RESEARCH ARTICLE

p75NTR-dependent Rac1 activation requires receptor cleavage and activation of an NRAGE and NEDD9 signaling cascade

Michele Zeinieh[1], Amir Salehi[2], Vijidha Rajkumar[1] and Philip A. Barker[1],*

ABSTRACT

The p75 neurotrophin receptor (p75NTR, also known as tumor necrosis factor receptor superfamily member 16) is implicated in diverse cellular events, but fundamental aspects of its signaling mechanisms remain unclear. To address this, we have established a novel bioassay to characterize signaling cascades activated by p75NTR. We show that in COS7 cells, p75NTR expression causes a large increase in cell surface area that relies on the activation of Rac1, and we demonstrate that the p75NTR-dependent COS7 phenotype is dependent on ADAM17- and γ-secretase-dependent cleavage of p75NTR and generation of the p75NTR intracellular domain (p75NTR^{ICD}). We show that the p75NTR adaptor protein NRAGE (also known as MAGED1) acts downstream of the p75NTR^{ICD} in this cascade and, through a yeast two-hybrid screen, identify NEDD9, a Cas family adaptor protein, as a novel NRAGE-binding partner that mediates p75NTR-dependent Rac1 activation and cell spreading. Our results demonstrate a crucial role for p75NTR cleavage in small GTPase activation and define a novel Rac1 activation pathway involving the p75NTR^{ICD}, NRAGE and NEDD9.

INTRODUCTION

The p75 neurotrophin receptor (p75NTR, also known as tumor necrosis factor receptor superfamily member 16), a member of the tumor necrosis factor receptor (TNFR) superfamily, participates in an array of cellular events that include apoptosis, survival signaling, differentiation, neurite outgrowth and growth cone collapse (reviewed in Schecterson and Bothwell., 2010). Some of these are activated by the neurotrophin binding to p75NTR and, in others, p75NTR acts as an accessory receptor and signaling component for other ligand-binding receptors (reviewed in Reichardt., 2006). Several studies have demonstrated the importance of small GTPases, most notably RhoA and Rac1 (Yamashita et al., 1999, Yamashita et al., 2002, Harrington et al., 2002, Yamashita and Tohyama., 2003, Domeniconi et al., 2005, Harrington et al., 2008, Coulson et al., 2008, Park et al., 2010, Sun et al., 2012), in p75NTR signaling

cascades but the precise mechanisms by which these and other downstream elements are activated remain uncertain.

In neuronal growth inhibition, a receptor complex containing p75NTR, Nogo (also known as reticulon-4) receptor and Lingo1 responds to myelin-based inhibitors (MBIs), and p75NTR functions as the receptor component that induces RhoA activation (Yamashita et al., 2002, Domeniconi et al., 2005, Harrington et al., 2008, Park et al., 2010, Sun et al., 2012). Kalirin-9, which is a dual Rho and Rac guanine exchange factor (GEF), and Rho GDP-dissociation inhibitor (Rho-GDI) share a binding site on p75NTR, and it has been proposed that MBIs shift the p75NTR binding preference from kalirin-9 to Rho-GDI (Harrington et al., 2008). This, in turn, acts to inhibit Rho-GDI activity and promote RhoA action. p75NTR can also mediate Rac1 regulation. In some settings, this links the activated receptor to the JNK signaling cascade and promotes apoptosis (Harrington et al., 2002), and in others, p75NTR collaborates with Par3 to localize Rac1 to the axon–glial interface and thereby promote myelination (Tep et al., 2012).

p75NTR undergoes a two-step cleavage event, known as regulated intramembrane proteolysis (RIP), in which the extracellular juxtamembrane domain is cleaved by ADAM17, followed by cleavage of the transmembrane domain through the γ-secretase complex. These sequential cleavage events release the intracellular domain (ICD) from its transmembrane tether. The untethered p75NTR^{ICD} fragment that is generated has been implicated in cell migration (Wang et al., 2008), enhancement of pro-survival signaling (Ceni et al., 2010, Matusica et al., 2013), induction of apoptosis (Kenchappa et al., 2006, Kenchappa et al., 2010) and changes in cell morphology (Domeniconi et al., 2005).

p75NTR is not catalytically active and therefore downstream signaling events rely on its interaction with cytosolic adaptor proteins. Previous studies have identified NRAGE (neurotrophin receptor interacting MAGE homolog, also known as MAGED1) as a p75NTR interactor that activates a JNK and caspase-3-dependent apoptotic pathway in vitro and in vivo (Salehi et al., 2000, Salehi et al., 2002, Bronfman et al., 2003, Bertrand et al., 2008). Interestingly, NRAGE has also been shown to play a role in regulating homotypic cell adhesion (Xue et al., 2005) and in cell movements that include epithelial to mesenchymal transitions (EMT) in mammary epithelia (Kumar et al., 2011).

Taken together, available data suggest that p75NTR and the soluble p75NTR^{ICD} fragment can participate in a wide array of functions in different contexts. The mechanisms that allow for the activation of distinct signaling paths remain uncertain, and specific signaling cascades that are selectively activated by the p75NTR^{ICD} have not been identified. This is, in part, due to a lack of reliable in vitro assays for analyzing p75NTR and p75NTR^{ICD} signaling cascades. Here, we describe a novel COS7-based cell spreading assay that provides a robust output for analyzing a

[1]Department of Neurology and Neurosurgery, Montreal Neurological Institute, McGill University, 3801 University Street, Montreal, Quebec H3A 2B4, Canada. [2]Department of Pathology, McGill University, 3801 University Street, Montreal, Quebec H3A 2B4, Canada.

*Author for correspondence (phil.barker@mcgill.ca)

subset of p75NTR-dependent signaling events. Using this assay, we show that p75NTR-dependent Rac1 activation only occurs after ADAM17-dependent cleavage of p75NTR and liberation of the p75NTR[ICD]. We demonstrate that activation of Rac1 by the p75NTR[ICD] relies on an NRAGE-dependent pathway and, through yeast-two hybrid screening, we identify NEDD9 as a novel NRAGE cofactor that is also required for p75NTR-dependent Rac1 activation and cell spreading. Our results demonstrate a crucial role for p75NTR cleavage in small GTPase activation and define a novel Rac1 activation pathway involving the p75NTR[ICD], NRAGE and NEDD9.

RESULTS
p75NTR induces COS7 cell spreading

To gain insight into the role of p75NTR cleavage and proximal signaling events, we established a bioassay based loosely on a COS7 contractility assay used previously to study semaphorin-induced signaling events (Takahashi et al., 1999, Takahashi and Strittmatter, 2001, Zanata et al., 2002, Mitsui et al., 2002). COS7 cells were transfected with EGFP together with p75NTR constructs (Fig. 1A,B), then plated at low density on glass coverslips that had been pre-coated with laminin. After 24 hours, cells were fixed, left non-permeabilized and exposed to wheat germ agglutinin conjugated to Cy3 to visualize cell surfaces. Fig. 1C,D shows that overexpression of full-length wild-type p75NTR or the p75NTR[ICD] resulted in significant

COS7 cell spreading, giving a mean cell surface area almost double that of control cells. Interestingly, overexpression of a cleavage-resistant form of p75NTR did not promote cell spreading, suggesting that COS7 cell spreading might provide a bioassay for p75NTR signaling activities that are dependent on receptor cleavage.

p75NTR acts through Rac1 to promote cell spreading

The Rho family of small GTPases plays a prominent role in transducing signals from plasma membrane receptors to the actin cytoskeleton, and p75NTR has previously been shown to activate RhoA and Rac1. Because Rac1 is necessary for fibroblast and macrophage cell spreading (Wells et al., 2004, Guo et al., 2006), we asked whether Rac1 was required for the cell spreading induced by p75NTR overexpression. To do this, COS7 cells were transfected with RacN17, a dominant-negative Rac1 isoform, and with full-length p75NTR or with the p75NTR[ICD]. Fig. 2A,B shows that cell spreading evoked by the p75NTR constructs was blocked in cells expressing RacN17.

As an alternative approach, we asked whether NSC 23766, a Rac1 inhibitor that blocks the interaction of Rac1 with its cognate GEFs, reduced the amount of cell spreading induced by p75NTR. COS7 cells transfected with full-length p75NTR or the p75NTR[ICD] were plated on glass coverslips precoated with laminin and, after 24 hours, were treated with NSC 23766 (100 µM) for 6 hours. Fig. 2C,D shows that the Rac1 inhibitor
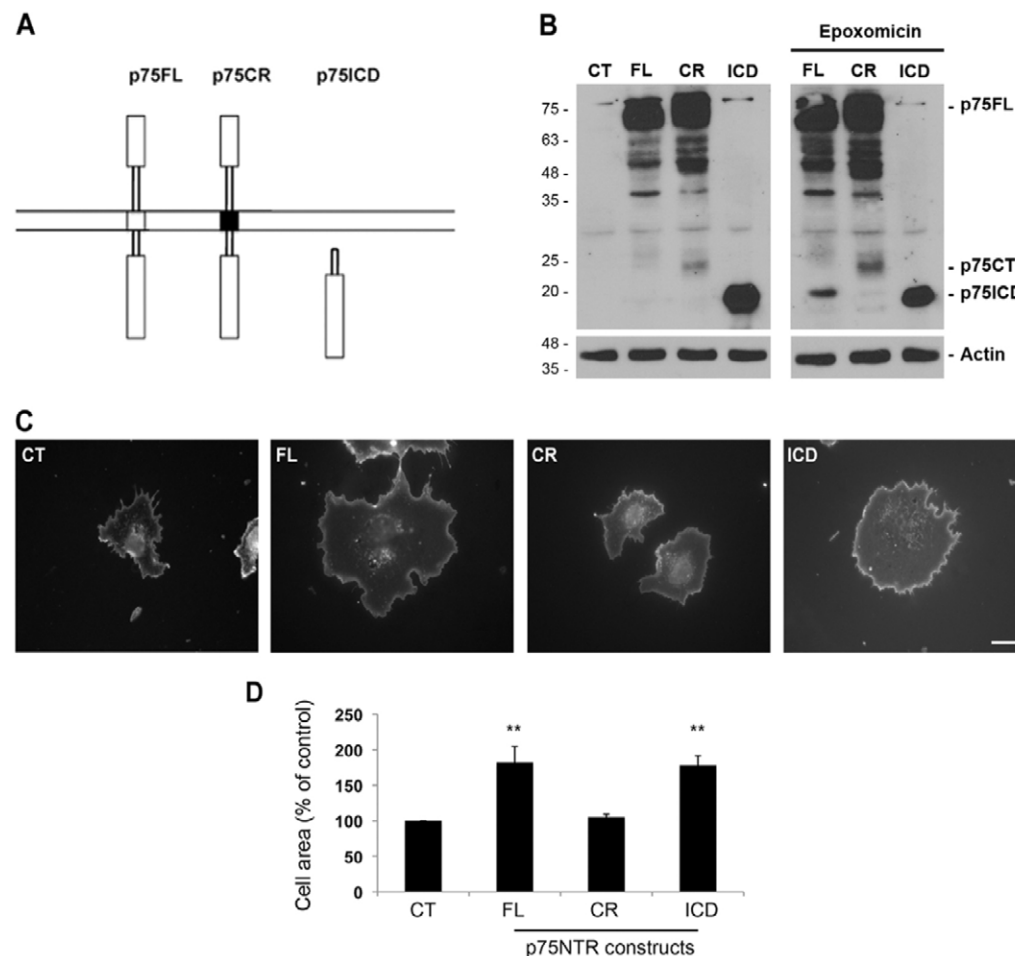

Fig. 1. p75NTR[ICD] mediates COS7 cell spreading. (A) Schematic showing the different p75NTR constructs overexpressed in COS7 cells: full-length p75 (p75FL), cleavage-resistant p75 (p75CR), in which the transmembrane domain (shown in black) is replaced with the transmembrane domain of Fas that is unable to be cleaved, and p75 intracellular domain (p75ICD). (B) Western blot showing the overexpression of the different p75NTR constructs in COS7 cells with and without epoxomicin to show the cleavage products. (C) Representative spreading assay of COS7 cells overexpressing EGFP alone (CT) or the different p75NTR constructs (p75FL, CR and ICD). Scale bar: 25 µm. (D) Quantification of the spreading assay showing a significant increase in cell area in p75FL- and p75ICD-expressing cells compared with that of the control (CT) and p75CR cells. Values are expressed as the percent change in cell area compared to that of controls and are shown as the mean±s.e.m. (n=3 independent experiments); **P<0.01.
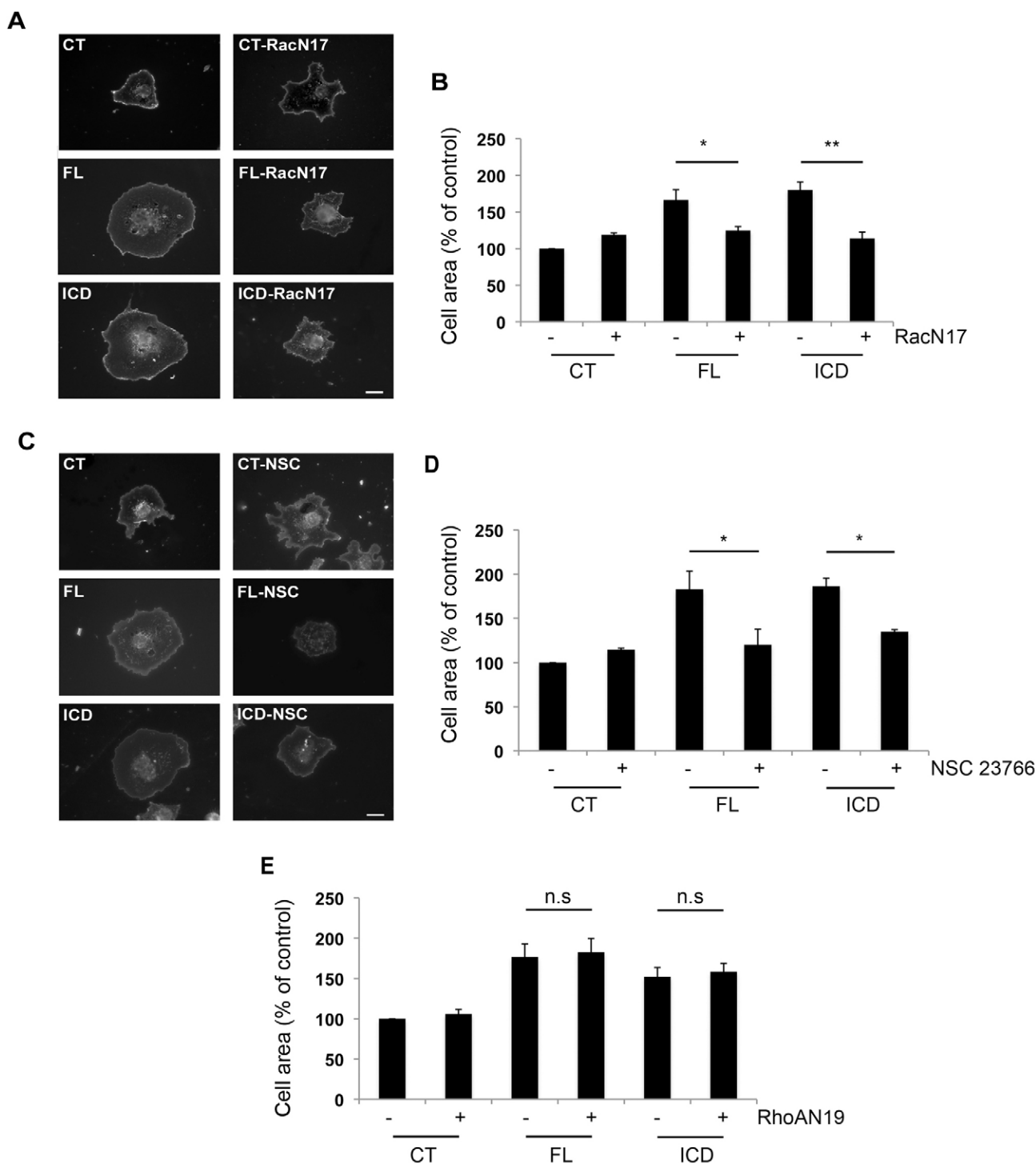
Fig. 2. p75NTR acts through Rac1 to induce cell spreading. (A) Representative spreading assay of COS7 cells overexpressing EGFP (CT), full-length p75 (FL) or p75ICD with or without dominant-negative Rac (RacN17). (B) Quantification of the spreading assay in A showing a significant decrease in the cell area of p75FL and p75ICD cells after overexpressing RacN17, but not in that of CT cells. Values are expressed as the percent change in cell area compared to that of controls and are shown as the mean±s.e.m. ($n=5$ independent experiments). (C) Representative spreading assay of COS7 cells expressing EGFP (CT), p75FL or p75ICD with or without the Rac inhibitor NSC 23766 for 6 hours. Scale bars: 25 μm. (D) Quantification of the spreading assay in C showing a significant decrease in the cell area of p75FL- and p75ICD-expressing cells treated with NSC 23766 that was not observed in CT cells. Values are expressed as the percent change in cell area compared to that of controls and are shown as the mean±s.e.m. ($n=3$ independent experiments). (E) Quantification of the spreading assay performed with cells overexpressing p75FL and ICD with or without dominant-negative RhoA (RhoAN19), showing no effect on cell spreading. Values are expressed as the percent change in cell area compared to that of controls and are shown as the mean±s.e.m. ($n=2$ independent experiments); *$P<0.05$; **$P<0.01$; n.s., not significant.

effectively blocked cell spreading evoked by full-length p75NTR and by the p75NTR^{ICD}.

In parallel experiments, we tested whether a dominant-negative form of RhoA (N19) altered spreading induced by full-length p75NTR or the p75NTR^{ICD}, but we found that it had no effect in this assay (Fig. 2E). Taken together, these data demonstrate that Rac1, but not RhoA, plays a crucial role in evoking p75NTR-induced cell spreading in COS7 cells.
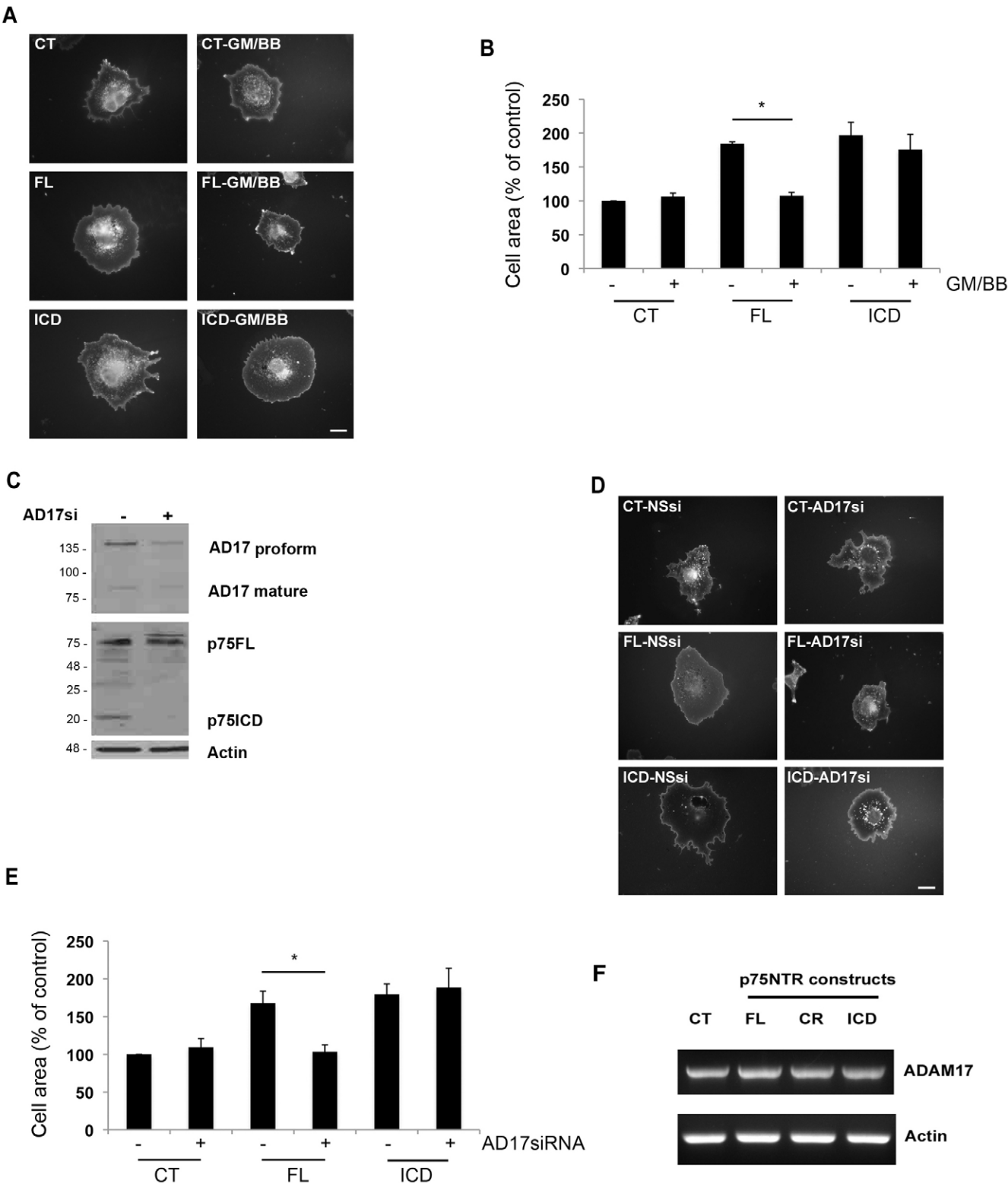

Fig. 3. See next page for legend.

Fig. 3. p75NTR cleavage by ADAM17 is necessary for cell spreading.
(A) Representative spreading assay of COS7 cells overexpressing EGFP (CT), full-length p75 (FL) and p75ICD with or without the broad matrix metalloprotease inhibitors GM6001 and BB94 (GM/BB) for 1 hour. (B) Quantification of the spreading assay in A showing a significant decrease in the cell area of p75FL cells when treated with GM6001 and BB94, but not in p75ICD cells or CT cells. Values are expressed as the percent change in cell area compared to that of controls and are shown as the mean±s.e.m. ($n=3$ independent experiments). (C) Western blot showing a decrease in ADAM17 expression in COS7 cells expressing ADAM17 siRNA (Ad17si) as well as a reduction in p75ICD generation upon ADAM17 knockdown. (D) Representative spreading assay of COS7 cells overexpressing EGFP (CT), p75FL or p75ICD with or without ADAM17 siRNA. Non-specific siRNA (NSsi) was used as control. Scale bars: 25 µm. (E) Quantification of the spreading assay in D showing a significant decrease in the cell area of p75FL cells after ADAM17 knockdown, but not in p75ICD cells or CT cells. Values are expressed as the percent change in cell area compared to that of controls and are shown as the mean±s.e.m. ($n=3$ independent experiments); *$P<0.05$. (F) RT-PCR of ADAM17 mRNA expression in control COS7 cells or in cells overexpressing the different p75NTR constructs.

ADAM17-mediated p75NTR cleavage is a prerequisite for cell spreading

p75NTR undergoes a two-step cleavage event, known as regulated intramembrane proteolysis (RIP), to generate the p75NTR^{ICD}. To determine whether RIP-dependent generation of the p75NTR^{ICD} is required for the changes in COS7 cell morphology, we first determined whether GM6001 and BB94, metalloprotease inhibitors capable of targeting ADAM proteases, blocked the cell spreading induced by overexpression of full-length p75NTR. COS7 cells transfected with full-length p75NTR or the p75NTR^{ICD} were plated on glass coverslips pre-coated with laminin and, after 24 hours, were treated with GM6001 (10 µM) and BB94 (200 nM) for 1 hour. Fig. 3A,B shows that GM6001 and BB94 sharply reduced spreading in COS7 cells expressing full-length p75NTR and that these compounds had no effect on cell spreading induced by the p75NTR^{ICD}. This suggests that p75NTR cleavage mediated by cell-surface ADAMs plays a crucial role in generating the cell spreading phenotype.

We have previously shown that ADAM17 mediates the initial extracellular cleavage of p75NTR (Kommaddi et al., 2011, Ceni et al., 2010), and therefore characterized its effect on cell spreading. Fig. 3C shows that ADAM17 mRNA is readily detected in COS7 cells and its levels are not altered by overexpression of full-length p75NTR, the cleavage-resistant form of p75NTR or the p75NTR^{ICD}. To determine whether ADAM17 is required for the effect of p75NTR on cell spreading, small interfering RNA (siRNA) was used to reduce endogenous ADAM17 levels in COS7 cells overexpressing full-length p75NTR and p75NTR^{ICD}. ADAM17 siRNA effectively reduced ADAM17 expression in COS7 cells, and accumulation of the p75NTR^{ICD} was blocked when ADAM17 levels were suppressed (Fig. 3D). To test the effect of ADAM17 knockdown on p75NTR-induced cell spreading, COS7 cells were transfected with ADAM17 siRNA or with control siRNA, together with p75NTR+EGFP or EGFP alone, and assessed for cell area 48 hours later. ADAM17 knockdown effectively blocked cell spreading induced by p75NTR overexpression but had no effect on spreading induced by the p75NTR^{ICD} (Fig. 3E,F). Taken together, these data demonstrate that by initiating p75NTR cleavage and allowing generation of the p75NTR^{ICD}, ADAM17 plays a crucial role in elaborating the cell spreading phenotype induced by p75NTR overexpression.

The p75NTR^{ICD} drives cell spreading

The p75NTR^{ICD} is generated in COS7 cells when the full-length receptor is overexpressed. The production of this fragment is blocked by the metalloprotease inhibitors GM6001 and BB94 as well as by compound XXI, a γ-secretase inhibitor (Fig. 4A). Consistent with previous results, γ-secretase inhibition resulted in accumulation of a 25-kDa C-terminal fragment (CTF) of p75NTR (Fig. 4A, lane 2). Several studies have suggested that the CTF and the p75NTR^{ICD} have distinct biological activities (Domeniconi et al., 2005, Kenchappa et al., 2006, Underwood et al., 2008, Kenchappa et al., 2010), and we therefore addressed whether the CTF could mediate p75NTR-dependent cell spreading. COS7 cells overexpressing p75FL, p75NTR^{ICD} or an EGFP control were plated on glass coverslips and treated with compound XXI (10 µM) or vehicle for 1 hour. Fig. 4B,C shows that compound XXI had no effect on cells expressing EGFP alone or expressing the p75NTR^{ICD} yet it blocked the spreading of cells expressing full-length p75NTR. Because compound XXI causes accumulation of the p75NTR CTF while preventing p75NTR^{ICD} accumulation, this indicates that the p75NTR^{ICD} is the relevant fragment required for p75NTR-dependent cell spreading in COS7 cells. Therefore, the p75NTR^{ICD}, generated through sequential ADAM17- and γ-secretase-dependent p75NTR cleavage, is required to drive changes in COS7 cell shape.

p75NTR binds to the four neurotrophins present in mammals and in our next experiments, we addressed whether these ligands have an impact on the COS7 spreading assay. The addition of nerve growth factor (NGF) or brain-derived neurotrophic factor (BDNF) (each at 25 ng/ml), for 1 hour or for 24 hours, had no effect on p75NTR cell spreading (Fig. 4D). To determine whether endogenous neurotrophins might play a role, we used RT-PCR to establish whether neurotrophins or their receptors are expressed in COS7 cells. Neither p75NTR nor the Trk receptor mRNA were detected (data not shown) but mRNA encoding each of the four neurotrophins was present in this line (Fig. 4E). The capacity of full-length p75NTR to drive cell spreading was not significantly changed when cells were maintained in REX (Fig. 4F), an antibody directed against the p75NTR extracellular domain that blocks ligand binding (Clary et al., 1994). We conclude that neurotrophin binding to p75NTR is not required for the cell spreading phenotype observed in this line and that constitutive, rather than ligand-induced, p75NTR cleavage drives the cell spreading phenotype.

NRAGE acts downstream of p75NTR to mediate cell spreading

The p75NTR^{ICD} does not have intrinsic enzymatic activity and therefore relies on interaction with cytosolic binding proteins to elicit downstream effects. One of these adaptors, termed NRAGE, mediates morphological changes in transformed human mammary epithelial cells and U2OS cells (Xue et al., 2005, Kumar et al., 2011), and we therefore asked whether NRAGE is required for p75NTR-dependent COS7 cell spreading. After confirming that NRAGE siRNAs suppressed NRAGE protein expression in COS7 cells (Fig. 5A), we asked whether NRAGE depletion altered cell spreading induced by overexpression of full-length p75NTR or the p75NTR^{ICD}. NRAGE knockdown had no effect on the area occupied by control cells but it eliminated the increase in cell spreading induced by p75NTR or the p75NTR^{ICD} (Fig. 5B,C). NRAGE could mediate these effects by binding to the p75NTR^{ICD} and playing a direct role in downstream signaling or by facilitating the generation or maintenance of the p75NTR^{ICD}. However, levels of full-length
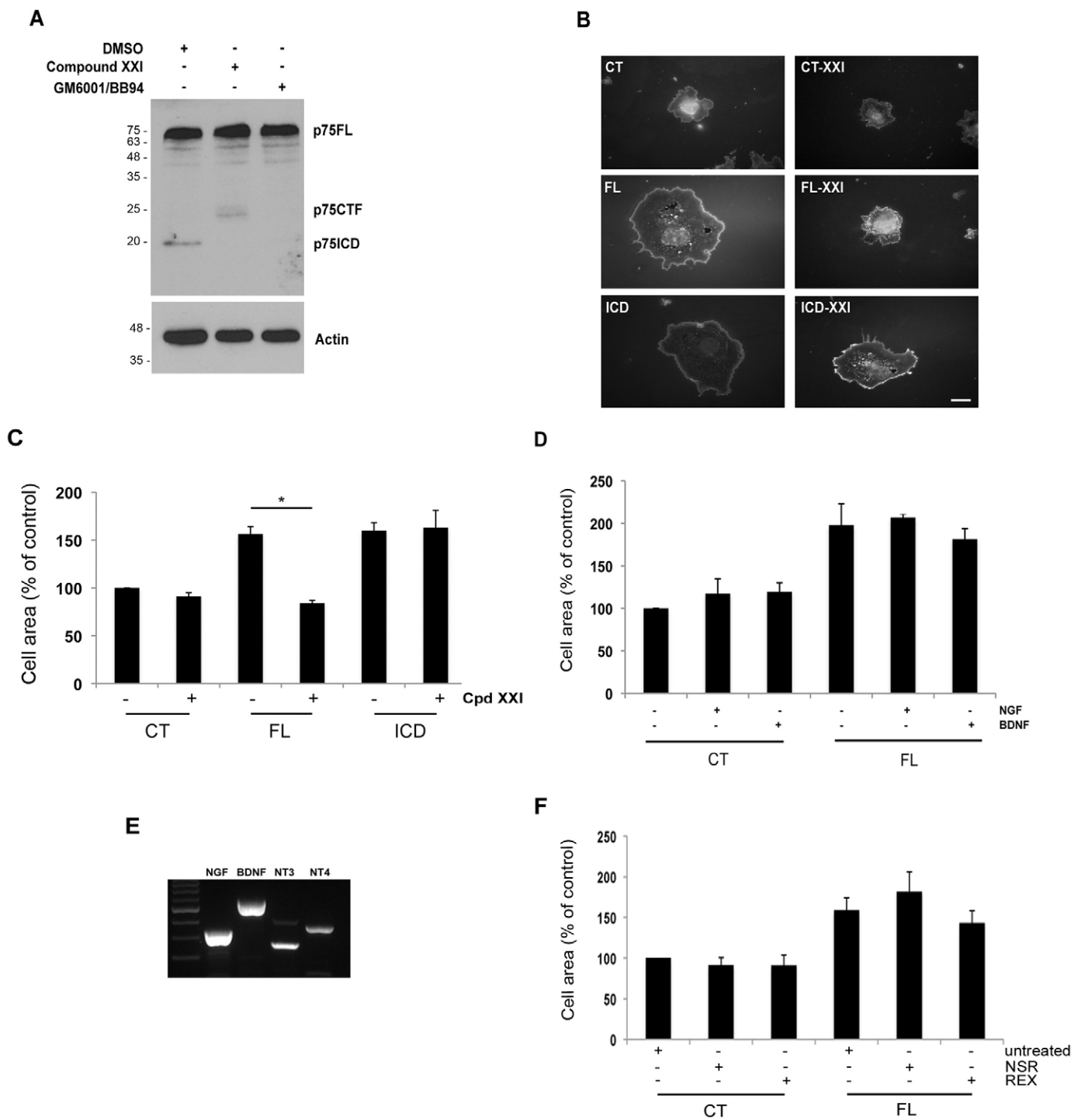
Fig. 4. p75NTR is cleaved by γ-secretase to induce cell spreading. (A) Western blot showing the inhibition of the generation of the p75NTR[ICD] but not CTF in COS7 cells when treated with compound XXI (lane 2) and the inhibition of both p75NTR[ICD] and CTF generation in cells treated with GM6001 and BB94 (lane 3). DMSO was used as vehicle control. (B) Representative spreading assay of COS7 cells overexpressing EGFP (CT), full-length p75 (FL) or p75ICD, with or without the γ-secretase inhibitor compound XXI for 1 hour. Scale bar: 25 μm. (C) Quantification of the spreading assay in B showing a significant decrease in the cell area of p75FL cells when treated with compound XXI, but not in p75ICD cells or CT cells. (D) Quantification of a spreading assay of COS7 cells overexpressing EGFP (CT) or p75FL, with or without NGF or BDNF (25 ng/ml) for 24 hours. Neurotrophin addition did not significantly affect p75NTR-induced spreading. (E) RT-PCR showing the expression of the different neurotrophins (NGF, BDNF, NT-3 and NT-4) in COS7 cells. (F) Quantification of spreading in cells exposed to REX, a p75NTR antibody that blocks ligand binding. Non-specific rabbit serum (NSR) was used as control. Antibody addition did not significantly affect p75NTR-induced spreading. In C,D,F values are expressed as the percent change in cell area compared to that of controls and show the mean±s.e.m. ($n=3$ independent experiments); *$P<0.05$ **$P<0.01$ and ***$P<0.001$.
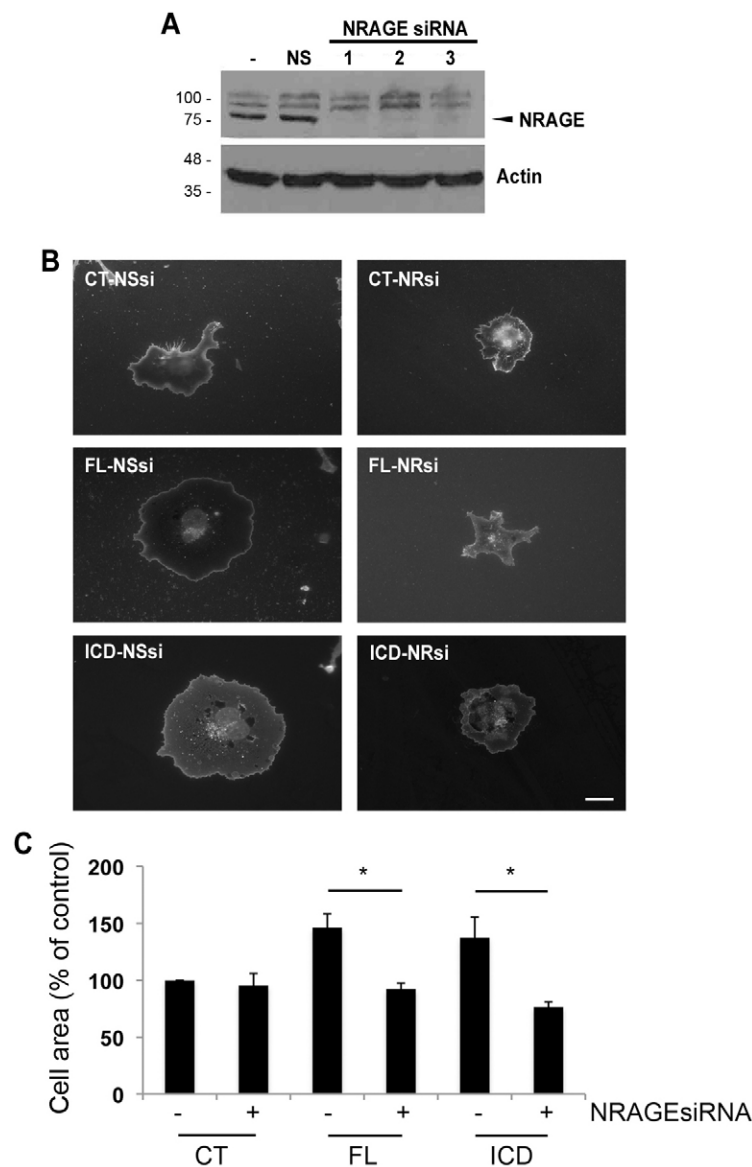
Fig. 5. NRAGE acts downstream of p75NTR in cell spreading.
(A) Western blot showing NRAGE knockdown in COS7 cells using three different siRNAs. Only NRAGE siRNA 1 was used in subsequent experiments. NS, nonspecific siRNA. (B) Representative spreading assay of COS7 cells overexpressing EGFP (CT), full-length p75 (FL) or p75ICD with or without NRAGE siRNA (NRsi). Nonspecific siRNA (NSsi) was used as control. Scale bar: 25 μm. (C) Quantification of spreading assay showing a significant decrease in the cell area of p75FL and p75ICD cells after NRAGE depletion but not in that of CT cells. Values are expressed as the percent change in cell area compared to that of controls and are shown as the mean±s.e.m. ($n=3$ independent experiments); *$P<0.05$.

p75NTR or the p75NTR^{ICD} were unchanged by NRAGE knockdown (data not shown), ruling out the latter possibility. We therefore focused our efforts on the hypothesis that NRAGE links the p75NTR^{ICD} to downstream signaling partners.

NRAGE interacts physically and functionally with NEDD9
To identify NRAGE-interacting proteins that could link the p75NTR^{ICD} to Rac1 activity, we performed a cytosolic yeast two-hybrid screen, using an NRAGE–SOS fusion protein as bait with a human fetal brain library of cDNAs fused to a myristoylated membrane-localization signal as a source of potential binding partners. Interestingly, four of the positive clones to emerge from this screen encoded distinct overlapping regions of NEDD9, a member of the Cas family (Fig. 6A). NEDD9 is a key player in the regulation of cell shape and cell migration and has recently emerged as a key player directing EMT in melanoma, lung and breast cancer (Kim et al., 2006, Izumchenko et al., 2009, Miao et al., 2013, Kondo et al., 2012, Little et al., 2014).

All four of the NRAGE-binding clones contained a NEDD9 fragment that started at amino acid 637 and ended at the

C-terminus of the protein (amino acid 834), and Fig. 6B shows that a GST–NEDD9 fusion protein containing this 198-amino-acid fragment was capable of associating with NRAGE expressed by *in vitro* translation, indicating that NRAGE directly binds to this NEDD9 fragment. To identify the minimal region within NEDD9 required for NRAGE binding, pulldowns were performed using progressively smaller fragments of NEDD9 and, from this, a fragment of NEDD9 stretching from amino acids 704 to 765 emerged as the NRAGE interaction domain (Fig. 6B). Examination of the primary sequence of this 62-amino-acid region revealed a putative helix-loop-helix domain. To test the relevance of the helical domains in this region, we disrupted each of the two helices by substituting proline residues in helix 1 (G722P) or in helix 2 (G744P). The G722P substitution had no effect on the NRAGE–NEDD9 interaction, whereas the G744P mutation abolished the interaction between the two proteins (Fig. 6C). Taken together, these data indicate that the helix-loop-helix present in NEDD9 is required to bind to NRAGE and that the second helix of this domain plays a crucial role in their association.
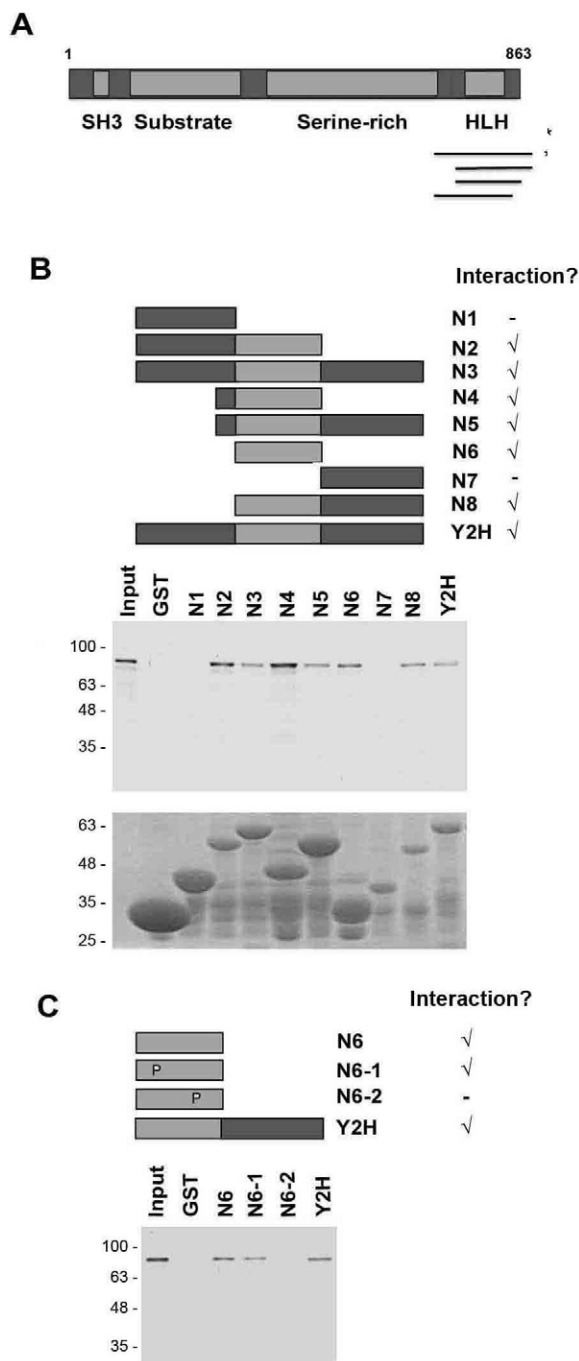
Fig. 6. NRAGE interacts with the C-terminal domain of NEDD9.
(A) Schematic showing the structure of NEDD9, which contains an SH3 domain, a substrate-binding domain, a serine-rich domain and a helix loop helix (HLH) domain. The four clones identified contain overlapping regions of the HLH spanning amino acids 637–834. (B) Different GST–NEDD9 fusion proteins containing the 198-amino-acid region were generated, and their interaction with NRAGE produced by in vitro translation was analyzed. In the schematic in B, the HLH region is represented in light gray. Regions outside of the HLH region are represented in dark gray. The interaction of NRAGE with GST–NEDD9 fusions was detected by NRAGE immunoblot. √ and − indicate the presence or absence of interaction, respectively. The lower panel shows a Coomassie-Blue-stained gel that confirms the expression of the different GST–NEDD9 mutants.
(C) Introduction of two proline residues in helix 1 and helix 2 of the NEDD9 HLH identifies helix 2 of NEDD9 as the NRAGE-binding domain. In the schematic in C, the HLH region is represented in light gray, and regions outside of the HLH region are represented in dark gray. In the lower panel, the interaction of NRAGE with GST–NEDD9 fusions was detected by immunoblotting for NRAGE.

To determine whether the NRAGE–NEDD9 interaction occurred in mammalian cells and to identify the domain in NRAGE that bound to NEDD9, we fused amino acids 637–834 of NEDD9 to GST and then expressed this with FLAG-tagged NRAGE, or with several FLAG-tagged NRAGE deletion mutants (shown schematically in Fig. 7A), in HEK293T cells and performed pull downs. Full-length NRAGE showed robust binding to the GST–NEDD9 fusion protein but not to GST alone (Fig. 7B). The main structural motifs present in this section of NRAGE are the interspersed repeat domain, the MAGE homology domain and a putative coiled-coil domain. Screening the NRAGE deletion mutants revealed that the NEDD9 interaction relied on a region within amino acids 666–775 that is C-terminal to the MAGE homology domain. Deletion of the coiled-coil domain or the C-terminal 48 amino acids reduced, but did not completely abrogate, the NRAGE–NEDD9 interaction. Therefore, much of this 110-amino-acid fragment of NRAGE is required to sustain a robust interaction with NEDD9.

We then asked whether NEDD9 is required for p75NTR-induced spreading, using NEDD9-specific siRNA to reduce its levels in COS7 cells (Fig. 8A). Interestingly, NEDD9 knockdown had no effect on cells transfected with EGFP alone but strongly inhibited cell spreading mediated by overexpression of full-length p75NTR or by the p75NTR^{ICD} (Fig. 8B,C). We conclude that NEDD9 is a downstream effector of a p75NTR–NRAGE pathway that mediates the cell spreading phenotype.

Finally, to determine whether p75NTR activates Rac1 through an NRAGE- and NEDD9-dependent pathway, we assessed Rac1 activity in COS7 cells using the fluorescence resonance energy transfer (FRET) biosensor described by Hodgson and colleagues (2010). p75NTR overexpression produced a robust increase in Rac1 activity and, significantly, the p75NTR-dependent increase was blocked when NRAGE or NEDD9 were depleted using siRNA (Fig. 8D). Taken together, these findings demonstrate that NRAGE and NEDD9 function downstream of p75NTR to activate Rac1.

DISCUSSION
Here, we show that p75NTR induces cell spreading through activation of the small GTPase Rac1. We demonstrate that p75NTR must be cleaved in a proteolytic process involving ADAM17 and γ-secretase for this effect to be manifested, and show that the p75NTR^{ICD} is the relevant signaling moiety in this context. We demonstrate that NRAGE participates downstream of the p75NTR^{ICD} in producing the cell spreading phenotype and identify NEDD9 as a novel NRAGE-binding protein that participates in this cascade. Thus, our data suggest that p75NTR-dependent cell spreading is dependent on generation of the p75NTR^{ICD}, which in turn drives NRAGE- and NEDD9-dependent activation of Rac1. Although generation of the p75NTR^{ICD}, NRAGE signaling and Rac1 activation have all been proposed to play important roles downstream of p75NTR (Salehi et al., 2002, Harrington et al., 2002, Domeniconi et al., 2005, Kenchappa et al., 2006, Bertrand et al., 2008, Wang et al., 2008, Kenchappa et al., 2010, Ceni et al., 2010, Tep et al., 2012, Matusica et al., 2013), this is the first study that links these events in a single cascade.

RIP is a conserved and well-established mechanism that affects the function of diverse membrane-anchored proteins, such as APP, Notch and Delta. RIP of p75NTR is characterized by the dual cleavage of the receptor by ADAM17 and γ-secretase, which
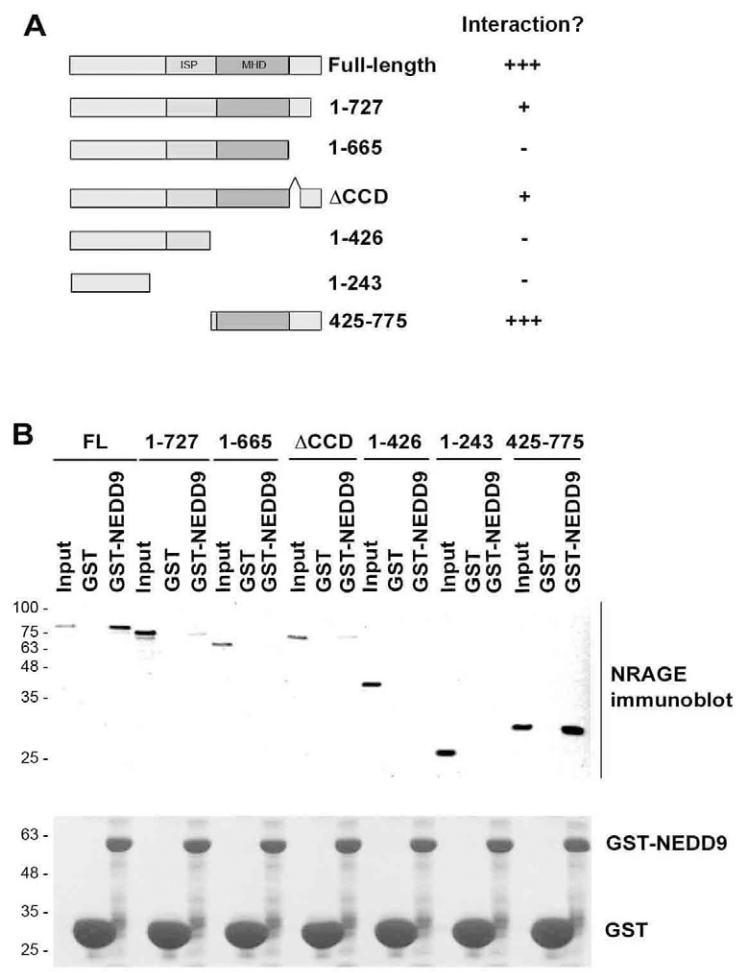
Fig. 7. NEDD9 interacts with the C-terminal domain of NRAGE.
(A) Schematic showing the different NRAGE deletion mutants that were generated. ISP, interspersed repeat domain; MHD, Mage homology domain; CCD, coiled-coil domain. − to +++ represent the interaction avidity from B. (B) The interaction of NRAGE fragments with GST–NEDD9 fusion proteins was detected using NRAGE immunoblotting. The lower panel shows a Coomassie-Blue-stained gel that confirms the expression of GST and GST–NEDD9. FL, full-length p75.

then releases the p75NTR^{ICD} into the cytosol (Kanning et al., 2003, Zampieri et al., 2005). Our data demonstrate that ADAM17 is responsible for the initial cleavage of p75NTR in COS7 cells and show that this is a prerequisite for subsequent γ-secretase-dependent proteolysis and release of the p75NTR^{ICD}. Because knockdown of ADAM17 inhibited production of the p75NTR^{ICD} and prevented cell spreading in cells transfected with full-length p75NTR, but did not block cell spreading of cells transfected with the p75NTR^{ICD}, we conclude that the p75NTR^{ICD} is the active signaling component required for Rac1 activation and cell spreading.

In previous work, we have shown that NGF-dependent TrkA (also known as NTRK1) activation activates an Erk-dependent signaling pathway to generate the p75NTR^{ICD}, which in turn facilitates NGF-dependent Akt signaling and cell survival (Kommaddi et al., 2011). Consistent with this, another recent study has shown that p75NTR cleavage facilitates Akt signaling and Erk signaling in sympathetic neurons (Matusica et al., 2013). Other works have shown that p75NTR cleavage is necessary for BDNF-induced sympathetic neuron death, for myelin-associated-glycoprotein-induced growth cone collapse (Kenchappa et al., 2006; Domeniconi et al., 2005) and for glioma migration (Wang et al., 2008). Here, we show that the addition of exogenous neurotrophin has no effect on p75NTR-induced cell spreading, suggesting that this effect might be ligand independent. In sympathetic neurons, expression of ADAM17 mRNA is induced

through a p75NTR-dependent signaling cascade but we found that, in COS7 cells, p75NTR overexpression does not alter ADAM17 mRNA levels. All four neurotrophins are expressed in COS7 cells, and it is conceivable that they bind to p75NTR and induce its cleavage in this setting. However, we found that incubation with REX, an antibody that blocks neurotrophin binding to p75NTR, had no effect on cell spreading. Therefore, consistent with our previous results (Ceni et al., 2010), we conclude that ligand binding to p75NTR is not required for the cleavage of the receptor. Determining the precise mechanisms that regulate p75NTR cleavage under physiological circumstances remains an interesting challenge.

NEDD9 (also called HEF1 or CasL) belongs to the Cas family of adaptor proteins that also includes p130Cas, Efs and HEPL (also known as BCAR1, Sin and CASS4, respectively). NEDD9 plays important roles in cell migration and cell adhesion (reviewed in Bouton et al., 2001, Guerrero et al., 2012), can stabilize focal adhesions and induce cell spreading (Bradshaw et al., 2011, Zhong et al., 2012, Baquiran et al., 2013), and has emerged as pro-metastasis factor in melanoma (Kim et al., 2006, Ahn et al., 2012), breast cancer (Izumchenko et al., 2009, Little et al., 2014), lung cancer (Miao et al., 2013, Kondo et al., 2012) and glioblastoma (Natarajan et al., 2006). This is the first study to establish a link between NRAGE and NEDD9 but it is interesting to note that other works have implicated NRAGE (Kumar et al., 2011) and NEDD9 (Kong et al., 2011, Ahn et al., 2012) in the
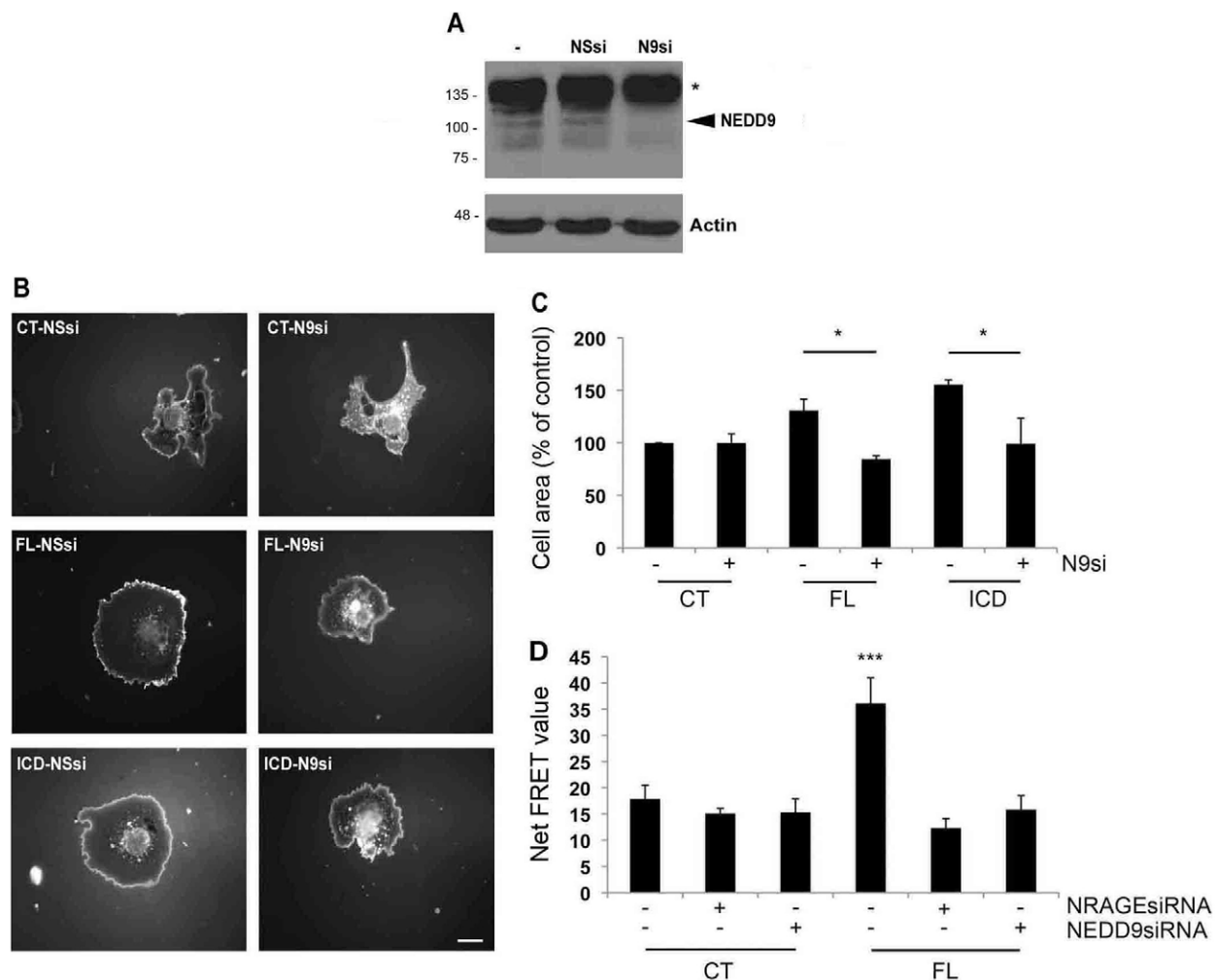
Fig. 8. NEDD9 acts downstream of p75NTR in cell spreading. (A) Western blot showing knockdown of NEDD9 in COS7 cells transfected with NEDD9 siRNA (N9si). The prominent band above NEDD9 that is marked with an asterisk represents a background band. (B) Representative spreading assay of COS7 cells overexpressing EGFP (CT), full-length p75 (FL) or p75ICD with or without NEDD9 siRNA. Nonspecific siRNA (NSsi) was used as control. Scale bar: 25 μm. (C) Quantification of the spreading assay in B showing a significant decrease in the cell area of p75FL and p75ICD cells after NEDD9 depletion but not in that of CT cells. Values are expressed as the percent change in cell area compared to that of controls and are shown as the mean±s.e.m. ($n=3$ independent experiments). (D) Rac1 activation assay performed by FRET analysis in COS7 cells overexpressing EGFP (CT) or p75FL with or without NRAGE or NEDD9 siRNA. Results show that NRAGE or NEDD9 depletion induced a significant decrease in Rac1 activity in cells overexpressing p75FL but not in CT cells. Values are represented as the normalized FRET signal and are shown as the mean±s.e.m. (three independent experiments); *$P<0.05$; ***$P<0.001$.

negative regulation of E-cadherin–β-catenin and in EMT. Given that other studies have demonstrated that Rac1 plays an essential regulatory role at the E-cadherin–β-catenin complex (Pujuguet et al., 2003, Wu et al., 2008, Frasa et al., 2010, Zhu et al., 2012), it will be important to determine whether an NRAGE–NEDD9–Rac1 cascade functions in EMT or similar contexts.

The precise mechanism by which the p75NTR[ICD]–NRAGE–NEDD9 cascade induces Rac1 activity is not known but activation of a Rac1 GEF seems likely to be important. Interestingly, a Rac1 GEF termed Trio has been reported to associate with an intact p75NTR–SorCS2 complex in hippocampal neurons and to facilitate Rac1 activity and neuronal growth. In this scenario, the association of proNGF with p75NTR results in Trio dissociation, which in turn results in Rac1 inactivation and hippocampal growth cone collapse (Deinhardt et al., 2011). Another Rac1 GEF, DOCK3, has been reported to directly bind to NEDD9 and activate Rac1-dependent melanoma migration (Sanz-Moreno et al., 2008).

In conclusion, we have used a novel cell-based assay to dissect the signaling events lying downstream of p75NTR. We show that generation of the p75NTR[ICD] plays a crucial role in the activation of Rac1, identify NEDD9 as a NRAGE-interacting protein and show that a p75NTR[ICD]–NRAGE –NEDD9 complex is required to effect morphological changes.

MATERIALS AND METHODS
Reagents and plasmids
Polyclonal antibodies directed against p75NTR and NRAGE were as described previously (Barker et al., 1994, Salehi et al., 2000), antibodies against ADAM17 were purchased from Cedarlane Laboratories Ltd (E8404, Burlington, ON, Canada), antibodies against NEDD9 were obtained from Thermo Fisher Scientific (MA1-5784, Ottawa, ON, Canada) and the M2 antibody (directed against the FLAG epitope) was obtained from Sigma (F3165, Oakville, ON, Canada). The monoclonal antibody against β-actin was purchased from MP Biomedicals (691001, Irvine, CA). The REX antibody was provided by Louis Reichardt (University of California at San Francisco, CA). NGF and BDNF were

obtained from Alomone laboratories (Jerusalem, Israel). Compound XXI, ilomastast (GM6001) and epoxomicin were from Calbiochem (San Diego, CA); batimastat (BB94) and NSC 23766 were from Tocris Bioscience (Ellisville, MO). Laminin was purchased from BD Biosciences (Mississauga, ON, Canada). Poly-D-lysine (PDL) was obtained from Sigma (Oakville, ON, Canada). Rhodamine-conjugated wheat germ agglutinin (WGA) was purchased from Vector Laboratories (Burlington, ON, Canada) and horseradish-peroxidase-conjugated secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA). All cell culture reagents were from Wisent Bioproducts (Saint Bruno, QC, Canada). Dako anti-fading mounting medium was purchased from Cedarlane Laboratories Ltd (Burlington, ON, Canada). The Rac biosensor was obtained from Addgene (Cambridge, MA) and was as described previously (Hodgson et al., 2010). Glutathione plasmids encoding full-length p75NTR, cleavage-resistant p75NTR and the p75NTR^{ICD} were as described previously (Kommaddi et al., 2011). Plasmids encoding RacN17 and RhoAN19 were kindly provided by Dr Peter McPherson (McGill University, QC, Canada).

Cell culture and transfection

COS7 cells and HEK293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 100 mg/ml penicillin-streptomycin, under 5% CO_2 at 37°C. COS7 cells were co-transfected with EGFP cDNA (0.5 µg) in the absence or presence of plasmids encoding the various p75NTR isoforms (2 µg) using the calcium phosphate transfection method. For experiments involving dominant-negative Rac (RacN17) or RhoA (RhoAN19), COS7 cells were co-transfected with p75NTR plasmids in the absence or presence of plasmids encoding the dominant-negative GTPases, with EGFP (0.5 µg) co-transfected in all cases. Cells transfected with the different constructs were maintained for 48 hours at 37°C before either plating on coverslips for the spreading assay or lysing in Laemmli sample buffer for analysis by immunoblotting.

NRAGE, NEDD9 and ADAM17 siRNA sequences directed against the respective simian mRNAs were designed using the Invitrogen Stealth RNAi^{TM} siRNAs prediction algorithm (specific sequences available on request). For knockdown experiments, cells were transfected with p75NTR constructs using the calcium phosphate transfection and, 48 hours later, were transfected with the different siRNAs in antibiotic-free medium using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), as per the manufacturer's instructions. A non-specific siRNA was used for control knockdowns. Cells were maintained for 48 hours at 37°C then plated on coverslips for the spreading assay or lysed in Laemmli sample buffer for analysis by immunoblotting.

Cell spreading assay

Glass coverslips (12 mm, Fisherbrand, Fisher Scientific, Ottawa, ON, Canada) were coated for 30 minutes with poly-D-lysine (0.5 µg/ml), washed with sterile water, coated with laminin (0.5 µg/ml) for 2 hours at 37°C and again washed with sterile water. Transfected COS7 cells were plated on coverslips (4000 cells/slip) then incubated at 37°C under 5% CO_2 for 24 hours. Medium was then removed and replaced with 4% paraformaldehyde in PBS for 15 minutes at 37°C. After washing with PBS (three 5-minute washes), cells were incubated with Rhodamine-tagged WGA (5 µg/ml) in PBS for 10 minutes and then washed with PBS (two 5-minute washes) and with water (one 5-minute wash). Coverslips were mounted in anti-fading mounting media (Dako), and kept at 4°C until imaging was performed. Imaging was performed using a 40× objective (NA 1.4) on a Zeiss Axio observer fluorescent inverted microscope equipped with Xenon illumination, and images were captured using Zen software (Zeiss) with an AxioCam MRm Rev.3 camera. The cell surface area of GFP-expressing cells was quantified with the NIH ImageJ software. At least 100 cells were counted per condition in each experiment.

In the case of treatment with GM6001, BB94 and compound XXI, cells were treated with the different compounds for 1 hour prior to fixation and staining. For NGF or BDNF, cells were serum starved for 2 hours then treated with the different neurotrophins (25 ng/ml) for 1 hour or 24 hours prior to fixation and staining. In the case of the REX treatment, cells were treated with the REX antibody (1:100) for 24 hours prior to fixation and staining.

RNA extraction and RT-PCR

mRNA was extracted from COS7 cells using Qiagen RNeasy Mini Kit according to the manufacturer's instructions (Valencia, CA). cDNA was produced using the Ominscript RT kit (Qiagen) with random hexamers (GE Healthcare, Mississauga, ON, Canada) as primers. PCR was then performed using the GoTaq green master mix reagent (Fisher, Ottawa, ON, Canada) with primers targeting the simian neurotrophins NGF, BDNF, NT-3 and NT-4, as well as the neurotrophin receptors, p75NTR, TrkA, TrkB and TrkC. In the case of ADAM17 mRNA expression, COS7 cells were transfected with p75FL, CR or ICD, or left untransfected, and mRNA was extracted as described above. PCR was then performed using GoTaq green master mix reagent with primers against simian ADAM17. The primer sequences are available upon request. The PCR run was performed at an annealing temperature of 55°C for 35 cycles in the case of neurotrophins and neurotrophin receptors and 28 cycles for ADAM17. Actin was used as an internal control. PCR products were run on a 1.5% agarose gel and bands were visualized with ethidium bromide.

Yeast two-hybrid screening

NRAGE-interacting proteins were identified through a cytosolic yeast two-hybrid screen. For this, the NRAGE open reading frame was cloned into the pSOS vector to produce a NRAGE–SOS fusion that was then expressed in a cdc25H yeast strain. Screening was performed using a library of human fetal brain cDNAs cloned into the pMyr vector, which anchors fusion proteins to the yeast plasma membrane, as per the manufacturer's instructions (Stratagene/Agilent, CA). From 2.5×10^6 clones analyzed, 70 supported growth of the cdc25H yeast strain at 37°C and four of these contained distinct fragments of human NEDD9 cDNAs. The longest of these encoded amino acids 637–834, which represents the terminal 198 amino acids of NEDD9.

In vitro translation and pulldown experiments

The 198-amino-acid NEDD9 fragment obtained from the yeast two-hybrid screen was cloned into a pGEX4-1 to produce a GST fusion protein. This region of NEDD9 contained a putative helix-loop-helix, and deletion and site-directed mutants of this region were produced using PCR overlap. Full-length NRAGE was produced using an in vitro transcription-translation kit (Promega, WI) and pulldowns were performed using the GST–NEDD9 fragments using the methodology described previously (Salehi et al., 2000). Levels of NRAGE or its deletion fragments that associated with the GST–NEDD9 fragment were determined by immunoblotting with anti-NRAGE antisera.

To identify the region of NRAGE that bound to NEDD9, the GST fusion containing the 198-amino-acid NEDD9 fragment (amino acids 637–834) was transferred to a mammalian expression vector and co-expressed with FLAG-tagged NRAGE or FLAG-tagged NRAGE deletion mutants in HEK293T cells, using the calcium phosphate transfection. Cells were lysed in NP40 buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP40, 10% glycerol) after 24 hours and then incubated with 20 µl glutathione-conjugated beads (GE Healthcare, Baie d'Urfe, QC, Canada) for 1 hour at 4°C. Beads were washed three times in NP40 buffer, resuspended in Laemmli sample buffer, and incubated at 100°C for 5 minutes. Levels of NRAGE or its deletion fragments that associated with the GST–NEDD9 fragment were determined by immunoblotting with M2.

Western blot analysis and immunoblotting

Cells were harvested in 2× Laemmli sample buffer and boiled for 5 minutes prior to loading on SDS-PAGE gels. In the case of ADAM17 detection, BB94 (0.2 µM) was added to the 2× Laemmli sample buffer before lysing the cells. For p75NTR^{ICD} detection, cells were pre-treated with epoxomicin (1 µM) for 6 hours before lysis to block proteasomal degradation of the ICD, as described previously (Ceni et al., 2010). After SDS-PAGE and transfer to nitrocellulose, membranes were rinsed in PBS then blocked in

TBST (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 2% Tween 20) that was supplemented with 5% (w/v) dried skimmed-milk powder. Primary and secondary antibody incubation was performed in TBST containing 2.5% (w/v) dried skimmed-milk powder blocking solution, with primary incubations performed overnight at 4℃ and secondary incubations performed for 1 hour at room temperature. Membranes were extensively washed in TBST after each incubation. Immunoreactive bands were detected using enhanced chemiluminescence solution kit (Perkin-Elmer Life Sciences, Norwalk, CT), as per the manufacturer's instructions.

Rac activity using FRET

Rac activity was assessed using the Rac FRET biosensors described previously (Hodgson et al., 2010). Briefly, the Rac FLAIR biosensor consists of a dual chain in which the donor CFP and the acceptor YFP are on different chains. The ECFP is replaced by CyPet (CyPet–Rac1) and EYFP is replaced by pYPet–PBD (PAK binding domain). Once Rac1 is activated, PBD interacts with Rac1 and this interaction results in a FRET signal.

COS7 cells were transfected or not with p75FL, together with CyPet–Rac1 and pYPet–PBD (2 µg) using calcium phosphate transfection and were then incubated for 48 hours at 37℃ under 5% CO_2. In order to correct for donor and acceptor bleed through, cells were transfected with either of the biosensors (CyPet–Rac1 or pYPet–PBD). For NRAGE and NEDD9 siRNA transfection, cells were transfected or not with p75FL as well as the Rac biosensors and, 48 hours later, NRAGE and NEDD9 were knocked down with NRAGE and NEDD9 siRNA transfected into cells using Lipofectamine 2000 in antibiotic-free medium. Cells were kept for 48 hours at 37℃ under 5% CO_2, then plated on glass coverslips precoated with poly-D-lysine for 30 minutes and laminin for 2 hours at a density of 10,000 cells/ml. Cells were kept for 24 hours at 37℃ under 5% CO_2, then fixed with 4% paraformaldehyde for 15 minutes at 37℃. They were then washed with PBS (two 5-minute washes) and sterile water (one 5-minute wash) and mounted on slides. Slides were kept at 4℃ until imaging.

Imaging was performed using a 40× objective (NA 1.4) on a Zeiss Axio observer fluorescent inverted microscope equipped with Xenon illumination, and images were captured using Zen software (Zeiss) with an AxioCam MRm Rev.3 camera. Images were taken for the three channels YFP, CFP and FRET. CFP and FRET were imaged at the same exposure time. For emission ratio imaging, the following filter sets (Chroma Technology) were used: CFP, 430/24 (excitation) and 470/20 (emission); FRET, 430/24 (excitation) and 535/30 (emission); YFP, 500/20 (excitation) and 540/40 (emission). FRET analysis was done using PixFRET plugin in NIH ImageJ software. This program takes into consideration the bleed-through intensities from each chain i.e the donor (CyPet–Rac1) and the acceptor (YPet–PBD), subtracts them from the FRET intensity and calculates the normalized FRET (NFRET) value according to the following formula:

$$NFRET = \frac{IFRET - BTDonor \times IDonor - BTAcceptor \times IAcceptor}{\sqrt{IDonor \times IAcceptor}}$$

where I represents the intensity and BT represents the bleed through (Feige et al., 2005).

Statistical analysis

Statistical analyses were performed using one-way ANOVA and Tukey post-hoc analysis. All values are expressed as the mean±s.e.m.

Competing interests

The authors declare no competing or financial interests.

Author contributions

P.A.B. conceived of the project, and P.A.B., A.S. and M.Z. designed the overall experimental plan. M.Z. performed all of the experiments except for the yeast two-hybrid screen, NEDD9 and MAGE-D1 mutagenesis and related interaction assays, which were performed by A.S. V.R. collected, analyzed and quantified microscopy data. M.Z. and P.A.B. wrote the manuscript.

References

Ahn, J., Sanz-Moreno, V. and Marshall, C. J. (2012). The metastasis gene NEDD9 product acts through integrin β3 and Src to promote mesenchymal motility and inhibit amoeboid motility. *J. Cell Sci.* **125**, 1814-1826.

Baquiran, J. B., Bradbury, P. and O'Neill, G. M. (2013). Tyrosine Y189 in the substrate domain of the adhesion docking protein NEDD9 is conserved with p130Cas Y253 and regulates NEDD9-mediated migration and focal adhesion dynamics. *PLoS ONE* **8**, e69304.

Barker, P. A., Barbee, G., Misko, T. P. and Shooter, E. M. (1994). The low affinity neurotrophin receptor, p75LNTR, is palmitoylated by thioester formation through cysteine 279. *J. Biol. Chem.* **269**, 30645-30650.

Bertrand, M. J., Kenchappa, R. S., Andrieu, D., Leclercq-Smekens, M., Nguyen, H. N., Carter, B. D., Muscatelli, F., Barker, P. A. and De Backer, O. (2008). NRAGE, a p75NTR adaptor protein, is required for developmental apoptosis in vivo. *Cell Death Differ.* **15**, 1921-1929.

Bouton, A. H., Riggins, R. B. and Bruce-Staskal, P. J. (2001). Functions of the adapter protein Cas: signal convergence and the determination of cellular responses. *Oncogene* **20**, 6448-6458.

Bradshaw, L. N., Zhong, J., Bradbury, P., Mahmassani, M., Smith, J. L., Ammit, A. J. and O'Neill, G. M. (2011). Estradiol stabilizes the 105-kDa phospho-form of the adhesion docking protein NEDD9 and suppresses NEDD9-dependent cell spreading in breast cancer cells. *Biochim. Biophys. Acta* **1813**, 340-345.

Bronfman, F. C., Tcherpakov, M., Jovin, T. M. and Fainzilber, M. (2003). Ligand-induced internalization of the p75 neurotrophin receptor: a slow route to the signaling endosome. *J. Neurosci.* **23**, 3209-3220.

Ceni, C., Kommaddi, R. P., Thomas, R., Vereker, E., Liu, X., McPherson, P. S., Ritter, B. and Barker, P. A. (2010). The p75NTR intracellular domain generated by neurotrophin-induced receptor cleavage potentiates Trk signaling. *J. Cell Sci.* **123**, 2299-2307.

Clary, D. O., Weskamp, G., Austin, L. R. and Reichardt, L. F. (1994). TrkA cross-linking mimics neuronal responses to nerve growth factor. *Mol. Biol. Cell* **5**, 549-563.

Coulson, E. J., May, L. M., Osborne, S. L., Reid, K., Underwood, C. K., Meunier, F. A., Bartlett, P. F. and Sah, P. (2008). p75 neurotrophin receptor mediates neuronal cell death by activating GIRK channels through phosphatidylinositol 4,5-bisphosphate. *J. Neurosci.* **28**, 315-324.

Deinhardt, K., Kim, T., Spellman, D. S., Mains, R. E., Eipper, B. A., Neubert, T. A., Chao, M. V. and Hempstead, B. L. (2011). Neuronal growth cone retraction relies on proneurotrophin receptor signaling through Rac. *Sci. Signal.* **4**, ra82.

Domeniconi, M., Zampieri, N., Spencer, T., Hilaire, M., Mellado, W., Chao, M. V. and Filbin, M. T. (2005). MAG induces regulated intramembrane proteolysis of the p75 neurotrophin receptor to inhibit neurite outgrowth. *Neuron* **46**, 849-855.

Feige, J. N., Sage, D., Wahli, W., Desvergne, B. and Gelman, L. (2005). PixFRET, an ImageJ plug-in for FRET calculation that can accommodate variations in spectral bleed-throughs. *Microsc. Res. Tech.* **68**, 51-58.

Frasa, M. A., Maximiano, F. C., Smolarczyk, K., Francis, R. E., Betson, M. E., Lozano, E., Goldenring, J., Seabra, M. C., Rak, A., Ahmadian, M. R. et al. (2010). Armus is a Rac1 effector that inactivates Rab7 and regulates E-cadherin degradation. *Curr. Biol.* **20**, 198-208.

Guerrero, M. S., Parsons, J. T. and Bouton, A. H. (2012). Cas and NEDD9 Contribute to Tumor Progression through Dynamic Regulation of the Cytoskeleton. *Genes Cancer* **3**, 371-381.

Guo, F., Debidda, M., Yang, L., Williams, D. A. and Zheng, Y. (2006). Genetic deletion of Rac1 GTPase reveals its critical role in actin stress fiber formation and focal adhesion complex assembly. *J. Biol. Chem.* **281**, 18652-18659.

Harrington, A. W., Kim, J. Y. and Yoon, S. O. (2002). Activation of Rac GTPase by p75 is necessary for c-jun N-terminal kinase-mediated apoptosis. *J. Neurosci.* **22**, 156-166.

Harrington, A. W., Li, Q. M., Tep, C., Park, J. B., He, Z. and Yoon, S. O. (2008). The role of Kalirin9 in p75/nogo receptor-mediated RhoA activation in cerebellar granule neurons. *J. Biol. Chem.* **283**, 24690-24697.

Hodgson, L., Shen, F. and Hahn, K. (2010). Biosensors for characterizing the dynamics of rho family GTPases in living cells. *Curr. Protoc. Cell Biol.* **Chapter 14**, Unit 14 11 1-26.

Izumchenko, E., Singh, M. K., Plotnikova, O. V., Tikhmyanova, N., Little, J. L., Serebriiskii, I. G., Seo, S., Kurokawa, M., Egleston, B. L., Klein-Szanto, A. et al. (2009). NEDD9 promotes oncogenic signaling in mammary tumor development. *Cancer Res.* **69**, 7198-7206.

Kanning, K. C., Hudson, M., Amieux, P. S., Wiley, J. C., Bothwell, M. and Schecterson, L. C. (2003). Proteolytic processing of the p75 neurotrophin receptor and two homologs generates C-terminal fragments with signaling capability. *J. Neurosci.* **23**, 5425-5436.

Kenchappa, R. S., Zampieri, N., Chao, M. V., Barker, P. A., Teng, H. K., Hempstead, B. L. and Carter, B. D. (2006). Ligand-dependent cleavage of the P75 neurotrophin receptor is necessary for NRIF nuclear translocation and apoptosis in sympathetic neurons. *Neuron* **50**, 219-232.

Kenchappa, R. S., Tep, C., Korade, Z., Urra, S., Bronfman, F. C., Yoon, S. O. and Carter, B. D. (2010). p75 neurotrophin receptor-mediated apoptosis in sympathetic neurons involves a biphasic activation of JNK and up-regulation of tumor necrosis factor-alpha-converting enzyme/ADAM17. *J. Biol. Chem.* **285**, 20358-20368.

Kim, M., Gans, J. D., Nogueira, C., Wang, A., Paik, J. H., Feng, B., Brennan, C., Hahn, W. C., Cordon-Cardo, C., Wagner, S. N. et al. (2006). Comparative oncogenomics identifies NEDD9 as a melanoma metastasis gene. *Cell* **125**, 1269-1281.

Kommaddi, R. P., Thomas, R., Ceni, C., Daigneault, K. and Barker, P. A. (2011). Trk-dependent ADAM17 activation facilitates neurotrophin survival signaling. *FASEB J.* **25**, 2061-2070.

Kondo, S., Iwata, S., Yamada, T., Inoue, Y., Ichihara, H., Kichikawa, Y., Katayose, T., Souta-Kuribara, A., Yamazaki, H., Hosono, O. et al. (2012). Impact of the integrin signaling adaptor protein NEDD9 on prognosis and metastatic behavior of human lung cancer. *Clin. Cancer Res.* **18**, 6326-6338.

Kong, C., Wang, C., Wang, L., Ma, M., Niu, C., Sun, X., Du, J., Dong, Z., Zhu, S., Lu, J. et al. (2011). NEDD9 is a positive regulator of epithelial-mesenchymal transition and promotes invasion in aggressive breast cancer. *PLoS ONE* **6**, e22666.

Kumar, S., Park, S. H., Cieply, B., Schupp, J., Killiam, E., Zhang, F., Rimm, D. L. and Frisch, S. M. (2011). A pathway for the control of anoikis sensitivity by E-cadherin and epithelial-to-mesenchymal transition. *Mol. Cell. Biol.* **31**, 4036-4051.

Little, J. L., Serzhanova, V., Izumchenko, E., Egleston, B. L., Parise, E., Klein-Szanto, A. J., Loudon, G., Shubina, M., Seo, S., Kurokawa, M. et al. (2014). A requirement for Nedd9 in luminal progenitor cells prior to mammary tumorigenesis in MMTV-HER2/ErbB2 mice. *Oncogene* **33**, 411-420.

Matusica, D., Skeldal, S., Sykes, A. M., Palstra, N., Sharma, A. and Coulson, E. J. (2013). An intracellular domain fragment of the p75 neurotrophin receptor (p75(NTR)) enhances tropomyosin receptor kinase A (TrkA) receptor function. *J. Biol. Chem.* **288**, 11144-11154.

Miao, Y., Li, A. L., Wang, L., Fan, C. F., Zhang, X. P., Xu, H. T., Yang, L. H., Liu, Y. and Wang, E. H. (2013). Overexpression of NEDD9 is associated with altered expression of E-Cadherin, β-Catenin and N-Cadherin and predictive of poor prognosis in non-small cell lung cancer. *Pathol. Oncol. Res.* **19**, 281-286.

Mitsui, N., Inatome, R., Takahashi, S., Goshima, Y., Yamamura, H. and Yanagi, S. (2002). Involvement of Fes/Fps tyrosine kinase in semaphorin3A signaling. *EMBO J.* **21**, 3274-3285.

Natarajan, M., Stewart, J. E., Golemis, E. A., Pugacheva, E. N., Alexandropoulos, K., Cox, B. D., Wang, W., Grammer, J. R. and Gladson, C. L. (2006). HEF1 is a necessary and specific downstream effector of FAK that promotes the migration of glioblastoma cells. *Oncogene* **25**, 1721-1732.

Park, K. J., Grosso, C. A., Aubert, I., Kaplan, D. R. and Miller, F. D. (2010). p75NTR-dependent, myelin-mediated axonal degeneration regulates neural connectivity in the adult brain. *Nat. Neurosci.* **13**, 559-566.

Pujuguet, P., Del Maestro, L., Gautreau, A., Louvard, D. and Arpin, M. (2003). Ezrin regulates E-cadherin-dependent adherens junction assembly through Rac1 activation. *Mol. Biol. Cell* **14**, 2181-2191.

Reichardt, L. F. (2006). Neurotrophin-regulated signalling pathways. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **361**, 1545-1564.

Salehi, A. H., Roux, P. P., Kubu, C. J., Zeindler, C., Bhakar, A., Tannis, L. L., Verdi, J. M. and Barker, P. A. (2000). NRAGE, a novel MAGE protein, interacts with the p75 neurotrophin receptor and facilitates nerve growth factor-dependent apoptosis. *Neuron* **27**, 279-288.

Salehi, A. H., Xanthoudakis, S. and Barker, P. A. (2002). NRAGE, a p75 neurotrophin receptor-interacting protein, induces caspase activation and cell

death through a JNK-dependent mitochondrial pathway. *J. Biol. Chem.* **277**, 48043-48050.

Sanz-Moreno, V., Gadea, G., Ahn, J., Paterson, H., Marra, P., Pinner, S., Sahai, E. and Marshall, C. J. (2008). Rac activation and inactivation control plasticity of tumor cell movement. *Cell* **135**, 510-523.

Schecterson, L. C. and Bothwell, M. (2010). Neurotrophin receptors: Old friends with new partners. *Dev. Neurobiol.* **70**, 332-338.

Sun, Y., Lim, Y., Li, F., Liu, S., Lu, J. J., Haberberger, R., Zhong, J. H. and Zhou, X. F. (2012). ProBDNF collapses neurite outgrowth of primary neurons by activating RhoA. *PLoS ONE* **7**, e35883.

Takahashi, T. and Strittmatter, S. M. (2001). Plexina1 autoinhibition by the plexin sema domain. *Neuron* **29**, 429-439.

Takahashi, T., Fournier, A., Nakamura, F., Wang, L. H., Murakami, Y., Kalb, R. G., Fujisawa, H. and Strittmatter, S. M. (1999). Plexin-neuropilin-1 complexes form functional semaphorin-3A receptors. *Cell* **99**, 59-69.

Tep, C., Kim, M. L., Opincariu, L. I., Limpert, A. S., Chan, J. R., Appel, B., Carter, B. D. and Yoon, S. O. (2012). Brain-derived neurotrophic factor (BDNF) induces polarized signaling of small GTPase (Rac1) protein at the onset of Schwann cell myelination through partitioning-defective 3 (Par3) protein. *J. Biol. Chem.* **287**, 1600-1608.

Underwood, C. K., Reid, K., May, L. M., Bartlett, P. F. and Coulson, E. J. (2008). Palmitoylation of the C-terminal fragment of p75(NTR) regulates death signaling and is required for subsequent cleavage by gamma-secretase. *Mol. Cell. Neurosci.* **37**, 346-358.

Wang, L., Rahn, J. J., Lun, X., Sun, B., Kelly, J. J., Weiss, S., Robbins, S. M., Forsyth, P. A. and Senger, D. L. (2008). Gamma-secretase represents a therapeutic target for the treatment of invasive glioma mediated by the p75 neurotrophin receptor. *PLoS Biol.* **6**, e289.

Wells, C. M., Walmsley, M., Ooi, S., Tybulewicz, V. and Ridley, A. J. (2004). Rac1-deficient macrophages exhibit defects in cell spreading and membrane ruffling but not migration. *J. Cell Sci.* **117**, 1259-1268.

Wu, X., Tu, X., Joeng, K. S., Hilton, M. J., Williams, D. A. and Long, F. (2008). Rac1 activation controls nuclear localization of beta-catenin during canonical Wnt signaling. *Cell* **133**, 340-353.

Xue, B., Wen, C., Shi, Y., Zhao, D. and Li, C. (2005). Human NRAGE disrupts E-cadherin/beta-catenin regulated homotypic cell-cell adhesion. *Biochem. Biophys. Res. Commun.* **336**, 247-251.

Yamashita, T. and Tohyama, M. (2003). The p75 receptor acts as a displacement factor that releases Rho from Rho-GDI. *Nat. Neurosci.* **6**, 461-467.

Yamashita, T., Tucker, K. L. and Barde, Y. A. (1999). Neurotrophin binding to the p75 receptor modulates Rho activity and axonal outgrowth. *Neuron* **24**, 585-593.

Yamashita, T., Higuchi, H. and Tohyama, M. (2002). The p75 receptor transduces the signal from myelin-associated glycoprotein to Rho. *J. Cell Biol.* **157**, 565-570.

Zampieri, N., Xu, C. F., Neubert, T. A. and Chao, M. V. (2005). Cleavage of p75 neurotrophin receptor by alpha-secretase and gamma-secretase requires specific receptor domains. *J. Biol. Chem.* **280**, 14563-14571.

Zanata, S. M., Hovatta, I., Rohm, B. and Püschel, A. W. (2002). Antagonistic effects of Rnd1 and RhoD GTPases regulate receptor activity in Semaphorin 3A-induced cytoskeletal collapse. *J. Neurosci.* **22**, 471-477.

Zhong, J., Baquiran, J. B., Bonakdar, N., Lees, J., Ching, Y. W., Pugacheva, E., Fabry, B. and O'Neill, G. M. (2012). NEDD9 stabilizes focal adhesions, increases binding to the extra-cellular matrix and differentially effects 2D versus 3D cell migration. *PLoS ONE* **7**, e35058.

Zhu, G., Wang, Y., Huang, B., Liang, J., Ding, Y., Xu, A. and Wu, W. (2012). A Rac1/PAK1 cascade controls β-catenin activation in colon cancer cells. *Oncogene* **31**, 1001-1012.