Netrin-1-induced activation of Notch signaling mediates glioblastoma cell invasion

Irene Ylivinkka1, Yizhou Hu1, Ping Chen2, Ville Rantanen2, Sampsa Hautaniemi2, Tuula A. Nyman3, Jorma Keski-Oja1 and Marko Hyytiäinen1,∗

1Departments of Pathology and Virology, The Haartman Institute, Translational Cancer Biology Research Program and Helsinki University Hospital, University of Helsinki, Finland
2Systems Biology Laboratory, Institute of Biomedicine and Genome-Scale Biology Research Program, University of Helsinki, FI-00014, Finland
3Research Program in Structural Biology and Biophysics, Institute of Biotechnology, University of Helsinki, FI-00014, Finland

∗Author for correspondence (marko.hyytiainen@helsinki.fi)

Accepted 11 March 2013
Journal of Cell Science 126, 2459–2469
© 2013, Published by The Company of Biologists Ltd
doi: 10.1242/jcs.120022

Summary
Glioblastoma multiforme is an aggressively invasive human brain cancer, which lacks effective treatment. The axonal guidance protein, netrin-1, is overexpressed in glioblastoma tumor biopsies. In Matrigel invasion assays we observed that experimental overexpression of netrin-1 increased cell invasiveness and its downregulation decreased invasiveness. Using tandem affinity purification and mass spectrometry protein identification we found that netrin-1 forms a complex with both Notch2 and Jagged1. Recombinant netrin-1 colocalized with Jagged1 and Notch2 at the cell surface and was also present in the intracellular vesicles with Jagged1, but not with Notch2. Netrin-1 activated Notch signaling and subsequent glioblastoma cell invasion. Interestingly, the recombinant central domain of netrin-1 counteracted the effects of the full-length netrin-1: it inhibited glioblastoma cell invasion and Notch activation by retaining the Notch signaling complex at the cell surface. This finding may give rise to therapeutic applications. These results reveal a new mechanism leading to glioblastoma cell invasion, in which netrin-1 activates Notch signaling.

Introduction
Gliomas are the most common human primary brain tumors. They are graded into four subtypes based on their malignancy as evaluated according to their level of glial cell differentiation. Of the four, glioblastoma multiforme (GBM) is the most aggressive type. The characteristics of GBM include rapid cell proliferation, diffusive invasion, extensive angiogenesis, necrosis and pseudopalisade structures in the tumor center (Brat et al., 2004). GBM is incurable, with a median survival time of 15 months after diagnosis (Stupp et al., 2005).

The main reason for the lethality of GBM is its highly invasive nature. However, GBM metastasizes very rarely outside the central nervous system. Owing to its intracranial location, surgical removal of the tumor is often very intricate and incomplete. The remaining tumor cells grow rapidly into a secondary tumor and cause relapses.

Netrin-1 (NTN1) is a laminin-related secreted pericellular protein. In man the netrin family consists of netrin-1, -3 and -4 and two GPI-anchored forms (Serafini et al., 1994). NTN1 was originally discovered as a regulator of axon guidance (Serafini et al., 1996). Recently it was observed to play a role in human GBM. We report here that NTN1 is upregulated in GBM biopsies using The Cancer Genome Atlas (TCGA) exon array datasets. Expression of NTN1 was upregulated in GBM biopsies compared with normal brain samples (Fig. 1A). To obtain a

Results
Overexpression of netrin-1 increases human glioblastoma cell invasion
NTN1 improves the survival of human neuroblastoma cells and regulates colorectal cancer tumorigenesis (Delloye-Bourgeois et al., 2009b; Paradisi et al., 2009; Rodrigues et al., 2007). To assess the possible role of NTN1 in human GBM we first analyzed the expression levels of NTN1 in human GBM tumor biopsies using The Cancer Genome Atlas (TCGA) exons. Expression of NTN1 was upregulated in GBM biopsies compared with normal brain samples (Fig. 1A). To obtain a
better insight into this, we compared the protein levels of NTN1 between normal human astrocytes and three human GBM cell lines. We observed that in U373MG and U251MG GBM cell lines NTN1 protein levels were higher than in astrocytes whereas in U87MG they were lower (Fig. 1B,C). Similarly, the mRNA expression of NTN1 was lower in U87MG compared with normal human astrocytes (Fig. 1D). In addition, we observed a trend of increased expression in cultured U251MG GBM cells. Previously it had been observed that U373MG and U251MG cells are more aggressive and invade more diffusively than U87MG cells (Belot et al., 2001). Taken together, our results suggested that NTN1 may play an important role in GBM tumorigenesis.

To explore this possibility further, we created U251MG GBM cells with either stably increased or decreased expression of NTN1 (Fig. 1E,F). The achieved stable knockdown levels were 60% of endogenous expression (Fig. 1F). Further reduction in the expression of NTN1 resulted in the apoptotic death of these cells (data not shown), indicating that NTN1 is a survival factor for human GBM, as also noted before (Miyamoto et al., 2010).

To characterize the effects of NTN1 on GBM cell invasion we used Matrigel invasion assays. U251MG cells overexpressing NTN1 displayed an almost twofold increase in invasiveness (Fig. 1G,H). Decreasing the expression of NTN1 reduced the invasiveness (Fig. 1I,J). Taken together, these results indicated that NTN1 enhances GBM cell invasiveness, similarly to the role of NTN1 in the induction of invasion of pancreatic and colorectal adenocarcinomas (Dumartin et al., 2010; Paradisi et al., 2009; Rodrigues et al., 2007).

**Netrin-1 interacts with Notch pathway proteins in human glioblastoma cells**

The U251MG cells express various classical netrin receptors (Hu et al., 2012). To elucidate NTN1 signaling mechanisms in these cells we searched for potential interacting protein complexes using tandem affinity purification and mass spectrometry identification of the binding proteins. For this we created cell lines stably expressing different FLAG- and HA-tagged (FH) NTN1 domains (Fig. 2A). As the parental cell line we used U251MG human GBM cells that express endogenous NTN1, as determined by western blotting and quantitative real-time PCR (Fig. 1B–D). The achieved expression levels of NTN1 were measured by qRT-PCR (E,F). The cells were seeded onto Matrigel-coated cell culture inserts and allowed to invade for 7 hours, fixed and stained. The area of the invaded cells was quantified (G,I). Representative images of the inserts are shown in H and J. Values are means ± s.d. of three experiments. Overexpression of NTN1 increased cell invasion whereas decreased expression inhibited cell invasion.
U251MG cell clones overexpressing the domains of NTN1 were harvested, and the obtained total cell lysates were used for tandem affinity purification (Fig. 2C). The interacting proteins were then identified by mass spectrometry (supplementary material Table S1). Among the identified proteins we found integrins α3β1 and β4, which are known receptors for NTN1 (Yebra et al., 2003), and therefore validated our screen. Pathway analysis of the identified proteins using KEGG (Kyoto Encyclopedia of Genes and Genomes) revealed that NTN1 associates with various cellular processes (supplementary material Table S2). One of the strongest candidate pathways implicated was the Notch signaling pathway: both NTN1(II)FH and NTN1(III)FH were linked to Notch signaling.

The identification data showed also that NTN1(I)FH interacted with several endocytosis-related proteins. NTN1(I)FH, in turn, interacted efficiently with E3 ubiquitin ligases, whereas NTN1(III)FH interacted with proteases (Fig. 3A). All these cellular processes are important steps in the regulation of Notch signaling (reviewed by Parks et al., 2000; Pratt et al., 2011). Most importantly, Notch1, 2 and 3 receptors and the known Notch ligand Jagged1 were identified (Shimizu et al., 1999). This suggests that the three domains of NTN1 may differentially contribute to the regulation of Notch signaling. We focused on Notch2 because it was identified in the mass spectrometry screen to interact with all NTN1 domains, with high confidence. In addition, both protein and mRNA expression levels of Notch2 were higher in U251MG cells than in human astrocytes (Fig. 3B,C). Furthermore, in U251MG cells the relative expression levels of Notch2 were higher than those of Notch1 (Fig. 3D).

The interaction between NTN1 and Notch-pathway proteins was validated by immunoprecipitation followed by immunoblotting (Fig. 3E). In this analysis we used tagged NTN1 because immunoprecipitation with endogenous NTN1 was not feasible because of low affinity of the antibodies available. NTN1FH as well as all the NTN1 domains interacted with anti-Notch2-antibody-reactive protein. Interestingly, NTN1FH and NTN1(II)FH pulled down Notch2 more efficiently than the other domains. Similarly, we validated the interaction of NTN1 with Jagged1. In immunoblotting we noticed that NTN1FH and NTN1(II)FH also pulled down Jagged1 more efficiently than the other domains (Fig. 3E). Because the Notch receptors and ligands have high affinity for EGF repeats (Rebay et al., 1991; Shimizu et al., 1999), we used a fragment of latent TGFβ binding protein 2 containing EGF repeats (designated as EGF ctrl in Fig. 3E and L2 XIV str-HA in Hyttiainen and Keski-Oja, 2003) as a control in the immunoprecipitation. This protein did not pull down Notch2 or Jagged1 and thus confirmed that not
all EGF-repeat-rich proteins pull down Notch components in our experimental system. These immunoprecipitation results confirmed our mass spectrometry results.

**Netrin-1 colocalizes with Notch2 and Jagged1 at the cell surface**

Because we observed differential binding of the NTN1 fragments to Notch signaling components, we next analyzed how the expression of different NTN1 fragments contributes to the invasiveness of the U251MG cells. We employed Matrigel invasion assays with U251MG cells expressing tagged NTN1 domains (Fig. 4). The expression of NTN1(II)FH significantly decreased the invasiveness whereas the expression of NTN1(I)FH and NTN1(III)FH slightly increased it. We therefore hypothesized that NTN1(II)FH might antagonize the effects of endogenously expressed NTN1, and focused on the effects of full-length NTN1 and the NTN1(II)FH in the following experiments.

To further validate the mass spectrometry results, we explored whether NTN1 and Notch2 or Jagged1 colocalize at the cell surface. U251MG cells were treated with conditioned medium containing NTN1FH or NTN1(II)FH. The levels of NTN1FH and NTN1(II)FH were confirmed by immunoblotting (Fig. 5A).

To characterize the stationary situation of NTN1 binding to glioblastoma cells we first treated U251MG cells with cold conditioned medium. We observed distinct colocalization of Jagged1 and NTN1FH at the cell–cell contacts (Fig. 5B). NTN1(II)FH also colocalized with Jagged1. However, the colocalization was not exclusively at the cell–cell contacts but more diffusely in the cells.
We then explored the possible colocalization between Notch2 and NTN1FH or NTN1(I)FH. Interestingly, the colocalization with NTN1FH was observed at cell edges (Fig. 5C). NTN1(I)FH and Notch2 colocalized only modestly. Similarly to Jagged1, colocalization of NTN1(I)FH and Notch2 was located more diffusively in the cells.

Netrin-1 localizes to intracellular vesicles together with Jagged1 but not with Notch2

Notch signaling is dependent on active endocytosis (reviewed by Baron, 2012). We therefore incubated U251MG cells with NTN1FH at 37°C to allow possible internalization. When U251MG cells were treated with NTN1FH-containing medium,
we observed that in addition to colocalization at the surface of cells, NTN1FH colocalized with Jagged1 in vesicle-like structures in the cytoplasm (Fig. 5D). In contrast, Notch2 colocalization in the cytoplasm was not observed (Fig. 5E), but colocalization was observed on the cell surface and in cell–cell contacts as with Jagged1. To explore the functions of the central domain of NTN1, we treated the cells with conditioned medium containing NTN1(II)FH. Internalization of this fragment was not observed. We observed its colocalization with Jagged1 and Notch2, but it occurred diffusively around the cell surface in both cases (Fig. 5D,E). To rule out the possibility that the colocalization was a consequence of the FLAG-HA tag, we repeated these experiments using conditioned medium containing FLAG- and HA-tagged soluble EphrinA1 (supplementary material Fig. S1A). We observed minor colocalization of EphrinA1 with Notch2 and with Jagged1 (supplementary material Fig. S1B,C). Soluble EphrinA proteins are efficiently endocytosed (Pitulescu and Adams, 2010). Therefore, the observed partial colocalization of EphrinA1 and Jagged1 or Notch2 is likely to be a consequence of localization to an endosomal compartment. However, the relative colocalization area of NTN1FH and Notch2 or Jagged1 was greater than with EphrinA1 and Notch2 or Jagged1 (supplementary material Fig. S1D,E). Furthermore, we did not observe internalization of the tagged NTN1(II)FH fragment. Therefore, the detected colocalization of NTN1FH with Notch2 and Jagged1 is very unlikely to be a consequence of the tag used. These findings suggest that NTN1FH but not NTN1(II)FH is internalized with Jagged1, and support our hypothesis that NTN1 and NTN1(II)FH have divergent effects on Notch signaling.

Netrin-1 overexpression increases Notch activity

Next, we analyzed how NTN1 and its domains might contribute to Notch signaling. We found that the overexpression of NTN1FH significantly increased Notch activity (Fig. 6A), whereas silencing of the expression of NTN1 clearly decreased Notch activity (Fig. 6B).

Overexpression of either NTN1(I)FH or NTN1(III)FH also increased Notch activity (Fig. 6C). In contrast, overexpression of NTN1(II)FH resulted in decreased Notch activity (Fig. 6C).
effects of NTN1FH and its fragments on Notch activation correlated with its effects on cell invasiveness. These results further supported our hypothesis that NTN1 and NTN1(II)FH modulate Notch signaling differently: NTN1 enhances Notch signaling whereas NTN1(II)FH decreases it.

Notch signaling has effects on cell morphology (Ingram et al., 2008). We therefore compared the effects of NTN1FH, NTN1(II)FH and the Notch signaling inhibitor, DAPT, on U251MG cell morphology. The cells were visualized by actin immunofluorescence staining. Interestingly, the cells treated with either NTN1(II)FH or DAPT were smaller and more rounded than the control cells (Fig. 6D). Similar morphological changes induced by NTN1(II)FH and DAPT further confirmed that NTN1(II)FH negatively regulates Notch signaling.

The role of Notch signaling in various types of cancer is controversial. Increased Notch signaling may either promote or restrict tumor progression (reviewed by Lobry et al., 2011). However, Notch signaling has been reported to promote glioma progression (Chigurupati et al., 2010; Sivasankaran et al., 2009; Stockhausen et al., 2010; Zhang et al., 2012). To confirm this, we treated the cells with DAPT, and used them in Matrigel invasion assays. When Notch signaling activity was reduced to 20% of the endogenous level, there was a significant decrease in the invasive capability of U251MG cells (Fig. 6E).

The central domain of netrin-1 increases the cell surface complex formation of Notch2 and Jagged1

We then analyzed, quantitatively, whether the cell surface localization of Notch2 or Jagged1 had been altered as a consequence of the expression of either NTN1FH or NTN1(II)FH. The cell surface proteins were biotinylated and then immunoprecipitated either with Notch2 or Jagged1 antibodies. We could not see any changes in the cell surface levels of Jagged1, and only minor, statistically insignificant increase in Notch2 levels (data not shown) in NTN1FH- or NTN1(II)FH-expressing cells. However, in immunoprecipitates of Notch2 we co-precipitated a protein of the size of Jagged1 in the cells overexpressing NTN1(II)FH (Fig. 7A). With immunoblotting we identified this protein as Jagged1 (Fig. 7B). This finding together with decreased Notch activation in luciferase reporter assays suggested that the presence of NTN1(II)FH causes Notch and Jagged1 to remain in complexes at the cell surface and thus decreases Notch signaling activation.

Fig. 7. NTN1(II)FH blocks localization of Notch2 and Jagged1 signaling complexes to the cell surface and prevents the colocalization of clathrin and the intracellular domain of Notch2 at the cell surface. (A) The cell surface proteins of U251MG cells expressing either NTN1FH or NTN1(II)FH were biotinylated. The cells were lysed and Notch2 or Jagged1 were immunoprecipitated. The Notch2 or Jagged1 expressed on the cell surface were detected by immunoblotting with streptavidin-conjugated horseradish peroxidase. Arrows indicate the correct molecular masses of Notch2 and Jagged1. Tubulin immunoblotting served as a loading control. No significant difference between the cell surface localizations of Jagged1 or Notch2 were observed. (B) The 150 kDa protein that co-immunoprecipitated with Notch2 in A (marked with white box in A), was further analyzed. The sample was again immunoblotted and the nitrocellulose was cut in half along the middle of the NTN1(II)FH lane. One half (on the left) was detected with anti-Jagged1 antibody and other half (on the right) with streptavidin. The 150 kDa protein was identified to be biotinylated Jagged1. (C) U251MG cells expressing either NTN1FH or NTN1(II)FH were used for immunofluorescence analysis to visualize the colocalization of the intracellular domain of Notch2 (Notch2-ICD) and clathrin. Red, clathrin; green, Notch2-ICD; yellow, the area of their colocalization. Enlarged images of the boxed areas are shown below. In cells expressing NTN1(II)FH the colocalization of clathrin and Notch2 was not observed.
We further examined whether the decrease in Notch signaling caused by NTN1(II)FH resulted from defects in the internalization of the intracellular domain of Notch2 (Notch2-ICD). The internalization occurs after the protease cleavage of Notch-receptor and takes place in clathrin-coated vesicles (Windler and Bilder, 2010). We therefore visualized the internalized Notch2-ICD by clathrin double immunofluorescence staining. This analysis revealed that in cells expressing NTN1(II)FH, Notch2-ICD and clathrin did not colocalize on the cell surface (Fig. 7C). In control cells and cells overexpressing NTN1FH, Notch2-ICD colocalized with clathrin in the leading edge of the cells. In NTN1(II)FH cells Notch2 was localized similarly to the leading edge of the cells, but clathrin was not recruited to the same locations.

Discussion

NTN1 was initially identified as an embryogenic axon guidance regulator. However, NTN1 was later found to be involved in the regulation of cancer cells. For example, NTN1 was recently proposed to act as a potential biomarker in GBM, and as a survival factor for glioma cells (Miyamoto et al., 2010; Ramesh et al., 2011). However, the functions and signaling mechanisms of NTN1 in GBM have remained largely unknown. Interestingly, NTN1 can induce the invasion of adenocarcinoma of the pancreas and colorectal cancer (Dumartin et al., 2010; Rodrigues et al., 2007). In this study we found that the expression levels of NTN1 are elevated in human GBM tumors and in aggressively invading glioblastoma cell lines, and that NTN1 acts as an invasion promoting factor for GBM cells.

To expand our understanding of the signaling of NTN1 in human GBM, we explored its binding partners in the GBM cells using tandem affinity purification combined with mass spectrometry identification of binding proteins. Pathway analysis of these interacting proteins revealed several cellular processes and signaling pathways where NTN1 may play a role, including phagocytosis, adherens junctions, the regulation of the actin cytoskeleton and ECM–receptor interactions.

One of the most prominent pathways identified in the interaction screen was the Notch signaling pathway. Notch signaling has been connected to enhanced GBM invasion (Chigurupati et al., 2010; Pierfelice et al., 2011; Sivasankaran et al., 2009; Zhang et al., 2012). Consistently, the inhibition of Notch signaling decreased the invasion of human GBM cells in our model system.

Current results indicate that NTN1 enhances Notch activity. Furthermore, both NTN1 and Notch signaling induced GBM cell invasion. NTN1 interacted physically with Notch pathway components, which led us to suggest that the Notch signaling pathway mediates NTN1-induced invasion. This was supported by our findings that NTN1 can pull down both Notch2 and Jagged1 and co-localizes with both Jagged1 and Notch2 on the cell surface. Furthermore, NTN1 colocalized with Jagged1 but not with Notch2 in the cytoplasm.

GBM patients are currently treated with various drugs, which often are not curative but provide only modest prolongation of the life of the patients. Therefore the discovery of new treatment options and enhanced targeting of the existing treatments are vital. Here we provide an insight into novel ways of targeting GBM. We report here that NTN1(II)FH specifically counteracts NTN1–Notch signaling and has therapeutic potential, as indicated by several results: NTN1(II)FH was able to pull down both Notch2 and Jagged1. In immunofluorescence analysis NTN1(II)FH colocalized with Notch2 and Jagged1 at the cell surface but did not colocalize with either of them in the cytoplasm. Exogenous NTN1(II)FH also altered the morphology of U251MG cells similarly to the Notch signaling inhibitor DAPT. Finally, we found that the Notch2–Jagged1 signaling complex was retained at the cell surface and that less colocalization of Notch2 and clathrin was observed on the cell surface in the presence of NTN1(II)FH. Based on these results we suggest that NTN1 facilitates Notch activation at cell surface.

This work provides new information of the mechanisms leading to aggressive GBM invasion – the main reason for its lethality. We describe a novel mechanism for activation of Notch signaling under pathological conditions. In several forms of human cancer, upregulation of the expression of NTN1 has been proposed as a means for tumor cells to escape apoptosis (Delloye-Bourgeois et al., 2009a; Delloye-Bourgeois et al., 2009b; Mazelin et al., 2004). Our results indicate that cancer cells can benefit from increased NTN1 expression also by activating the Notch signaling pathway, which provides a promising treatment opportunity for GBM.

Materials and Methods

Reagents and antibodies

Polyclonal antibodies recognizing the extracellular domain of Notch2 (25–255) and intracellular domain of Jagged1 (C-20) were obtained from Santa Cruz Biotechnology. A monoclonal antibody (D6/78) recognizing the intracellular domain of Notch2 was purchased from Millipore, a monoclonal antibody recognizing amino acids 28–264 of NTN1 was obtained from Enzo Biosciences, and a monoclonal antibody (X22) recognizing clathrin heavy chain was obtained from Thermo Scientific. Anti-hemagglutinin (HA-11) antibody (Covance) was used to recognize tagged NTN1. Anti- GAPDH (clone 71.1) antibody (Sigma) and anti-β-tubulin (H-235) antibody (Santa Cruz Biotechnology) were used as loading controls in immunoblotting. The fluorescein-conjugated Alexa Fluor secondary antibodies used for immunofluorescence microscopy were obtained from Invitrogen. IRDye™ 800CW secondary antibodies used in Li-Cor quantification of western blots were purchased from Li-Cor Biosciences. Horseradish-peroxidase-conjugated mouse, rabbit and goat immunoglobulins and streptavidin were purchased from DAKO. The Notch signaling inhibitor LY-374973 (E-[N-(3,5-difluorobenzenoyl)-L-alanyl]-S-phenylglycine t-butyl ester) (DAPT) was from Sigma-Aldrich.

Cell culture

U251MG cells (Health Sciences Research Resources Bank, Japan), U373MG cells (obtained from Dr Bengt Westermark, Uppsala) and U87MG cells (ATCC) were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplied with 10% heat-inactivated fetal calf serum (FCS; Gibco), 100 IU/ml penicillin, 50 mg/ml streptomycin and 1% L-glutamine. 293FT cells (Invitrogen, USA) were grown in DMEM supplied with 4.5 µg/ml glucose, 10% heat-inactivated FCS, 100 IU/ml penicillin, 50 mg/ml streptomycin and 1% L-glutamine. Human normal astrocytes were obtained from Lonza and cultured according to manufacturer’s instructions.

Plasmids

For the silencing of NTN1 expression, a series of shRNAs containing five different NTN1 targeting shRNAs were obtained from RNAi consortium (Broad Institute). After testing the efficiency of the shRNAs the two most efficient clones (designated as sh2 and sh3 which correspond to TRCN0000061944 and TRCN0000061945, respectively) were used in further experiments. To overexpress NTN1 or its domains the mouse cdNA of the full-length protein or its fragments were cloned into pLVX Puro vector (Clontech). First, the signal fragment of CD33 was cloned into the multiple cloning site of the pLVX vector containing C-terminal FLAG- and HA-tag-coding cDNAs were amplified with primers containing either

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>GenBank Accession</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTN1</td>
<td>NM_008744</td>
</tr>
<tr>
<td>CD33</td>
<td>NM_000070</td>
</tr>
<tr>
<td>Notch2</td>
<td>NM_001133</td>
</tr>
<tr>
<td>Jagged1</td>
<td>NM_004552</td>
</tr>
</tbody>
</table>

...and NTN1(III)FH 485–604 of NTN1. GenBank sequence number NM_008744.
C-terminal EGF-like repeats, and corresponding to IgG-tagged fragment L2-XIV (Hyttingen and Kojima, 2003) was transferred to a vector containing C-terminal streptag-HA tags. The ephrinA1FH plasmid was constructed similarly to that for NTN1. First, the sequence corresponding to the extracellular domain of the Ephrin A1 with no stop codon was amplified with PCR and cloned into pPKS-FH plasmid. Next, the corresponding sequences were cleaved from the pPKS-FH plasmid and inserted into the pLVX Puro plasmid (Clontech).

To monitor Notch activity, a Notch response element containing plasmid TPI- luc(981-6) and a Notch2-intracellular-domain-encoding plasmid, pEF-BOSneoSMNotch2 RAMIC, were obtained from RIKEN Bioresource Center DNA (Kato et al., 1996; Kurooka et al., 1998). pRL-TK Renilla luciferase vector (Promega) was used as an internal control for luciferase assays. pCR.1 vector was obtained from Invitrogen.

Primers were as follows: NTN1 forward, 5’-GAATATGGACATCAGGCGGCGGTTTG-3’; NTN1(II) FH reverse, 5’-GAAGTGTATCCGCTTCAGCTTG-3’; NTN1(III) FH forward, 5’-GAATATCCAGAATAAGAATTCGGGTG-3’; NTN1(III) FH reverse, 5’-GAAGTGTATCCGCTTCAGCTTG-3’.

**Lentivirus production and titration**

Lentiviruses were produced in 293FT cells using Lipofectamine 2000 (Invitrogen) transfection reagent. After the manufacturer’s instructions, 48 hours after transfection, virus-containing supernatants were collected. Viruses were titered by adding various volumes of virus supernatant to target cells and incubated for 24 hours. After this the virus-containing culture medium was replaced with fresh medium and the cells were incubated for the additional 24 hours. The cell culture medium was removed with medium containing 5 μg/ml of puromycin (Calbiochem). The cells exposed to puromycin selection were then monitored for 48 hours. The minimum volume of virus-containing supernatant that kept all the target cells alive was used in further experiments.

**Lentiviral gene silencing or overexpression**

Target cells were seeded on a 24-well plate and grown overnight. The next day the medium was replaced with the lentivirus supernatant and the cells were incubated for 24 hours. The culture medium was replaced with a fresh one. After 24 hours of incubation, the culture medium was replaced with selection medium. The efficiency of the knockdown or overexpression of NTN1 was evaluated by quantitative real-time PCR and immunofluorescence staining.

**Real-time quantitative PCR**

Total RNA from cells of interest was purified using NucleoSpin® RNA II kit (Macherey-Nagel). CDNAS were manufactured with iScript cDNA Synthesis Kit (Bio-Rad). The real-time levels of the indicated genes was measured by quantitative real-time PCR using Taqman primers (GAPDH: Hs99999905_m1; NTN1: Hs00180355_m1; Jagged1: Hs01070032_m1; Notch2: Hs00171086_m1; Notch1: Hs00171122_m1). The relative expression of each gene was calculated using the 2^-ΔΔCt formula. The results were analyzed using Odyssey 2.1 software.

**Immunofluorescence analysis**

The cells were grown on glass coverslips and at the end of the incubation period were fixed with 4% PFA for 5 minutes. The cells were then permeabilized with 0.1% Triton X-100 in PBS. After blocking, the cells were probed with fluorescent antibodies (1:100 dilution) at room temperature for 1 hour. The secondary antibodies were visualized by incubating the cells with Alexa Fluor 488-conjugated secondary antibodies (Molecular Probes). The images were acquired with Zeiss digital AxioCam grayscale camera and AxioVision 4.6 software.

### SDS-PAGE and immunoblotting

The isolated polypeptides were separated using 4–20% gradient Tris–glycine SDS-PAGE. After separation, the proteins were transferred to a nitrocellulose filter by semi-dry western blotting. The transfer efficiency was confirmed with Ponceau staining. After incubation the filter was washed with TBST. Primary antibody diluted in 5% non-fat milk in TBST was incubated with the filter at +4°C overnight. The excess antibody was then removed with the same washing buffer. The filter was then incubated with horseradish-peroxidase-conjugated secondary antibody diluted in 5% non-fat milk in TBST. Finally, the filter was washed and the immunoreactive proteins were visualized by ECL-reagent (GE Healthcare) and exposed to X-ray film.

### Quantification of western blots

After transferring the proteins to nitrocellulose membranes, immunoblotting was performed according to the protocol suggested by Li-Cor Biosciences. Briefly, the protein binding sites of the membrane were blocked in 5% nonfat milk in PBS at room temperature for 1 hour. The primary antibodies were incubated for 1 hour in 1% milk, 0.1% Tween-20, PBS and membrane was incubated with gentle mixing at +4°C overnight. After that the membrane was washed with 0.1% Tween in TBS. Next, the membrane was incubated with fluorochrome-conjugated secondary antibodies in 1% milk, 0.1% Tween-20, 0.01% SDS in PBS for 1 hour. After incubation, the membrane was washed with 0.1% Tween in TBS. Finally the membrane was scanned with the Odyssey Infrared Imaging System. Intensities of the bands were analyzed using Odyssey 2.1 software.

### Matrigel invasion assay

Cell culture inserts with 8.0 μm pore size (Becton Dickinson) were coated with 3 μg/ml growth-factor-reduced Matrigel® (BD Biosciences) in sterile water and dried under a cell culture laminar hood for 12 hours. Next, 20,000 cells in 300 μl of DMEM were applied to the upper chamber and 750 μl of complete medium was added to the lower chamber. The cells were then incubated for 7 hours in a humidified cell culture incubator. After incubation the cells were fixed with a fixing solution containing 40% methanol, 5% CH3COOH. The cells were stained with a filtered solution containing 40% methanol, 5% CH3COOH, 0.2% Coomassie Brilliant Blue. After staining, the inserts were washed with the fixing solution. Finally, the cells that had not invaded were removed from the upper chambers by scraping with cotton swabs. The invaded cells were analyzed under an AxioVert 200 microscope (Zeiss) equipped with a Plan-Neofluar 5x objective, NA 0.15 (Zeiss). Images were acquired with AxioCam HR (Zeiss) and AxioVision 3.1 (Zeiss) software. The area covered by invaded cells was quantified with ImageJ software. Images were converted to an 8-bit format, the background was subtracted, images were thresholded and the area of cells was selected and measured.

### Tandem affinity purification and mass spectrometry identification of binding proteins

Cells expressing tagged NTN1 fragments were grown to 80% confluency. For the purification, 150 mm cell culture dishes were used per clone. The cells were lysed with lysis buffer containing Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS and complete protease inhibitors (Roche). Collected cell lysates were incubated with FLAG-Sepharose on a shaker at +4°C for 12 hours. After incubation the Sepharose particles were collected by centrifugation and washed three times with the lysis buffer. After washing the particles were detached from the FLAG-Sepharose by incubation with 150 ng/μl FLAG peptide at +4°C for 2 hours. The eluted proteins were then incubated with anti-HA Sepharose in an end-over-end rotating shaker for 1 hour. After incubation the suspension was transferred to a Bio-Spin mini-chromatography column (Bio-Rad) and the Sepharose particles were washed three times with the lysis buffer. The attached proteins were eluted by incubating the Sepharose particles with non-reducing Laemmli sample buffer at 100°C for 5 minutes. The polypeptides of the samples were separated by SDS-PAGE (10–20% gradient gels) and visualized by silver staining. The identification, proteins were in-gel digested with trypsin and the resulting peptides were identified by liquid chromatography–tandem mass spectrometry analysis as previously described (Ohman et al., 2010).

### Luciferase reporter assays

A plasmid containing the Notch response element controlling the transcription of the luciferase gene (TPH-luc) was co-transfected by Fugene 6 transfection to target cells together with constitutive expression of Renilla luciferase (pRL-TK) and pCR3.1 empty vector (Kurooka et al., 1998). In the positive control pCR 3.1 vector was replaced with the intracellular domain of Notch2 containing pEF-BOSneoSMNotch2 RAMIC (Kato et al., 1996). After 48 hours the cell lysates were collected and luciferase activity was measured with the Dual Luciferase Assay Kit (Promega) using a Digene DCR-luminometer.

### Biotinylation of the cell surface proteins

The cells were first washed three times with cold phosphate-buffered saline. EZ-Link Sulfo-NHS-LC-LC-Biotin (0.5 mg/ml) in PBS (Pierce Biotechnology) was allowed to react with the cell surface proteins on ice for 1 hour. Non-specific binding of excess biotin was prevented by washing the cells three times with 150 mM glycine in Tris-buffered saline, pH 7.5. After blocking, the cells were
lysed with 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate (DOC), 0.1% SDS supplemented with Complete protease inhibitor (Roche). The nuclei were removed from lysates by centrifugation (1000 rpm for 10 minutes). The cell lysates were preabsorbed with protein-G-Sepharose (GE healthcare) at +4°C for 1 hour. The lysates were then incubated with anti-Notch2 extracellular or anti-Jagged1 antibodies at +4°C for 1 hour. The antibodies and their binding proteins were precipitated with protein-G-Sepharose. The Sepharose particles were subsequently washed and the bound proteins were eluted with Laemmli sample buffer at +100°C for 5 minutes. The biotinylated Notch2 or Jagged1 proteins were separated by SDS-PAGE and visualized by western blotting, and detected with horseradish-peroxi-oxide-conjugated streptavidin.

Statistical analysis
Error bars represent the standard deviation or standard error of mean of the 3–5 independent repeats performed for each experiment. Statistical significance was assessed with non-parametric Mann–Whitney U-tests for independent samples.

Bioinformatics analysis
Exon array data of 425 primary GBM samples and 10 normal brain tissues from The Cancer Genome Atlas (TCGA) (Cancer Genome Atlas Research Network, 2008) were preprocessed at the gene expression level using the Multiple Exon Array Preprocessing (MEAP) algorithm (Chen et al., 2011). Downstream data analyses were performed within the Anduril framework (Ovaska et al., 2010). Candidate pathways were analyzed using the Moksiskaan database to determine comprehensive signaling pathway networks from canonical pathways (Laakso and Hautaniemi, 2010).

Colocalization analysis
To quantify the colocalization in immunofluorescence micrographs, a cocoeistence analysis was performed. The background was subtracted from the acquired micrographs by low pass filtering the images with an averaging filter of dimensions 29×29 for the red, and 9×9 for the green signal, after which the averaged image was subtracted from the original. The subtracted signals were thresholded by constant values of 2% for red, and 4% for green signal. The values were estimated by visual inspection of signal expression. The relative area of colocalization was expressed as the area of overlapping signals divided by the total area of signals. The processing and analysis were carried out in the Anduril framework (Ovaska et al., 2010).

Acknowledgements
We thank Anne Remes and Sami Starast for excellent technical assistance and the Biomedical Imaging Unit at the University of Helsinki for providing microscopy and imaging facilities. We also express our gratitude for RIKEN BRC and Dr Tanoue Honjo for providing us the TP1-luc(981-6) and pEF-BOSneoSE-mNotch2 RAMIC plasmids.

Author contributions
I.Y. performed and designed most of the experiments, Y.H. carried out experiments and bioinformatics analyses, P.C. conducted the bioinformatics analyses, V.R. performed immunofluorescence quantifications, S.H. supervised bioinformatics analyses, T.A. performed mass spectrometry identifications, M.H. designed the research, J.K.-O., I.Y., Y.H. and M.H. analyzed the data and wrote the paper.

Funding
This research was supported by grants from the Finnish Cultural Foundation (M.H.); the Academy of Finland [grant number 218145 to S.H.; grant numbers 135628, 140950 and 255842 to T.N.; grant number 139236 to J.K.-O.]; the Finnish Cancer Foundation (S.H. and J.K.-O.); the Sigrid Juselius Foundation (S.H. and J.K.-O.); Finska Läkarelinnakanet (J.K.-O.); the Finnish Society of Sciences and Letters (J.K.-O.); Biocentrum Helsinki (J.K.-O.); Helsinki University Hospital Fund and the University of Helsinki (J.K.-O.); Helsinki Biomedical Graduate Program (I.Y. and Y.H.); and the Finnish Doctoral Programme in Computational Sciences (P.C.).

Supplementary material available online at http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.120022/-/DC1

References
Castets, M. and Mehlen, P. (2010). Notrin-1 role in angiogenesis: to be or not to be a proangiogenic factor? Cell Cycle 9, 1466-1471.


NTN1 induces Notch signaling 2469


Fig. S1. NTN1FH colocalizes with Jagged1 and Notch2 more efficiently than control protein. (A) The secretion of NTN1FH and EphrinA1FH was validated by immunoblotting. Culture medium from U251MG cells overexpressing either NTN1FH or EphrinA1FH was harvested and subjected to immunoblotting using HA antibody. Both NTN1FH and EphrinA1FH were secreted efficiently. (B) U251MG cells were treated with NTN1FH or EphrinA1FH containing conditioned medium at +37°C for 60 minutes. The colocalization with Jagged-1 was visualized as in Fig. 5D. Colocalization of NTN1FH and Jagged1 was observed both on cell surface and cytoplasm of the cells. Minor intracellular colocalization was observed also with EphrinA1FH. (C) The colocalization of Notch2 with NTN1FH or EphrinA1FH was explored as in Fig. 5E. NTN1FH and Notch2 (red) colocalized on cell surface. (D,E) The relative area of colocalization was analyzed by measuring the overlapping area of green and red signal and by normalizing to the area where either green or red signal was detected. NTN1FH colocalized with Jagged 1 (D) and with Notch2 (E) on a larger area than EphrinA1FH.

Table S1. A list of proteins interacting with NTN1 fragments

Download Table S1

Table S2. List of KEGG pathways interacting with NTN1(I)FH

Download Table S2