of

PDLIM1 with p75^{NTR} was confirmed in genetically diverse patient-derived glioma cells (Figure 5e).

PDLIM1 is critical for glioma cell invasion both *in vitro* and *in vivo*
As PDLIM1 possesses two functional domains, PDZ and LIM, we next assessed the requirement of these domains for the interaction with p75^{NTR}. To address this, HA-tagged PDLIM1 constructs lacking either the PDZ domain (ΔPDZ) or the LIM domain (ΔLIM) were generated. U87p75 glioma cells were
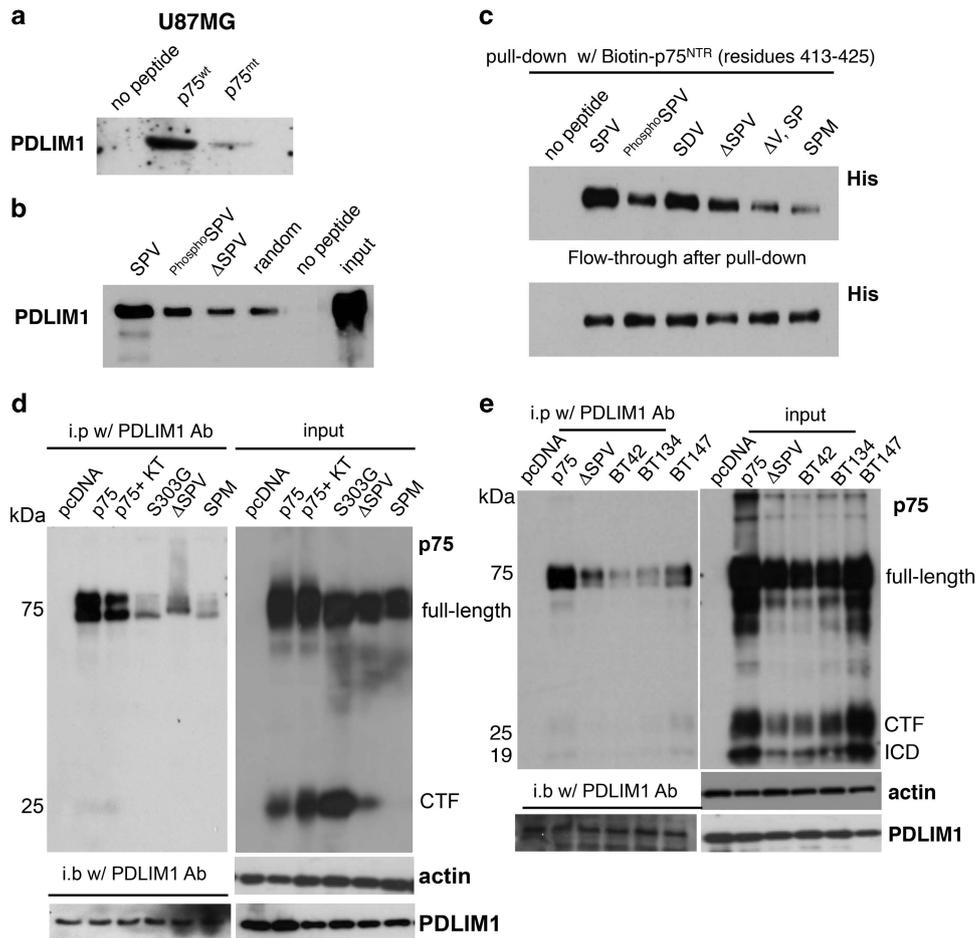
Figure 5. PDLIM1 associates with p75^NTR via the C-terminal PDZ-binding motif, an interaction regulated by phosphorylation of S425. (**a**) Using a biotinylated peptide to the C-terminus of either p75^NTR (p75^wt peptide, Biotin-N-LCSESTAT*SPV*-COOH, residues 417–427) or a Valine deletion mutant of p75^NTR (p75^MT peptide, biotin-N- LCSESTAT*SP*-COOH, residues 417–426) as affinity reagents, PDLIM1 was confirmed as a p75^NTR-interacting protein by western blot analysis. (**b**) To assess the physiological relevance of the phosphorylation of S425 within the PDZ-binding motif, biotin-labeled peptides corresponding to the p75^NTR wild-type C-terminal (SPV), the S425 phosphorylated C-terminal (^PhosphoSPV) and the PDZ-binding motif deleted C-terminal (ΔSPV) were used as affinity reagents. PDLIM1 was shown to interact with maximum affinity to the wild-type unphosphorylated peptide. (**c**) Biotin-labeled p75^NTR peptides (SPV, ^PhosphoSPV, ΔSPV, ΔV, SP and SPM) were incubated with His-tagged recombinant PDLIM1 protein, respectively. Pull-down assays were performed to determine the amount of PDLIM1 protein bound to each peptide. Flow-through shows unbound proteins after incubation. (**d**) Immunoprecipitation with PDLIM1 antibodies co-precipitated p75^NTR and PDLIM1 while the mutant alleles of p75^NTR (S303G, ΔSPV and SPM) or p75^NTR cells treated with the PKA inhibitor KT5720 (KT; 200 nM) were severely compromised for PDLIM1 binding. (**e**) Interaction of PDLIM1 with p75^NTR was confirmed in patient-derived glioma stem cells/tumor-initiating cells (BT42, BT134, BT147) by immunoprecipitation using anti-PDLIM1. Input cell lysates were assessed by western blot analysis for expression of p75^NTR and PDLIM1.

transfected with HA-PDLIM1-ΔPDZ, HA-PDLIM1-ΔLIM or full-length HA-PDLIM1 (Figure 6a) and assessed for their ability to interact with p75^NTR. Although removal of either the PDZ or LIM domain of PDLIM1 compromised the ability of the protein to interact with p75^NTR, the removal of the PDZ domain resulted in a nearly complete loss of p75^NTR binding (Figure 6b).

Next, to directly assess the role of PDLIM1 in glioma behavior, U87p75 cells were stably transfected with PDLIM1 shRNA (pLK0.1-puro shPDLIM1) or a control shRNA (pLK0.1-puro). Western blot analysis and RT–PCR showed that PDLIM1 expression was dramatically reduced in U87p75 cells transfected with PDLIM1 shRNA as compared with the control shRNA (Figure 6c). The cells stably expressing PDLIM1 shRNA were then assessed for their invasive ability *in vitro* where knockdown of PDLIM1 was found to significantly reduce p75^NTR-mediated invasion (Figure 6d). To confirm this *in vivo*, cells were implanted into the brains of SCID mice and allowed to grow for 4 weeks. The mice were killed, and paraffin-embedded brain sections were stained with antibodies against human nuclei (Figure 6e; brown, left panel) or PDLIM1 (Figure 6e; brown, right panel). As predicted, knockdown of PDLIM1 resulted in complete abrogation of p75^NTR-mediated glioma invasion *in vivo*.

These data identify the PDZ-domain containing protein PDLIM1 as a novel p75^NTR-interacting protein that is essential for p75^NTR-dependent glioma invasion, an interaction regulated by phosphorylation of S425 within the PDZ-binding domain.

DISCUSSION

The highly invasive nature of glioblastoma renders them incurable by surgery and radiotherapy, thus warranting attention to the molecular determinants regulating their invasive behavior. We previously demonstrated that the p75^NTR signaling axis is one such determinant in glioma invasion.[18,19] Using pharmacological and loss-of-function approaches, we defined a specific role for serine phosphorylation of p75^NTR (CD271) in glioblastoma tumorigenesis
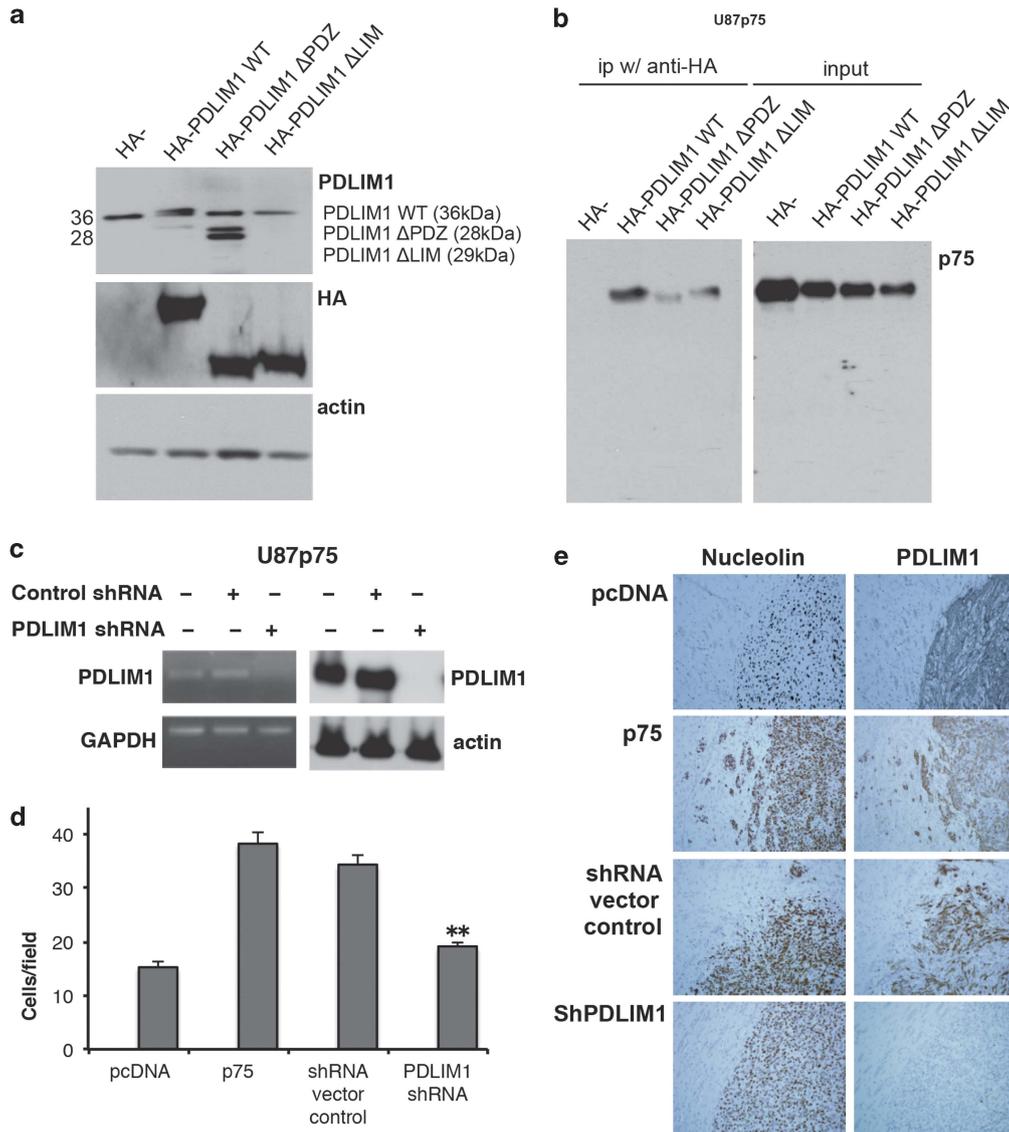
Figure 6. PDLIM1 is critical for glioma cell invasion. (**a**) U87p75 cells were transfected with full-length HA-PDLIM1 or HA-PDLIM1 lacking either the PDZ domain (ΔPDZ) or the LIM domain (ΔLIM). Shown are western blot analyses for PDLIM1 (top), HA- (middle) and actin (bottom). The PDLIM1 antibody was developed against the LIM domain and thus does not recognize the HA-PDLIM1- ΔLIM therefore the expression of the ΔLIM construct was confirmed by the HA-specific antibody. HA and actin were used to confirm similar protein expression and protein loading, respectively. (**b**) Shown is p75[NTR] western blot analysis of cell lysates from U87p75 cells expressing HA-PDLIM1-WT, HA-PDLIM1-ΔPDZ or HA-PDLIM1-ΔLIM immunoprecipitated with anti-HA (left panel). Right panel shows the level of p75[NTR] in each input cell lysate. Immunoprecipitation with HA antibodies co-precipitated p75[NTR]. (**c**) To generate PDLIM1 knockdown cells, U87p75 cells were transfected with PDLIM1shRNA or a control shRNA. RT–PCR (left panels) and western blot analysis (right panels) show reduced or absent PDLIM1 expression when cells are expressing PDLIM1 shRNA. GAPDH and actin were used as loading controls. (**d**) Inhibition of PDLIM1 expression in U87p75 human glioma cells by shRNA results in a significant decrease of p75[NTR]-mediated invasion through collagen-coated transwells. Histogram shows the mean ± s.e.m. from five independent experiments; asterisks (**) indicate $P < 0.01$ as compared with U87 shRNA vector control (one-way ANOVA with the Neuman-Keuls post-test). (**e**) U87pcDNA, p75[NTR] and p75[NTR] shPDLIM1 glioma cells (5X10^{4} cells) were implanted intracerebrally into SCID mice and allowed to grow for 28 days. The mice were killed, and paraffin brain sections were stained with human nucleolin (brown) to visualize the tumors (left panel) or PDLIM1 (brown, right panel). Sections were counterstained with hematoxylin (blue).

and invasion. We demonstrate that phosphorylation of p75[NTR] on S303 is required for glioma invasion both *in vitro* and *in vivo* and that this regulation requires the subsequent dephosphorylation of a second serine site present in the PDZ-binding motif, S425. We provide evidence that dephosphorylation of S425 is essential for the interaction of p75[NTR] with the PDZ and LIM domain containing protein PDLIM1. Moreover, we show for the first time that S425 within the canonical PDZ-binding motif of p75[NTR] is phosphorylated and this regulates the interaction of p75[NTR] with PDLIM1. Although it has been shown in other proteins

that phosphorylation within the PDZ-binding motif increases the interaction with PDZ-containing proteins,[72,73] we provide evidence that phosphorylation of p75[NTR] on S425 prevents its binding to PDLIM1, a negative regulation that has also been observed between the β2-adrenergic receptor and NHERF1 to control recycling of the receptor.[74] In addition, we demonstrate that crippling the PDZ-binding domain, or downregulating PDLIM1, abrogates p75[NTR]-mediated invasion. Importantly, our study was not limited to established cell lines, as a role for PDLIM1 in glioma invasion was also confirmed in primary patient-derived

cultures grown and maintained under neurosphere conditions, an environment that promotes the isolation of glioma cells with a cancer stem-like phenotype[60,75,76] and maintains the highly invasive nature of the patient tumor.

It is now clear that the spatial localization of receptors and/or downstream effector molecules is necessary for appropriate recognition and subsequent transmission of extracellular signals (for example, see reviews by Gauthier and Robbins,[77] Casaletto and McClatchey,[78] Gold et al.[79] and Hupalowska and Miaczynska[80]). For instance, the localization of p75^{NTR} to lipid rafts has been shown to be important for at least some aspects of its biological activity.[39,81–85] We have previously shown that regulated intramembrane proteolysis of p75^{NTR} is required for p75^{NTR}-mediated glioma invasion.[19] Herein, we found that although disruption of phosphorylation at S303, or mutation of the PDZ-motif (ΔSPV or SPM) results in abrogation of p75^{NTR}-mediated invasion, it does not compromise regulated intramembrane proteolysis of the receptor as these mutant receptors still undergo cleavage to generate both C-terminal fragment and intracellular domain fragments (Figures 2b, 3b, 4d and e). These data therefore suggest that processing of the receptor, although previously shown to be necessary, is not sufficient to mediate the invasive behavior of glioma cells and supports a model where either the localization of the receptor and/or its cleavage products (intracellular domain), or the posttranslational modification of these products, may be required to induce glioma invasion, possibilities that are not mutually exclusive and all may be involved in some context.

The role of PDLIM1 in invasion is not limited to glioma and is involved in breast cancer cell migration, invasion and metastasis through interaction with the actin cytoskeleton,[70] a key function of many LIM domain-containing proteins.[44–48] Furthermore a role for p75^{NTR} has been established in melanoma cell migration and tumorigenesis[28,30,55,56] for which we have shown an interaction of PDLIM1 with p75^{NTR} (Supplementary Figure S8). In addition PDLIM1 is highly expressed in dorsal root ganglion neurons especially after nerve injury and may regulate the actin cytoskeleton during neurite outgrowth[86,87] a function also shown to involve neurotrophins and p75^{NTR}.[23,24]

Although there are numerous LIM domain-containing proteins in the mammalian genome,[88] there are only 10 proteins that contain a PDZ and LIM domain. It remains to be determined whether other PDZ/LIM family members have a role in glioma invasion. Furthermore, whether other PDZ domain proteins interact with the C-terminus of p75^{NTR} in glioma cells is undetermined, however, it is interesting that the PDLIM PDZ domains may have unique binding specificity with their respective substrates.[89] Of note, a recent study suggests that MDA-9/syntenin a PDZ domain-containing protein, is important in glioma invasion and pathogenesis.[90]

The requirement for PDLIM1 in p75^{NTR}-mediated glioma invasion with its regulated binding to the receptor suggests a logical therapeutic axis for treatment of glioblastoma. Development of reversible small-molecule inhibitors that target neuron-specific PDZ domains have been proposed as a means to specifically target compartmentalized signaling within the CNS.[91] In addition, peptides targeting the PDZ domain of PTPN4, a tyrosine phosphatase, are efficient inducers of glioma cell death.[92] Thus, targeting the PDZ domain interaction of p75^{NTR} may provide a tissue and receptor specific therapeutic approach for patients with malignant glioma and may have global application in other cancers (that is, metastatic melanoma and breast) where p75^{NTR} and/or PDLIM1 have also been implicated.[28–30,55,93,94]

MATERIALS AND METHODS

Cell culture and treatments
U87 glioma cell lines were grown in Dulbecco's modified eagle's medium (Gibco-BRL, Bethesda, MD, USA) supplemented with 10% fetal bovine serum (Gibco-BRL) at 37 °C in a humidified 5% CO$_2$ incubator. Stable transfectants were maintained in identical media with 400 µg/ml of G418 (Sigma, St. Louis, MO, USA). Brain tumor-initiating cells were established within the Brain Tumor Stem Cell Core at the University of Calgary and maintained as previously described.[19,60] Human Metastatic melanoma cells 3S5 and 70W[97,98] were obtained from Dr Robert Kerbel Mount Sinai Research Institute, Toronto Canada. U87 or brain tumor-initiating cells were treated with the indicated concentrations of KT5720 (1h; KT, Calbiochem, Gibbstown, NJ, USA), 2′5′-dideoxyadenosine (1 h, 30 µм; Sigma) or forskolin (30mins, 1 µм, Calbiochem), and cultured at 37 °C. Control cells were treated with 1% dimethyl sulfoxide (vehicle). All cell lines were tested for mycoplasma contamination.

Mutagenesis and transfection
The human p75^{NTR} expression vector was constructed as described previously.[19] To generate the p75^{NTR} serine phosphorylation site mutant (p75S303G), the p75^{NTR} C-terminal PDZ-binding motif truncation mutant (p75ΔSPV) and the p75^{NTR} C-terminal PDZ-binding motif substitution mutant (p75SPM), site-directed mutagenesis was performed (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions. Constructs were inserted into pcDNA 3.1 vectors (Invitrogen, Carlsbad, CA, USA). Human PDLIM1 cDNA was purchased from Open Biosystems (Cat. no. MHS6278-202755473, Huntsville, AL, USA). Vectors encoding HA-tagged full-length PDLIM1 (residues1–329), ΔPDZ (residues 68-329) or ΔLIM mutant (residues 1–250) were cloned into the HA-pcDNA3.1 vector. ΔPDZ or ΔLIM mutants were obtained by PCR using the pcDNA 3.1 HA-PDLIM1plasmid as a template and verified by DNA sequencing. Transfection and selection of glioma cell lines was performed as described previously.[19] Flow cytometry and western blotting confirmed appropriate expression.

Invasion assay
Cells (2.5×10^4) were seeded on collagen-coated transwell inserts (8 µm pore size; Corning Costar, Corning, NY, USA) and incubated for 3 h at 37 °C in a 5% CO$_2$ incubator. Media was aspirated and the cells were stained in 1% crystal violet in 95% ethanol for 1 min, rinsed in phosphate-buffered saline (PBS) and cells on the upper membrane surfaces were removed with a cotton swab. The number of cells that migrated were counted by light microscopy, and the sum of cells in five microscopic fields was recorded.

Reporter gene assay
To examine cAMP activity, transcription of a CRE-promoter was evaluated in U87 cells transfected with a CRE-luciferase reporter plasmid. U87 cells were plated in a six-well tissue culture plate at 1×10^4 cells per well and transfected with a pCRE-Luc reporter plasmid using lipofectamine 2000 (Invitrogen). Three days after transfection, cells were treated as outlined in each experiment. For analyses, cells were lysed in 0.5 ml of lysis buffer (Dual-Luciferase Reporter Assay System, Promega, Madison, WI, USA) and cell lysate (10 µl) was combined with 50 µl of luciferase assay reagent. Luciferase activity was measured with a GloMax-multi detection system (Promega).

Immunoprecipitation and immunoblot
Cells were lysed with RIPA buffer (20 mм Tris-HCl (pH 7.4), 150 mм NaCl, 0.5% (v/v) NP-40, protease inhibitor cocktail (Roche, Indianapolis, IN, USA), phosphatase inhibitor (Sigma)), protein quantification was determined, and proteins were resolved by sodium dodecyl sufate–polyacrylamide gel electrophoresis. Western blot analysis was performed using rabbit polyclonal anti-human p75^{NTR} intracellular domain (Promega), mouse monoclonal anti-β-actin (Millipore, Billerica, MA, USA) or mouse monoclonal PDLIM1 (Abcam, Cambridge, MA, USA) with the appropriate HRP-conjugated secondary antibodies (SantaCruz Biotechnology, Santa Cruz, CA, USA). For immunoprecipitation experiments, whole-cell lysates were incubated with the indicated antibody in the presence of Sepharose-Protein A/G (Sigma). Immunoprecipitates were boiled with 2× Laemmli buffer, resolved by sodium dodecyl sufate–polyacrylamide gel electrophoresis

and western blotting were performed using anti-p75^{NTR} (Promega), anti-PDLIM1 (Abcam) or anti-HA (Abcam) antibodies.

Reverse transcription–polymerase chain reaction (RT–PCR)
RNA from U87 cells was purified using Trizol (Invitrogen) according to the manufacturer's instructions. cDNA synthesis and analysis by PCR were performed as previously described.[18,53] These references also list primer sequences.

Peptide affinity assay and mass spectrometric analysis
For the detection of p75^{NTR}-interacting proteins, wild-type and mutant p75^{NTR} peptides, or a random peptide (control), were labeled on the amino terminus with biotin (synthesized by CanPeptide, Montréal, QC, Canada). U87 glioma cells were lysed as described above and incubated with biotin-labeled peptides for 1 h at 4 °C. NeutrAvidin agarose (Thermo Scientific, Rockford, IL, USA) was added and incubated for 1 h. Peptide bound to the agarose beads was centrifuged, washed with PBS and boiled with 2× Laemmli buffer. Proteins that bound to each peptide were resolved on a 10% sodium dodecyl sufate–polyacrylamide gel electrophoresis gel and silver-stained as described previously[95] or transferred to nitrocellulose for western blot analysis. Proteins that bound to the wild-type p75^{NTR} peptide and not to the mutant peptides were excised from the gel, and processed LC-MS/MS analysis as described.[95] A similar procedure was followed for the detection of serine phosphorylation on the p75^{NTR} mutant and wild-type receptors following immunoprecipitation with anti-p75^{NTR} (Promega).

Animals
Six- to 8-week-old female SCID mice (Charles River Laboratories, Shrewsbury, MA, USA) were housed in groups of three to five and maintained on a 12-h light/dark schedule with a temperature of 22 °C ± 1 °C and a relative humidity of 50% ± 5%. Food and water were available *ad libitum*. All procedures were reviewed and approved by the University of Calgary Animal Care Committee.

In vivo studies using an intracranial glioma model
Glioma cells harvested using Puck's EDTA were resuspended in PBS and implanted intracerebrally into SCID mice (5×10^4 cells/mouse) as previously described.[18,19,96] Animals were randomized prior to the injection of tumor cells. At 4 weeks, BrdU was given by intraperitoneal injection 24 h prior to killing of the mice, brains were removed, fixed in 4% formalin and paraffin-embedded. Whole brain tissue sections from all animals in each group were examined by immunohistochemistry and assessed by two independent investigators, including one blinded assessment. To ensure adequate power to detect invasion, all analysis included five animals per experimental group and three independent experiments were performed.

Immunohistochemistry
Whole brain sections were fixed with cold acetone, and rehydrated through an ethanol gradient. Endogenous peroxidases were inactivated with 0.075% H_2O_2/methanol, and nonspecific binding blocked with Rodent Block M (Biocare Medical, Concord, CA, USA). Sections were incubated with 1:100 diluted rabbit polyclonal anti-p75^{NTR} antibody (Promega), mouse monoclonal anti-human nucleolin (Abcam) or anti-Tenascin C (Novus Biologicals, Littleton, CO, USA) in antibody diluent (Biocare Medical). Following washing with PBS, the appropriate biotinylated secondary antibody (Vector Laboratories, Burlingame, CA, USA) was applied and detected using the VECTASTAIN Elite ABC kit (Vector Laboratories) and SIGMAFAST DAB (Sigma). Hematoxylin was used as a counterstain and sections were mounted with Entellan (Electron Microscopy Sciences, Hatfield, PA, USA).

Pull-down analysis of p75^{NTR}-PDLIM1 direct interaction
One microgram of C-terminal His-tagged recombinant PDLIM1 (Fitzgerald, Acton, MA, USA) and biotin-labeled p75^{NTR} C-terminal peptides (1mg/ml) (described above) were incubated in 300 µl of binding buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.05% NP-40) overnight at 4 °C. NeutrAvidin agarose (Thermo Scientific) was added for 1 h, beads were centrifuged, washed with PBS and boiled with 2× Laemmli buffer. Proteins bound to each peptide were resolved by sodium dodecyl sufate–polyacrylamide gel

electrophoresis and bound PDLIM1 was detected by western blot analysis using anti-His (Abcam) or anti-PDLIM1.

PDLIM1 shRNA
pLKO.1-shPDLIM1 vector TRCN0000161271 (shPDLIM1; GCCTTGGTTAATT-GACTCACA) as well as pLKO.1-shControl SHC002 were purchased from Sigma and used according to the manufacturer's instructions. shPDLIM1 and shControl were transfected into U87p75^{NTR} cells using FuGENE 6 transfection reagent (Roche) according to the manufacturer's instructions and cells expressing the shRNA were selected in media containing Puromycin (5 µg/ml). Inhibition of PDLIM1 expression was confirmed by RT–PCR and western blot analysis.

Statistical analysis
Data were analyzed by one-way ANOVA and Student's *t*-test comparison, using GraphPad Instat3 software (La Jolla, CA, USA). Statistical significance was reached at $P < 0.05$.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS
BYA, RSG, SMR and DLS conceived the overall project and designed all experiments. BYA, RSG, JJR, XH, JZ, N-HD and MA performed all of the experiments. BYA, RSG, SMR and DLS analyzed the data. BYA, RSG, SMR and DLS wrote the paper.

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