A p75NTR signaling cascade activates small GTPases to regulate cytoskeletal dynamics

Michele P. Zeinieh

Department of Neurology and Neurosurgery
Montreal Neurological Institute
McGill University
Montreal, Quebec, Canada

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ABSTRACT

The p75 neurotrophin receptor (p75NTR) is a multifunctional receptor that conveys several physiological functions such as cell death and survival, neurite outgrowth, differentiation, and cell migration, and it is also implicated in many pathological conditions. p75NTR belongs to the tumor necrosis factor superfamily (TNFRs) and as all members of this family, p75NTR lacks an intrinsic catalytic activity and the diverse signaling pathways it transduces are due to its interaction with different cytosolic adaptor proteins. Signaling of p75NTR through Rho GTPases has been described in many different processes such as death of oligodendrocytes, myelination, as well as inhibition of neurite outgrowth in response to myelin inhibitory proteins. However, the link between p75NTR and Rho GTPases activation is not well defined, mainly due to the lack of a robust assay that allows the study of p75NTR signaling. In this work we used the COS7 spreading assay as a heterologous system to study the regulation of Rho GTPases downstream of p75NTR. This assay is widely used as a model to study growth cone dynamics in response to semaphorin signaling. We find that p75NTR mediates COS7 cell spreading on laminin through activation of Rac1 and that cleavage of p75NTR by ADAM17 and γ-secretase is an essential step in this process. We have previously identified NRAGE, a MAGE family adaptor protein, as a p75NTR interactor through which p75NTR induces cell death. Here we show that NRAGE acts downstream of p75NTR to mediate Rac1 activation and cytoskeletal rearrangement. To gain more insight on how NRAGE mediates the spreading effects downstream of p75NTR we performed a yeast two-hybrid screen and identified NEDD9, a Cas family member as a potent NRAGE interactor. NEDD9 is widely studied as a prometastatic factor acting downstream of integrins and focal adhesion kinase. Here we show that NEDD9 acts downstream of p75NTR to mediate Rac1 activation and cell spreading.

Methods available for the measurement of Rho GTPases activity do not take into account subtle changes in Rho GTPases activation and underestimate the importance of the spatiotemporal changes in their activity. In order to study how p75NTR regulates the activation of Rho GTPases we took advantage of the Rho biosensors that allow the measurement of Rho GTPases activity through FRET. Using this method we find that p75NTR constitutively activates Rac1 but does not change RhoA activity although results show a trend towards a decrease in RhoA activation. Importantly, we show that this balance is altered when coreceptors such as NogoR1 and myelin inhibitory proteins such as Nogo66 are present.

p75NTR has been shown to play an important role in cell migration and cancer metastasis. In particular, p75NTR is a potent mediator of glioma invasion *in vitro* and *in vivo*. Here we show that p75NTR is overexpressed in high grade human glioma, and that p75NTR

increases migration of U87MG cells *in vitro* using the agarose drop assay which is a 2D migration assay on laminin, as well as by following the random motility of U87MG cells. Importantly, we show that cleavage of p75NTR is required for U87MG migration. However, NRAGE and NEDD9 act independently of p75NTR in this process, possibly through distinct pathways.

In conclusion, this work defines a new pathway downstream of p75NTR in the regulation of Rac1 activation involving NRAGE and NEDD9 and that the role of p75NTR in Rho GTPases activation highly depends on coreceptors. Importantly, we show that p75NTR cleavage and the release of the $p75^{ICD}$ plays a crucial role in Rho GTPases signaling as well as glioma migration. Using the FRET bioassay it will be possible now to study the signaling mechanism linking p75NTR to Rho GTPases especially the role of GEFs or GAPs. It will also be important to investigate the relevance of the p75NTR-NRAGE-NEDD9-Rac1 pathway in physiological conditions.

RÉSUMÉ

Le récepteur neurotrophique p75 (p75NTR) joue un rôle important dans plusieurs fonctions physiologiques telles que la mort, la survie, la différentiation, et la migration cellulaire, ainsi que la croissance des neurones. p75NTR contribue de même à différentes pathologies. Une des voies de transduction par laquelle p75NTR agit est par le biais des Rho GTPases. Ce phénomène est observé au cours de la mort des oligodendrocytes, la myélinisation, et la rétraction des neurones en réponse aux protéines inhibitrices de la myéline. Par contre, la régulation des Rho GTPases par p75NTR n'est pas complètement explorée, dûe en particulier à l'absence d'un modèle d'étude adéquat. Notre travail consiste en l'élaboration d'un système artificiel basé sur l'expansion des cellules COS7 sur laminine. Cette méthode est largement utilisée pour l'étude de la réponse du cône de croissance aux sémaphorines. Nous démontrons que p75NTR induit l'expansion des cellules COS7 via Rac1. Nous montrons aussi que p75NTR subit un double clivage par ADAM17 et γ-secretase résultant en la génération du domaine intracellulaire de la protéine ($p75^{ICD}$), une étape essentielle dans l'activation de Rac1. NRAGE, membre de la famille des protéines adaptatrices MAGE, intéragit avec p75NTR et permet l'expansion des cellules COS7 ainsi que l'activation de Rac1. Un criblage à double hybride des levures nous a permis d'identifier NEDD9, membre de la famille des proteines adaptatrices Cas, comme interacteur de NRAGE. NEDD9 est un facteur prométastase qui fait partie de la voie de signalisation médiée par les intégrines. Notre travail démontre que p75NTR agit par le biais de NEDD9 pour induire l'expansion des cellules ainsi que l'activation de Rac1.

Les méthodes disponibles pour la mesure de l'activité des Rho GTPases ne tiennent pas en compte les variations modestes de leur activation et sous-estiment l'importance de leur distribution celllaire. Nous avons utilisé les biocapteurs Rho GTPases qui permettent la mesure de leur activité par la méthode FRET. Par cette méthode nous montrons que p75NTR active Rac1 tandis que RhoA montre une faible tendance vers une diminution quoique non significative. Nous montrons aussi que l'activation des Rho GTPases par p75NTR est influencée par la présence de corécepteurs tels que NogoR1 et celle de protéines inhibitrices de la myéline.

Etant donné le rôle de p75NTR dans la migration des cellules cancéreuses et en particulier des gliomes, nous avons examiné les signaux de transduction impliqués dans ce processus. Le taux de p75NTR est élevé dans les échantillons humains de gliomes de haut grade. Utilisant un modèle de migration à deux dimensions *in vitro* nous démontrons que p75NTR facilite la migration des cellules U87MG et que le clivage protéolitique de la protéine est essentiel dans ce processus. De même p75NTR augmente le mouvement cinétique spontané des

cellules sur laminine. NRAGE et NEDD9 ne semblent pas directement impliqués dans la voie de signalisation de p75NTR dans ce processus.

En conclusion, ce travail nous permet d'établir une nouvelle voie de transduction impliquant p75NTR-NRAGE-NEDD9 dans l'activation de Rac1. La régulation des Rho GTPases par p75NTR dépend largement de la présence de corécepteurs. Nous définissons un rôle majeur pour $p75^{ICD}$ pour l'activation des Rho GTPases et la migration des gliomes. La méthode FRET nous permettra de mieux comprendre comment p75NTR contrôle l'activation des Rho GTPases. Il serait aussi intéressant d'étudier l'importance de la cascade p75NTR-NRAGE-NEDD9-Rac1 dans un contexte physiologique.

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LIST OF ABBREVIATIONS

AATF/Che-1	Apoptosis Antagonizing Transcription Factor
Abi2	Abl interactor 2
Abl	Abelson murine leukemia
ADAM	A disintegrin and metalloproteinase
ADF	Actin Depolymerizing Factor
Akt	Protein kinase B
AMIGO3	Adhesion molecule with immunoglobulin domain 3
APP	Amyloid precursor protein
ARMS	Age-related maculopathy susceptibility
Arp2/3	Actin related protein 2/3
BDNF	Brain derived neurotrophic factor
BMP	Bone morphogenic protein
BRCA2	Breast Cancer Gene 2
BTIC	Brain tumor initiating cells
C3G	Cyanidin-3-glucoside
CA1	Cornus Ammonis
Cas	Crk associated substrates
CASS4	Cas scaffolding protein family member 4
Cdc42	Cell division cycle 42
Cdk1	Cyclin dependent kinase 1
cDNA	Complementary DNA
CFP	Cyan fluorescent protein
Che-1	Abnormal chemotaxis protein
CGN	Cerebellar granule neurons
cIAP	Cellular inhibitor of apoptosis protein
CNS	Central nervous system
CRD	Cysteine rich domain
Crk	C10 regulator of kinase
DHR2	Dock180 Homology Region 2
Dim1p	18S rRNA dimethyltransferase
DOCK	Dedicator of cytokinesis
DRG	Dorsal root ganglia
ECM	Extracellular matrix
Efs/Sin	Embryonal Fyn-associated substrate
EGF	Epidermal growth factor
EGFP	Enhanced green fluorescent protein
EMT	Epithelial to mesenchymal transition
Erk	Extracellular regulated kinase
FAK	Focal adhesion kinase
FMNL3	Formin like protein 3
FRET	Fluorescence resonance energy transfer
GAP	GTPase activating enzyme
GBM	Glioblastoma multiforme
GDI	Guanine dissociation inhibitor
GDP	Guanosine diphosphate
GEF	Guanine exchange factor
GRB2	Growth factor receptor-bound protein 2
GTP	Guanosine-5'-triphosphate

GST	Glutathione-S-transferase
HEF1	Human enhancer of filamentation
HEK 293	Human embryonic kidney cells 293
HIF1α	Hypoxia inducible factor 1α
HSPC300	Heat shock protein C300
IRSp53	Insulin receptor substrate protein of 53 kDa
JNK	c-jun amino terminal kinase
LIM	LIM homeobox gene
LINGO1	Leucine rich repeat and immunoglobulin domain containing 1
LRR	Leucine rich repeat
LTP	Long term potentiation
MAG	Myelin associated glycoprotein
MAGE	Melanoma associated antigen
MAGI-1	Membrane associated guanylate kinase with inverted organization 1
MAGUK	Membrane associated guanylate kinase
MAPK	Mitogen-activated protein kinase
mDia	Mammalian diaphanous
MEF	Mouse embryonic fibroblasts
MEK	MAPK kinase
MHD	MAGE homology domain
MIB	Myelin inhibitory proteins
miRNA	Micro RNA
MLC	Myosin light chain
MLCK	Myosin light chain kinase
MMP	Matrix metalloproteinase
MMTV-PyVmT	Mouse mammary tumor virus-polyoma virus middle T antigen
mRNA	Messenger RNA
mTORC2	Mechanistic target of rapamycin 2
NADE	Neurotrophin receptor associated death effector
Nag-1	Nonsteroidal anti-inflammatory drug-activated gene-1
Nap125	Nck associated protein
NEDD9	Neural Precursor Cell Expressed Developmentally Downregulated 9
NFκB	Nuclear factor κ B
NGF	Nerve growth factor
NgR	Nogo receptor
NMR	Nuclear magnetic resonance
NRAGE	Neurotrophin receptor interacting MAGE homolog
NRH2	Neurotrophin receptor homolog 2
NRIF	Neurotrophin receptor interacting factor
NT3	Neurotrophic factor 3
NT4/5	Neurotrophic factor 4/5
OMgp	Oligodendrocyte myelin glycoprotein
P14ARF	p14 alternate reading frame
P21WAF1	p21 Wild-type p53-activated Fragment 1
p75CR	p75 cleavage resistant
p75CTF	p75 C terminal fragment
p75ECD	p75 extracellular domain
p75ICD	p75 intracellular domain
p75NTR	p75 neurotrophin receptor
PAK	P21 protein (cdc42/rac)-activated kinase 1

PBD	PAK binding domain
PBS	Phosphate buffered salien
PC12	Pheocromocytoma cells 12
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PDL	Poly-*D*-lysine
PEST	sequence rich in proline (P), glutamic acid (E), serine (S), and threonine (T)
PDZ	PSD95/Dlg/ZO1
PI (3,4,5) P3	Phosphatidylinositol 3, 4, 5-triphosphate
PI (4,5) P2	Phosphatidylinositol 4, 5-biphosphate
PI3K	Phosphatidylinositol 3 kinase
PIR121	p53-inducible mRNA
PKA	Protein kinase A
PMA	Phorbol-12 myristate 13-acetate
PTEN	Phosphatase and tensin homologue
PTP-PEST	Protein tyrosine phosphatase-PEST
PTP1B	Protein tyrosine phosphatase 1B
Pyk2	Protein tyrosine kinase 2
Ras	Rat sarcoma
Rb	Retinoblastoma protein
Rac	Ras-related C3 botulinum toxin substrate 1
RFP	Red fluorescent protein
RGC	Retinal ganglion cells
RhoA	Ras homologue A
RING	Really Interesting New Gene
RIP	Regulated intramembrane proteolysis
RIP2	Receptor interacting kinase 2
ROCK	Rho Kinase
ROR a	Retinoid-related orphan receptor a
Ror2	Receptor tyrosine kinase-like orphan receptor 2
Sall2	Sal-like protein 2
SC-1	Schwann cell factor 1
SCG	Superior cervical ganglia
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SERT	Serotonin transporter
SH2	Src homology domain 2
SH3	Src homology domain 3
Shc	Src homology domain containing protein
siRNA	Small interference RNA
Smurf2	Smad ubiquitination regulatory factor 2
SorCS2	Sortilin related Vps10p domain containing receptor 2
SOX2	Sex determining region Y-box 2
Src kinase	Sarcoma family kinase
TAB1	TAK-1-binding protein
TAK	Transforming growth factor-β-activated kinase 1
TBST	Tris buffered saline with Tween20
TBX2	T-box transcription factor 2
TGF-β	Transforming growth factor β
TNFR	Tumor necrosis factor receptor

TNFα	Tumor necrosis factor α
TRAF	TNFR-associated factor
TRAIL	TNF-related apoptosis inducing ligand
TRIP6	Thyroid hormone receptor interactor 6
Trk	Tropomyosin regulated kinase
TROY	TNFR superfamily 19 (TNFRSF19)
U87MG	U87 malignant glioma
UNC5	Uncoordinated family member 5
uPA	Urkinase plasminogen activator
VEGF	Vascular endothelial growth factor
WASP	Wiskott–Aldrich Syndrome protein
Vps10p	Vacuolar protein sortin 10 protein
WAVE	Wiskott-Aldrich syndrome protein family verprolin-homologous protein
XIAP	X linked inhibitor of apoptosis
YFP	Yellow fluorescent protein
ZU5	present in zonula occludens-1 (ZO-1) and unc5-like netrin receptors and ankyrin

CONTRIBUTION OF AUTHORS

Chapter 2

I did all the experiments presented in this chapter except the yeast two-hybrid experiment and the characterization of the NRAGE-NEDD9 interaction (figures 2.6 and 2.7) that were done by Amir Salehi. Vijidha Rajkumar helped in imaging and quantification of the cell spreading assay. Vincent Soubannier helped in designing the macro for quantification of the spreading assay and in developing the FRET assay. I wrote the first draft of the manuscipt and this was corrected and edited by Dr. Barker.

Contribution to figures:

Figures 2.1, 2.2, 2.3, 2.4, 2.5, 2.8: Michele Zeinieh

Figures 2.6 and 2.7: Amir Salehi

Chapter 3

I generated all the data presented in this chapter. Inna Ermeichouk helped in the quantification of FRET. Vincent Soubannier helped in setting up the FRET assay as well as quantification methods. The Rho biosensor plasmids were purified by Genevieve Dorval. I wrote the first draft of the chapter and this was corrected and edited by Dr. Barker.

Contribution to authors:

Figures 3.1, 3.2, and 3.3: Michele Zeinieh

Chapter 4

I did all the migration experiments presented in this chapter (figures 4.2, 4.3, and 4.4). The glioma biopsies were obtained from Dr. Kevin Petrecca at the Montreal Neurological Insitute. I produced the protein expression data and the immunostaining for p75NTR in tumor tissues were done by Dr. Charles Hao at the MNI. Quantification of migration assay was done by me and Ilke Geladi. The 'Ring' macro for quantification of the agarose drop assay was designed by Vincent Soubannier. Lentiviruses were geneerated by Kathleen Daigneault and Genevieve Dorval. I wrote the first draft of the chapter and this was corrected and edited by Dr. Barker.

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CHAPTER 1 - LITERATURE REVIEW

1. THE P75 NEUROTROPHIN RECEPTOR

1.1 Introduction

Development of the nervous system requires the fine tuning of neuronal connections and the correct targeting of axons to their respective tissues. Appropriate patterning is governed by neurotrophins secreted by the target tissue that act as trophic factors for the extending axons. The neurotrophin hypothesis proposes that the foremost axons that respond to these factors survive and are able to innervate the tissue whereas axons that fail to compete for the available neurotrophins retract and die. This ensures a well-regulated and organized construction of the nervous system that results in the appropriate innervation of target tissues. In addition, neurotrophins contribute to cell survival and proliferation, differentiation, apoptosis, and also participate in many pathological conditions. Nerve growth factor (NGF) is the most characterized member of the neurotrophin family, which also includes brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT3) and NT4/5. NGF binding assays were able to identify two types of receptors on the surface of neuronal cells and PC12 cells: the tropomyosin-related kinase (Trk) tyrosine kinase receptors that selectively bind the different neurotrophins with high affinity characterized by a dissociation constant (K_d) of ~10^{-11}M, and the p75 neurotrophin receptor (p75NTR) that binds all neurotrophins with equal low affinity (K_d~10^{-9}M) (Vale and Shooter, 1985, Roux and Barker, 2002, Schor, 2005).

Neurotrophins are produced as immature forms, the proneurotrophins, that are cleaved intracellularly by furin and proconvertases, and extracellularly by matrix metalloproteinases (MMPs), to form the mature forms (Lee et al, 2001, Mowla et al, 2001). Interestingly, p75NTR can also bind the proneurotrophins with high affinity. Thus, p75NTR can induce signaling downstream of pro and mature neurotrophins (Lee et al, 2001).

p75NTR is widely expressed in the central and peripheral nervous system during development, but its expression becomes more restricted in the adult, with the exception of an induced expression upon injury. p75NTR is also expressed in several types of non neuronal tissues (Roux and Barker, 2002, Schor, 2005, Reichardt, 2006).

Signaling downstream of p75NTR mediates a myriad of functions, ranging from cell death to cell survival, cell differentiation, axonal growth, and cell migration. The role of p75NTR in cell death and survival mechanisms is well established although many details about its signaling are still lacking. Little is known about how p75NTR mediates signals to the

cytoskeleton in functions such as neurite outgrowth and growth cone dynamics, as well as cell migration during development or in cancer progression.

In this section, we provide insights on the role of p75NTR in different signaling outcomes with a particular focus on its role in cytoskeletal remodeling and cancer migration. Finally, we will place a special emphasis on the interplay between p75NTR and Rho GTPases.

1.2 Structure of the p75NTR

p75NTR is the founding member of the tumor necrosis factor superfamily (TNFR) that now encodes more than 25 members. The *p75ntr* gene is composed of six exons spanning regions 17q12-17q22 of the human chromosome region (Huebner et al, 1986, Sehgal et al, 1988). The protein sequence of p75NTR is highly conserved between various species and it is composed of 399 amino acids, and represents a type I transmembrane protein. The extracellular domain (ECD) is *N*-glycosylated and contains four repeats of six cysteine rich domains (CRD1-CRD4) that constitute the putative binding sites for neurotrophins. These domains are also found in the other TNFRs (Yan and Chao, 1991, Baldwin et al, 1992). The juxtamembrane or "stalk" domain is *O*-glycosylated at several sites. The intracellular domain (ICD) is palmitoylated at cysteine 279, and is phosphorylated at multiple serine and threonine sites in the mature protein (Grob et al, 1985, Taniuchi et al, 1986). The ICD contains an 80 amino acid "death domain" module that constitutes a prominent feature of the TNFRs, first identified in proapoptotic receptors of this family but later shown to be implicated in multiple signaling events (Roux and Barker, 2002, Schor, 2005). The death domain of p75NTR presents many differences with other TNFRs, and its NMR structure revealed the presence of a globular death domain flanked by flexible regions that likely represent binding sites with downstream molecules (Liepinsh et al, 1997). All these posttranslational modifications are proposed to modulate protein-protein interaction as well as to allow proper folding and intracellular localization of the receptor. Unlike other TNFRs, p75NTR binds homodimeric NGF whereas TNFRs bind trimeric ligands (He and Garcia, 2004). (Figure 1.1)

Like other TNFRs, p75NTR lacks intrinsic catalytic activity, and the multitude of functions it fulfils arise from the fact that it binds to multiple adaptor proteins in the cell, thus activating several distinct signal transduction pathways that will be discussed later.

1.3 Functions of p75NTR

p75NTR was originally regarded as a facilitator for TrkA signaling, contributing in increasing the affinity of NGF to TrkA for cell survival (Makkerh et al, 2005, Ceni et al, 2010). However, p75NTR is more than a signaling facilitator. A growing body of evidence shows that p75NTR induces a plethora of diverse functions ranging from cell survival to cell death, differentiation, migration, and neurite outgrowth among others. Most importantly, p75NTR associates with different coreceptors on the cell surface or ultimately it can drive signaling without additional coreceptors (Yamashita et al, 2005, Reichardt, 2006). As mentioned earlier, p75NTR like other TNFRs binds cytosolic adaptor proteins necessary for its signaling. The association of p75NTR with different co-receptors as well as diverse signaling molecules engages this protein in a multitude of signaling cascades making from p75NTR a multifunctional protein.

One important aspect of p75NTR signaling is that it can be processed through a dual cleavage event known as regulated intramembrane proteolysis (RIP), a process shared by many receptors such as amyloid precursor protein (APP), Notch, Syndecan-3, ErbB4, and N-cadherin among others. Processing of these receptors through RIP is essential for their signaling. p75NTR proteolytic cleavage is characterized by a first cleavage step by the α-secretase tumor necrosis factor converting enzyme/a disintegrin and metalloproteinase enzyme 17 (TACE/ADAM17), that sheds the ectodomain leaving behind a membrane tethered C- terminal fragment (CTF). The CTF is subsequently cleaved by presenilin-dependent γ-secretase that releases the ICD (Kanning et al, 2003, Weskamp et al, 2004, Zampieri et al, 2005). p75NTR processing occurs in response to both neurotrophins and proneurotrophins (Urra et al, 2007, Kenchappa et al, 2010, Ceni et al, 2010, Kommaddi et al, 2011). The importance of cleavage in p75NTR signaling will be discussed in detail later.

1.3.1 p75NTR and regulation of cell surface receptors

p75ntr -/- mice have proven to be a useful tool to decipher the function of p75NTR *in vivo.* These studies, coupled with *in vitro* work, showed that p75NTR is a potent mediator of cell death in sensory and sympathetic neurons (Yeo et al, 1997, Majdan et al, 1997, Bamji et al, 1998, Frade and Barde, 1999, Friedman, 2000, Majdan et al, 2001, Troy et al, 2002), motoneurons (Wiese et al, 1999), as well as Schwann cells after axotomy (Syroid et al, 2000). In many situations and depending on the cell context, p75NTR antagonizes the survival signaling mediated by Trk receptors, switching the response to an apoptotic signal (Barrett and Bartlett, 1994, Majdan et al, 2001, Song et al, 2010). For example, neurotrophins or proneurotrophins

binding to p75NTR induces death of sympathetic neurons and hippocampal neurons. However, in *p75ntr-/-* neurons, neurotrophins interact with Trk receptors and induce survival (Bamji et al, 1998, Friedman, 2000, Majdan et al, 2001, Troy et al, 2002, Song et al, 2010). Similarly, during synapse formation in the superior cervical ganglia (SCG), NGF-TrkA allows the formation of postsynaptic densities and this pathway is restricted by p75NTR to balance synapse formation (Sharma et al, 2010).

However, it is difficult to reconcile the notion that p75NTR antagonizes Trk signaling with findings showing that p75NTR is a positive regulator of TrkA signaling. Indeed, NGF binding to p75NTR increases its affinity to TrkA leading to TrkA phosphorylation and activation as well as induction of downstream signaling in PC12 cells (Berg et al, 1991, Barker and Shooter, 1994, Ryden et al, 1997, Esposito et al, 2001, Ceni et al, 2010, Matusica et al, 2013). Treatment of PC12 cells with MC192, a monoclonal antibody that binds p75NTR and competes with NGF binding, results in a dramatic decrease in TrkA phosphorylation and a subsequent inhibition of downstream signaling (Barker and Shooter, 1994). The activation of the p75NTR-TrkA pathway is essential for cell survival (Ceni et al, 2010) as well as neurite outgrowth (Negrini et al, 2013). Further studies aiming at deciphering the mechanism through which p75NTR enhances Trk signaling, show that p75NTR inhibits the internalization and degradation of Trks upon neurotrophin binding, thus extending the cell-surface dependent signaling downstream of Trks (Makkerh et al, 2005). This mechanism may involve activation of mTORC2 and PI3K upon NGF treatment; PC12-27 cells, that lack p75NTR, show a defect in TrkA activation due to a decrease in mTORC2 activity (Negrini et al, 2013).

p75NTR-Trk signaling is likely bidirectional and cooperative. NGF binding to TrkA is essential for cleavage of p75NTR by α-secretase and the activation of downstream mechanisms (Urra et al, 2007, Ceni et al, 2010, Forsyth et al, 2014) and this is believed to be mediated by MEK (Ceni et al, 2010).

As mentioned above, p75NTR is also a receptor for neurotrophin precursors. Binding of proneurotrophins to p75NTR requires the formation of a receptor complex between p75NTR and sortilin, a Vps10p domain-containing transmembrane protein. ProBDNF and proNGF induce developmental cell death through this complex in SCG neurons, basal forebrain neurons and Schwann cells (Nykjaer et al, 2004, Teng et al, 2005, Volosin et al, 2006, Song et al, 2010). Proneurotrophins also induce cell death after injury and seizures (Beattie et al, 2002, Harrington et al, 2004, Volosin et al, 2006, Song et al, 2010), as well as in non-neuronal cells (Rogers et al, 2010). ProNGF binding to p75NTR-sortilin complex antagonizes the Trk survival signaling through activation of PTEN that dephosphorylates and inactivates Akt, thus inhibiting the

survival signaling promoted by Trk as seen in basal forebrain and hippocampal neurons (Song et al, 2010). ProNGF is also a mediator of cell cycle arrest through p75NTR and was shown to inhibit oligodendrogenesis through blocking neural stem cells proliferation (Guo et al, 2013). Interestingly, it was recently reported that proNGF mediates neurite outgrowth of SCG neurons that are dependent on NGF for their survival. This effect is mediated by p75NTR and appears to be selective for subpopulations of neurons that depend on NGF (Howard et al, 2013). Hence, the effect of proNGF could be influenced by p75NTR coreceptors as well as downstream signaling mechanisms.

In addition to its role in developmental apoptosis and survival, p75NTR is also a regulator of neurite outgrowth. p75NTR mediates the inhibitory effect of myelin inhibitory proteins (MIBs) on neurite outgrowth through association with the Nogo receptor (NgR) (Wang et al, 2002). Importantly, neurite retraction downstream of this complex involves activation of RhoA and can be reversed by neurotrophins (Yamashita et al, 1999, Yamashita et al, 2002, Yamashita and Tohyama, 2003, Park et al, 2010). However, p75NTR-mediated growth cone collapse and neurite retraction can also occur in the absence of MIBs. ProNGF binding to p75NTR/SorCS2 complex, a sortilin related protein, leads to neurite retraction through a mechanism involving inactivation of Rac1 and actin depolymerization (Deinhardt et al, 2011). Similarly, proBDNF binding to p75NTR/sortilin complex induces neurite collapse through a RhoA/Rho kinase (ROCK) signaling pathway in the absence of MIBs (Sun et al, 2012).

The studies mentioned above define an inhibitory role for p75NTR on neurite outgrowth. However, evidence shows that *p75ntr-/-* mice present a loss in sensory innervation correlated with heat insensitivity and development of ulcers at the distal extremities (Lee et al, 1992). These surprising results suggest that the role of p75NTR is context dependent and is highly influenced by the coreceptors it associates with. For instance, p75NTR interacts with the semaphorin 3A receptor and disrupts the formation of the neuropilin/plexin A4 receptor complex thus preventing the repellent activity of Sema3A and promoting outgrowth. In the absence of p75NTR there is a release of inhibition from this complex that leads to neurite retraction (Ben-Zvi et al, 2007).

Thus, all the studies mentioned above show the multifaceted functions of p75NTR that are influenced by the coreceptors it is associated with as well as the ligands it binds. The functions of p75NTR are further complicated by its multiple downstream effectors that mediate its diverse biological functions.

1.3.2 p75NTR and downstream signaling

As mentioned earlier, p75NTR lacks an intrinsic catalytic activity, thus requiring its interaction with multiple scaffolding and signaling molecules in the cytosol. A number of adaptor proteins have been identified as p75NTR binding partners that mediate the diverse p75NTR signaling pathways. Their association with the p75NTR intracellular domain, and notably its death domain or juxtamembrane domain, allows the triggering of multiple cascades. Although a structure-function map for p75NTR is still lacking, identification of different scaffolding proteins as well as their interaction domain with $p75^{ICD}$ gave us a broader understanding of p75NTR signaling in many biological events.

One of the most studied aspects of p75NTR signaling is its involvement in apoptosis. As mentioned earlier, p75NTR can trigger apoptosis in a ligand dependent or independent manner through its interaction with different coreceptors as well as downstream adaptor molecules. NRAGE (neurotrophin receptor interacting MAGE homolog), a MAGE family adaptor protein, was identified as a p75NTR binding partner that interacts with the juxtamembrane domain of the receptor. Interestingly, binding of NRAGE to p75NTR displaces TrkA from the p75NTR/TrkA complex and triggers apoptosis in PC12 cells and in sympathetic neurons through activation of JNK and caspase 3 (Salehi et al, 2000, Salehi et al, 2002, Bertrand et al, 2008). Interestingly, other members of the MAGE family also bind to p75NTR, and notably necdin, MAGE-H1, and MAGE-G1 that interact with p75NTR to regulate the cell cycle and cell differentiation (Tcherpakov et al, 2002, Kuwako et al, 2004). In addition to NRAGE, NADE (p75NTR associated death executioner) is another p75NTR binding protein that associates with the p75NTR death domain. NADE promotes apoptosis of oligodendrocytes as well as zinc-induced neuronal death downstream of p75NTR (Mukai et al, 2000, Park et al, 2000). Interestingly, p75NTR and NADE accumulate in the CA1 hippocampal region after kainate-induced seizures leading to death of these neurons (Yi et al, 2003).

Interestingly, p75NTR can act as a transcriptional modulator depending on the adaptor protein it binds to. NRIF (neurotrophin receptor interacting factor) binds to $p75^{ICD}$ and triggers apoptosis of sympathetic neurons and retinal ganglion cells through a JNK dependent mechanism (Casademunt et al, 1999, Linggi et al, 2005, Kenchappa et al, 2006). In addition, NRIF mediates death of hippocampal neurons after pilocarpine-induced seizures through association with p75NTR (Volosin et al, 2008). It is believed that this interaction is necessary for the NRIF nuclear translocation where it binds DNA and mediates apoptotic signaling (Kenchappa et al, 2006, Volosin et al, 2008, Parkhurst et al, 2010). A similar event is observed for p75NTR binding to Sall2, a transcription factor that acts as a tumor repressor by blocking

DNA synthesis. Sall2 binds the p75NTR death domain and translocates to the nucleus in response to NGF. This leads to inhibition of cell proliferation and induction of cell differentiation and neurite outgrowth in PC12 cells as well as primary hippocampal neurons (Pincheira et al, 2009).

The different signaling partners of p75NTR link the receptor to downstream effectors. For example, binding of p75NTR to the tumor necrosis factor receptor-associated factor (TRAF) proteins and receptor-interacting protein kinase 2 (RIP2) links p75NTR to the NFκB signaling pathway (Krajewska et al, 1998, Khursigara et al, 1999, Khursigara et al, 2001, Wooten et al, 2001, Charalampopoulos et al, 2012). In addition to these factors p75NTR also associates with other partners such as ARMS, SC-1, RhoA, ceramide, caveolin, and ranBPM (Roux and barker, 2002, Bai et al, 2003).

Identification of p75NTR binding partners and the study of *p75ntr-/-* mice show that p75NTR is multifunctional and promotes context-dependent apoptosis, cell survival, neurite outgrowth or myelination, as well as cell migration and cancer progression.

1.4 Importance of the $p75^{ICD}$ in p75NTR signaling

Cleavage of p75NTR has been studied in many different events and a growing body of evidence attributes an essential role for $p75^{ICD}$ in different biological outcomes ranging from cell death, to cell survival, differentiation, as well as cancer progression (Urra et al, 2007, Kenchappa et al, 2006, Wang et al, 2008, Parkhurst et al, 2010, Ceni et al, 2010, Kenchappa et al, 2010, Provenzano et al, 2011, Forsyth et al, 2014). p75NTR-mediated apoptosis requires p75NTR cleavage and ICD release. SCG sympathetic neurons die in response to BDNF in a process involving the $p75^{ICD}$ (Kenchappa et al, 2006, Kenchappa et al, 2010), and it is believed that $p75^{ICD}$ binds NRIF and leads to its translocation to the nucleus where it activates apoptotic signaling (Kenchappa et al, 2006). Cleavage of p75NTR appears to be essential for binding with its downstream partners. Besides NRIF, $p75^{ICD}$ associates with RhoGDI in response to myelin inhibitory proteins. This is turn releases RhoA and activates it to promote neurite retraction (Domeniconi et al, 2005, Yamashita and Tohyama, 2003).

Cleavage of p75NTR is not only important for the receptor mediated cell death since p75NTR cleavage can also facilitate Trk survival signaling in PC12 cells, cortical neurons, and cerebellar granule neurons upon neurotrophin treatment (Urra et al, 2007, Ceni et al, 2010). Although cleavage of p75NTR is induced by, and important for, TrkA activation in PC12 cells (Urra et al, 2007, Ceni et al, 2010), it concomitantly decreases the ability of p75NTR to associate

with TrkA, suggesting that TrkA associated with the membrane tethered transmembrane receptor (Jung et al, 2003).

One interesting aspect of $p75^{ICD}$ in p75NTR functions is that it promotes the ability of p75NTR to act as a transcriptional modulator. Although, p75NTR is not a transcription factor, several studies have shown that the $p75^{ICD}$ is capable of translocating to the nucleus in response to neurotrophins where it modulates transcription of genes by binding gene promoters (Frade, 2005, Parkhurst et al, 2010, Provenzano et al, 2011). $p75^{ICD}$ can affect the cell cycle through either repressing the transcription of cell cycle genes such as cyclin E1 in HeLa cells (Parkhurst et al, 2010), or it induces gene transcription and proliferation such as in spiral ganglion Schwann cells (Provenzano et al, 2011).

Cleavage of p75NTR also occurs under pathological conditions. It is observed that p75NTR is cleaved under hypoxic conditions *in vitro* and *in vivo*. Generation of $p75^{ICD}$ in this context leads to an increase in the hypoxia inducible factor (HIF1α) and promotes angiogenesis in the retina (Le Moan et al, 2011). $p75^{ICD}$ also mediates the tumorigenic effects of p75NTR. In fact, $p75^{ICD}$ is a key mediator of glioma migration and invasion *in vitro* and *in vivo*. $p75^{ICD}$ is detected in high grade gliomas (Johnston et al, 2007, Wang et al, 2008) and in brain tumor initiating cells (BTICs) (Forsyth et al, 2014) and is required for proliferation of BTICs (Forsyth et al, 2014).

Thus, taken together, all these findings attribute an important role for p75NTR cleavage and in particular $p75^{ICD}$ in signaling. It appears that the $p75^{ICD}$ constitutes the signaling component of p75NTR probably through its interaction with signaling proteins or its translocation to the nucleus where it modulates transcriptional events.

1.5 p75NTR in the regulation of the cytoskeleton

The implication of p75NTR in the regulation of the cytoskeleton and its association with cytoskeletal proteins is not well understood. Although some findings attribute an important role for p75NTR in the activation of Rho GTPases there is no clear evidence of how p75NTR conveys signals to the cytoskeleton. The role of p75NTR in neurite outgrowth in association with different axon guidance molecules, suggests that p75NTR acts to reassemble cytoskeletal proteins, actin and microtubules, to direct the extension of neurites or conversely it mediates the depolymerization of these proteins for neurite retraction (Walsh et al, 1999a and b, Bentley and Lee, 2000, Ben-Zvi et al, 2007, Lim et al, 2008). Studies show that NGF induces differentiation of PC12 cells by promoting a rapid distribution of actin at the tips of growing neurites through a Rac1 dependent mechanism (Yamaguchi et al, 2001, Yasui et al, 2001, Spillane et al, 2012). This

is achieved through the recruitment of the actin nucleating complex Arp2/3, WAVE1 and cortactin to filopodia tips in sensory neurons (Spillane et al, 2012). However, these studies do not show a direct effect of p75NTR in this process, and it is possible that NGF is acting through TrkA or p75NTR or both in PC12 cells. However, studies in chick retinal neurons show that BDNF induces growth cone extension by stimulating filopodial dynamics through p75NTR (Gehler et al, 2004a). This is achieved through dephosphorylation and stabilization of the actin severing protein complex ADF/cofilin that ensures actin turnover and the addition of new actin monomers (Gehler et al, 2004b). However, it is not known what activates ADF/cofilin downstream of BDNF-p75NTR although Rho/ROCK signaling was shown to be involved. One study has indicated that p75NTR can associate with the actin bundling protein fascin in melanoma cells in a ligand dependent manner. Neurotrophins dephosphorylate fascin and increase its binding to actin. Fascin associates with p75NTR in lamellipodia and filopodia, driving migration of melanoma cells (Shonukan et al, 2003). Conversely, in hippocampal neurons, proNGF phosphorylates and inactivates fascin through p75NTR/SorCS2 complex thus mediating actin disassembly and growth cone collapse (Deinhardt et al, 2011). It was recently found that p75NTR interacts with MAGI-1 (membrane-associated guanylate kinase with inverted organization-1) a member of the membrane-associated guanylate kinase proteins (MAGUK) in PC12 cells. p75NTR and MAGI-1 colocalize at the tips of PC12 neurites upon NGF treatment (Ito et al, 2013). Interestingly, MAGI-1 is localized at cell-cell junctions in epithelial cells and it associates with PTEN that stabilizes the E-cadherin/β-catenin complex thus increasing adhesion (Kotelevets et al, 2005). TRIP6 (thyroid receptor interacting protein 6) interacts with MAGI-1 and displaces PTEN leading to disruption of cell-cell junctions and of the actin cytoskeleton (Chastre et al, 2009). It is hence possible that p75NTR regulates the cytoskeleton through recruitment of MAGI-1-PTEN complex or alternatively MAGI-1-TRP6 complex. Hence, it appears that the role of p75NTR on the actin cytoskeleton depends on the cell context as well as on p75NTR binding partners that can convey distinct signaling mechanisms to the cytoskeleton downstream of this receptor.

Cell polarization is an important aspect known to require profound rearrangement of the cytoskeleton. Recent evidence show that p75NTR acts as a polarity protein in differentiating neurons (Zuccaro et al, 2014), as well as in Schwann cells where it localizes at the axon-glia interface (Chan et al, 2006, Tep et al, 2012). p75NTR polarization specifies axonal fate in differentiating hippocampal and cortical neurons (Zuccaro et al, 2014). The asymmetrical distribution of p75NTR is governed by its association with the polarity protein Par3 upon stimulation with BDNF in Schwann cells, a process that activates Rac1 at the axon-glia interface

and enhances myelination (Chan et al, 2006, Tep et al, 2012). Many of the aspects of p75NTR-mediated cytoskeleton changes depend on its interaction with Rho GTPases, particularly studied in the context of neurite outgrowth. The role of p75NTR in the regulation of Rho GTPases will be discussed in more detail later.

1.6 p75NTR and non-cancer cell migration

1.6.1 p75NTR and cell migration in the nervous system

During development of the nervous system, progenitor cells migrate from their site of origin to their final target by following a chemotactic gradient. Once they reach their final target, these progenitor cells differentiate yielding the different types of cells in the nervous system. Neurotrophins constitute an important source of chemotactic factors. They promote migration of the different cells in the nervous system during development as well as after injury and p75NTR has been shown to contribute to this migratory behavior. p75NTR contributes to the correct development of the olfactory system by governing the migration of neuronal precursor cells from the subventricular zone to the olfactory bulb along the rostral migratory stream (Snapyan et al, 2009). Similarly p75NTR governs the migration of GnRH neurons to the forebrain (Raucci et al, 2013). It also contributes to the migration of Schwann cells and the correct outgrowth of sensory neurons (Anton et al, 1994, Bentley and Lee, 2000). NGF binding to p75NTR drives the migration of oligodendroglial progenitor cells to the site of injury in a model of experimental autoimmune encephalomyelitis in mice (Oderfeld-Nowak et al, 2009). It appears that the migration of Schwann cells is tightly regulated by neurotrophin binding to p75NTR. Whereas NGF binding to p75NTR mediates Schwann cell migration, BDNF inhibits migration and promotes myelination (Yamauchi et al, 2004). So p75NTR acts as a balance in this process that controls migration and myelination in a well-organized manner depending on the neurotrophin available. In line with this, p75NTR blocks the migration of cerebellar granule cells by binding proBDNF (Xu et al, 2011).

Similarly, p75NTR is also involved in the migration of epidermal cells during wound healing and the levels of p75NTR are greatly decreased in the epidermal cells of patients with skin ulcer (Iwata et al, 2013). Thus, p75NTR appears to play an important regulatory role in cell migration during development and under pathological conditions, including cancer migration that will be discussed in details in the section below.

1.7 p75NTR in cancer

It has been well documented that p75NTR plays an important role in cancer, contributing to many aspects of cancer biology ranging from proliferation (Vanhecke et al, 2011, Forsyth et

al, 2014), migration (Johnston et al, 2007, Wang et al, 2008, Truzzi et al, 2008), resistance to apoptosis (Marchetti et al, 2004, Verbeke et al, 2010), and increased self renewal of cancer stem cells (Boiko et al, 2010, Morrison et al, 2013). This section covers the multiple functions of p75NTR in tumorigenesis as well as the downstream signaling mechanisms with a particular focus on cancer cell migration.

1.7.1 Role of p75NTR in tumorigenesis

Cancer cell migration is an inherent process of tumorigenesis that ensures dissemination of the tumor reminiscent with increased aggressivity. Cancer invasion and metastasis constitute a major obstacle for efficient therapy urging the search for new players in this process that could eventually be used as therapeutic targets. The role of p75NTR in cancer cell migration remains controversial. Although it is regarded as promigratory, an antimetastatic role has also been attributed to p75NTR. This is not completely surprising in view of the different interactors p75NTR associates with and the different signaling pathways it can elicit. Nevertheless, p75NTR is a proinvasive factor in many different types of cancer such as melanoma (Hermann et al, 1993, Truzzi et al, 2008), glioma (Johnston et al, 2007, Wang et al, 2008), medulloblastoma (Wang et al, 2010a) and pancreatic carcinoma (Zhu et al, 2002, Wang et al, 2009). Conversely, p75NTR is also shown to inhibit migration of gastric cancer cells (Jin et al, 2007a and b, Jin et al, 2010), and prostate cancer cells (Nalbandian and Djakiew, 2006, Wynne and Djakiew, 2010), suggesting that p75NTR could have opposite functions in tumor progression depending on the type of cancer.

Using an *in vivo* selection procedure, Johnston and colleagues selected for highly invasive glioma cells from the non-invasive U87 malignant glioma (U87MG) cell line implanted in immunocompromised mice brain. The invasive cells were reintroduced in the brain of the mice where they were able to form highly invasive tumors compared to the non-invasive well-defined tumors formed by the original non-invasive cells. Gene screening of the highly invasive cells demonstrate the upregulation of *p75ntr* gene among other genes (Johnston et al, 2007). Further, p75NTR is highly expressed in invasive glioma and its proteolytic cleavage through RIP is indispensable for glioma invasion *in vitro* and *in vivo* (Johnston et al, 2007, Wang et al, 2008). Interestingly, p75NTR is also upregulated in brain tumor initiating cells (BTICs) where it undergoes cleavage, and this is an essential step for BTICs migration and proliferation (Wang et al, 2008, Forsyth et al, 2014). Neurotrophins binding to p75NTR plays an important role in migration and proliferation of glioma. They are believed to act via an autocrine/paracrine loop (Weis et al, 2002, Johnston et al, 2007, Wang et al, 2008, Forsyth et al, 2014). In addition to its

role in gliomagenesis, p75NTR also contributes to the tumorigenic effect in other cancers. Indeed, frequent gain of function mutations of p75NTR are found in melanoma (Papandreou et al, 1996) and these mutations promote the survival and migration of melanoma cells (Shonukan et al, 2003, Marchetti et al, 2004, Truzzi et al, 2008), in particular melanoma known to metastasize to the brain (Marchetti et al, 2004). Importantly, high levels of p75NTR are found, particularly in spindled melanoma cells compared to epithelioid melanoma (Iwamoto et al, 1996, Iwamoto et al, 2001, Lazova et al, 2010, Sigal et al, 2012). Spindled melanoma cells are characterized by increased invasiveness in the perineurium and p75NTR was reported to play an important role in perineural invasion (Chan et al, 2010).

In contrast to its role as a prometastatic factor, p75NTR has been reported as an antimetastatic player in gastric and prostate cancer. Interestingly, p75NTR is downregulated in highly metastatic gastric cancer (Jin et al, 2007a and b) and prostate cancer (Nalbandian and Djakiew, 2006). It is proposed that p75NTR inhibits migration of these two cancer types through preventing the expression of matrix metalloproteinases such as uPA, MMP2, and MMP9 (Nalbandian and Djakiew, 2006, Jin et al, 2007a and b), as well as through activation of the TGF-β family member, Nag-1. Nag-1 activates the p38MAPK pathway and inhibits migration of prostate cancer cells in a p75NTR dependent manner (Wynne and Djakiew, 2010). Thus, the role of p75NTR in cancer cell migration is context and cell type dependent, and these studies further emphasize on the multifunctional role of p75NTR in cancer.

Besides its role in cancer migration, p75NTR also confers cancer cells with resistance to apoptosis. Proteomic analysis of MCF-7 breast cancer cells overexpressing p75NTR show an increase in cytokeratin -8, -18, and -19, proteins involved in resistance to apoptosis and cell survival (Wilmet et al, 2011). In line with this finding, MDA-MB-231 cancer cells overexpressing p75NTR are resistant to TRAIL-induced apoptosis with a concomitant increase in cIAP1 expression and $p21^{WAF1}$, a mediator of cell cycle arrest (Verbeke et al, 2010).

Cancer stem cells have been identified in many tumor types. These cells are capable of unlimited self renewal *in vivo* and can induce *de novo* tumor formation, and generate tumors that resemble the parental one (Clarke et al, 2006, Sakariassen et al, 2007, Ward and Dirks, 2007). The expression of stem cell markers correlates with enhanced self renewal, multilineage differentiation, and high tumorigenicity. Interestingly, one of the most-widely used markers is CD271, which is p75NTR itself. CD271 is one of several markers on self-renewing cells and the precise role of p75NTR in this process remains obscure. However, it is certain that melanoma stem cells taken from surgical patient samples and that express CD271/p75NTR have high self renewal capacity and are able to reproduce the parental tumor *in vivo* (Boiko et al, 2010, Civenni

et al, 2011). Interestingly p75NTR positive cells are found in melanoma stem cells that have high propagation capacity *in vivo*, and in cell lines derived from metastatic tumors but not from primary tumors (Civenni et al, 2011). p75NTR was also identified on the surface of medulloblastoma cells characterized by high self renewal (Morrison et al, 2013), as well as in brain tumor initiating cells where it is associated with increased proliferation (Wang et al, 2008, Forsyth et al, 2014).

1.7.2 p75NTR signaling and tumorigenesis

Although p75NTR is implicated in diverse aspects of tumorigenesis, it is not known how p75NTR regulates these different events. Evidence showing the association of p75NTR with the migration/adhesion machinery, including the integrins or the actin cytoskeleton, is lacking. Integrins are extracellular matrix (ECM) adhesion receptors that transduce bi-directional signals between the ECM and the cytoskeleton and are important for the regulation of functions such as cell morphology, migration, proliferation, differentiation, and survival (Nagano et al, 2012). Activation of integrins upon ECM ligand binding or through their interaction with cell surface receptors induces their clustering, leading to the formation of focal adhesions that recruit multiple cell adaptor as well as signaling proteins that convey signals to the cytoskeleton (Nagano et al, 2012, Gupta et al, 2013). Integrin $\alpha 9\beta 1$ is associated with increased invasion of SW480 colon carcinoma cells *in vitro* and *in vivo* (Gupta et al, 2009, Gupta et al, 2013), and melanoma cells (Lydolph et al, 2009), through induction of epithelial to mesenchymal transition (EMT) in a Src and Rac dependent pathway. Interestingly, studies show that NGF can bind integrin $\alpha 9\beta 1$, and NGF and integrin $\alpha 9\beta 1$ are both highly expressed in high grade glioma (Brown et al, 2008, Staniszewska et al, 2008). This interaction increases the proliferation and migration of glioma cells *in vitro* and *in vivo* through an Erk1/2/MAPK dependent pathway (Brown et al, 2008). NGF and integrin $\alpha 9\beta 1$ are also highly expressed in endothelial cells and drive angiogenesis by promoting migration and proliferation of endothelial cells (Walsh et al, 2012). The above mentioned studies do not directly implicate p75NTR in the NGF- integrin $\alpha 9\beta 1$ signaling cascade in glioma invasion although they do show that p75NTR is upregulated in high grade glioma (Brown et al, 2008, Staniszewska et al, 2008). Thus, it is possible that p75NTR is part of the integrin signaling pathway although this process is not fully elucidated. However, few studies have addressed this issue. In fact, p75NTR is shown to facilitate the trophic effect of NGF and integrin $\alpha 9\beta 1$ on muscle cells during development and after ischemia through the Erk1/2/MAPK pathway (Ettinger et al, 2012). Moreover, TNFR-1, another TNFR family member that harbors a death domain similar to p75NTR, is increased in endothelial cells

associated with glioblastoma multiforme (GBM). Interestingly, TNFR-1 inhibits the death of endothelial cells when plated on laminin via integrin α6β1, a laminin receptor (Huang et al, 2012). Interestingly, integrin α6β1 is increased in glioma stem cells that display enhanced self renewal (Lathia et al, 2010), a role also attributed to p75NTR (Wang et al, 2008, Civenni et al, 2011, Morrison et al, 2013).

As mentioned earlier, p75NTR associates with the scaffolding protein MAGI-1 (Ito et al, 2013). It is reported that MAGI-1 induces invasiveness in Madin-Darby canine kidney (MDCK) cells as well as prostate cancer cells through interaction with TRP6 which disrupts the cell-cell junctions (Kotelevets et al, 2005, Chastre et al, 2009). It is possible that p75NTR employs this pathway downstream of MAGI-1 to induce invasiveness, through phosphorylation of Shc and Erk as it was reported in neurite extension (Ito et al, 2013). On the other hand, TROY, a TNFR member that is highly homologous to p75NTR promotes glioma migration through association with the non-receptor protein tyrosine kinase Pyk2, and activation of Rac1 (Paulino et al, 2010). It is possible that p75NTR could be activating the same pathway in glioma invasion through Rho GTPases as reported in the nervous system during neurite outgrowth (Yamashita et al, 1999, Harrington et al, 2002, Deinhardt et al, 2011).

Polarization of cells during cell migration is a key process that allows directional migration of cells towards a source of attractive cue. In particular, directional migration of cancer cells drives their motility ensuring their correct targeting of specific sites where they form new tumors, or towards the blood stream for their dissemination. Polarization of cancer cells is ensured by many players that contribute to the extensive changes in cytoskeletal dynamics leading to front-rear asymmetry that is essential for detecting extracellular cues (Friedl and Gilmour, 2009). As mentioned previously, p75NTR associates with the polarity protein Par3 in Schwann cells to promote the migration and myelination process (Chan et al, 2006, Tep et al, 2012). Although a role for p75NTR in cancer cell polarity is not yet certain, it is possible that p75NTR associates with the same polarity proteins machinery to drive migration of cancer cells.

The diverse roles for p75NTR in tumorigenesis, demonstrate the multifaceted functions of p75NTR. The association of p75NTR with multiple cell coreceptors as well as downstream signaling partners confers this receptor with different functions in cancer although the complete understanding of the role of p75NTR in tumorigenesis is still lacking. Further investigation of p75NTR signaling in tumorigenesis coupled with genetic studies *in vitro* and *in vivo* is essential to decipher the role of p75NTR in this process.

1.8 THE RHO FAMILY OF SMALL GTPASES

The Ras superfamily of GTPases is a family of small monomeric GTPases that regulate diverse biological activities. They switch between two conformational states: an active "GTP" bound state that leads to their interaction with downstream signaling partners, and an inactive "GDP" state that terminates the signal. The Ras family of GTPases is composed of five major groups: Ras, Rho, Rab, Arf, and Ran (Etienne-Manneville and Hall, 2002, Burridge and Wennerberg, 2004, Hall, 2012). Among these families, the Rho family of GTPases is of great interest in our work and will be discussed in detail.

Twenty genes encoding proteins of the Rho GTPases are present in humans and members of this family are found in all eukaryotic species and are highly conserved. The Rho family of GTPases is divided in five groups: the Rho-like, Rac-like, Cdc42-like, Rnd, and RhoBTB. Three additional proteins were also described but they do not belong to any of these groups: RhoD, Rif, and RhoH/TTF. Two recent GTPases were also identified, MIRO-1 and MIRO-2, that bear little homology with the other Rho GTPases and lack the Rho-specific insert loop in their GTPase domains (Fransson et al, 2003).

Rho GTPases activation is regulated by guanine exchange factors (GEFs) that switch the GTPases to the "ON" state, and by GTPase activating proteins (GAPs) that induce the intrinsic GTPase activity of Rho GTPases leading to their inactivation. Rho GTPases are also regulated by guanine nucleotide dissociation inhibitors (GDIs). RhoGDIs modulate the cellular localization of Rho GTPases, an important factor for their activation (Burridge and Wennerberg, 2004).

Rho GTPases transduce signals from growth factors and their receptors at the cell surface as well as from adhesion structures to regulate cytoskeletal dynamics. They can also be activated by intracellular signals including cytosolic free calcium and lipid raft trafficking (Del Pozo and Schwartz, 2007, Grunicke, 2009). Hence, Rho GTPases play key roles in cell adhesion, migration, cell polarity, endocytosis, vesicle trafficking, cell cycle progression, differentiation, oncogenesis, and gene transcription (Ridley et al, 1992, Etienne-Manneville and Hall, 2002).

This section will discuss the upstream and downstream signaling mechanisms involved in Rho GTPases functions with a particular focus on their role in cell adhesion and migration. We will also describe what is known about the importance of p75NTR-Rho GTPases signaling. (Figure 1.2)

1.8.1 Role of Rho GTPases in cytoskeletal remodeling and adhesion

It is well established that Rho GTPases are master regulators of the actin cytoskeleton, integrating extracellular signals that converge into multiple biological functions such as adhesion, migration, endocytosis, regulation of the cell cycle and oncogenesis.

Rho GTPases are activated by extracellular signals as well as by adhesion of cells to ECM. Fibroblasts cultured in the absence of serum show a round morphology, however, serum induces cell adhesion and formation of protrusions (Ridley and Hall, 1992). Once activated, Rho GTPases induce actomyosin contractility characterized by the formation of two actin structures: stress fibers and membrane ruffles. The pioneer work by Ridley and colleagues showed that overexpression of a constitutively active form of RhoA is responsible for the formation of stress fibers, whereas Rac contributes to the formation of membrane ruffles and lamellipodia. In addition, Cdc42 mediates the formation of filopodia (Ridley and Hall, 1992, Ridley et al, 1992). The activation of Rho GTPases by adhesion of cells to ECM, results in the clustering of integrins at the cell surface. Rho activation leads to the formation of focal adhesions that result in an enhanced clustering of integrins. In addition, Rac and Cdc42 activation lead to the formation of focal complexes that are smaller than focal adhesions, and occur after the recruitment of integrins to regions where new protrusions are formed (Huveneers and Danen, 2009).

1 .8.2 Upstream regulators of Rho GTPases: roles of GEFs, GAPs, and GDI

Rho GTPases are modulated by three major classes of regulators: GEFs, GAPs, and GDIs. These regulators ensure the right control of Rho GTPases activation in a spatiotemporal manner. Rho GTPases are posttranscriptionally modified by the addition of a lipid moiety at the C-terminus. This lipid moiety interacts with RhoGDI through its insertion into the hydrophobic pocket formed by the immunoglobulin-like β-sandwich of GDI. This interaction brings the N-terminal regulatory portion of GDI against the effector-binding interface of Rho GTPases, preventing its interaction with downstream effectors. It is likely that the phosphorylation of RhoGDI and Rho GTPases modulates their relative affinities for each other (Spiering and Hodgson, 2011). Once RhoGDIs are bound to Rho GTPases, the latter are sequestered to the cytoplasm where they remain in an inactive state. Dissociation of RhoGDI from Rho proteins allows targeting of Rho GDP to the plasma membrane where it is activated by GEFs (Boulter et al, 2010). The presence of RhoGDI is essential for the stabilization of Rho GTPases, as depletion of RhoGDI leads to their proteosomal degradation while keeping the newly synthesized Rho GTPases in association with the endoplasmic reticulum (Boulter et al, 2010). Moreover, RhoGDIs ensure a correct balance between activated and inactivated pools of Rho GTPases. In fact, depletion of RhoGDI induces an upregulation of Rac1 and RhoA that impairs cell migration (Boulter et al, 2010). RhoGDIs can interact with many signaling molecules and cell surface receptors. In many situations interaction of RhoGDI with cell surface receptors displaces RhoGDI from Rho GTPases leading to their activation. In other contexts, this association is

essential for keeping a correct balance between the available pools of the different GTPases. For example, it has been reported that RhoGDI associates with integrin β8 in glioma cells and disruption of this complex increases the levels of Rac1, impairing the balance between the available pools of RhoA and Rac1 and leading to decreased cell invasiveness (Reyes et al, 2013). On the other hand, the RhoGDI association with p75NTR or TROY, displaces it from RhoA. The resulting RhoA activation ultimately leads to growth cone collapse of hippocampal neurons or cerebellar granule neurons (Yamashita and Tohyama, 2003, Lu et al, 2013). In addition, in glioma cells semaphorin 5A dependent-inhibition of migration requires the association of its receptor plexin B3 with RhoGDI. This association in turn recruits Rac1 to RhoGDI and subsequently inactivates Rac1 (Li and Lee, 2010).

Thus RhoGDI regulates Rho GTPases through different mechanisms involving: 1- stabilization of Rho GTPases and inhibition of their proteosomal degradation, 2- tight control of the balance between the different pools of activated Rho GTPases available and 3- regulation of Rho GTPases shuttling between the cytosol and the plasma membrane where they associate with downstream effectors.

Guanine nucleotide exchange factors (GEFs) activate Rho GTPases by mediating the switch of the GTPases from GDP to GTP bound state. All GEFs share a common tandem domain, composed of pleckstrin homology domain (PH) and catalytic Dbl homology domain (DH). Sixty members of this family have been identified to date. Another Rho-GEF family lacking the PH-DH tandem domain was later described, with DOCK180 being the founding member, and ten members of this family have been characterized to date (Burridge and Wennerberg, 2004, Hanna and Sibai, 2013). They mediate their GEF activity through a DHR2 domain. DOCK 180 was shown to associate with Rac1 and induce spreading of NIH-3T3 cells (Kiyokawa et al, 1998a, Kiyokawa et al, 1998b). There is no DOCK GEF described for Rho activation. A third subfamily consists of the zizimin family composed of zizimin1/DOCK9, zizimin2/DOCK11, and zizimin3/DOCK10. They are identified as GEFs for Cdc42 (Gadea et al, 2008). The PH-DH containing GEFs are associated with PI(4,5)P2 at the cell membrane in a closed conformation where the PH domain interacts with the DH domain and inactivates it. Upon PI3K activation, PI(4,5)P2 is phosphorylated producing PI(3,4,5)P3 that has a high affinity to the PH domain, which releases the inhibition of the DH domain and activates the GEF. Interestingly, several external stimuli activate Rho GTPases in a PI3K dependent manner. NGF dependent activation of Rac relies on PI3K and Akt activation (Yasui et al, 2001). E-cadherin mediated activation of Rac in MDCK cells and the gastrointestinal L cells depends on the activation of the Rac GEF Vav2. Activation of Vav2 is c-Src and PI3K dependent, since blockade of PI3K or c-

Src prevents Vav2 phosphorylation and activation thus inhibiting Rac activation (Fukuyama et al, 2006). PI3K is also activated downstream of Rac and Cdc42 enhancing activation of GEFs through a positive feedback loop, as was described in mammary tumors (Keely et al, 1997).

It is now well established that activation of Rho GTPases is not a global event but instead it is restricted spatially and temporally. Their selective activation is under the control of GEFs that recruit specific effectors to different domains of the cell membrane, restricting signaling to a particular cell region during events such as polarization and cell migration. During cell migration, the leading edge is polarized and directed towards the direction of the movement, whereas the trailing edge ensures actomyosin contractility. Polarization is ensured by the differential activation of GTPases at the leading edge versus the trailing edge. For example, a complex containing the Rac and Cdc42 GEF, βPix together with the effector PAK and polarity proteins is recruited to the leading edge of the cell during migration (Pertz, 2010). Similarly Syx, a RhoGEF, is also localized and activated at protruding edges of cancer cells. Syx is an important mediator of cell polarity that signals through mDia1 (mammalian homolog of diaphanous) downstream of RhoA. Activation of mDia1 induces microtubule bundling and establishment of cell polarity and migration of cancer cells (Dachsel et al, 2013). Thus, the fine tuning of Rho GTPases activation in a spatiotemporal manner is ensured by specific interactions with GEFs and effectors that permit a well defined biological process.

Another level of regulation of Rho GTPases is mediated through the GTPase activating proteins (GAPs) that inactivate the Rho GTPases by inducing their intrinsic GTPase activity. About eighty genes encoding a Rho GAP domain have been characterized to date and they present a wide variety of different domains, denoting their diverse functions. It is important to note that some GAPs do not use their GAP domains but rather act as Rho effectors. Interestingly, the p85 subunit of PI3K harbors a Rho GAP homology domain through which it binds the Cdc42 effector domain and regulates its activity (Zheng et al, 1994). Another effector, N-chimaerin also binds to Rac GTP and regulates its activity (Burridge and Wennerberg, 2004). Rho GAPs are also important for localized cycling of GTPases during cell movement as was described for GEFs. The Rho GAP, StarD13, mediates rapid cycling of RhoA at the leading and trailing edge of astrocytoma cells contributing to cell migration. Depletion of StarD13 stabilizes focal adhesions and inhibits cell migration (Khalil et al, 2014). Similarly to what is described for GEFs, GAPs are also activated spatiotemporally, allowing the recruitment of specific complexes to one cell compartment. The interplay between GEFs, GAPs, and effectors in time and space is essential for the cell response to a specific cue or external stimulus.

Another level of regulation of Rho GTPases is driven by their phosphorylation status at specific sites. The phosphorylation of RhoA by PKA on Ser188 has been observed in T lymphocytes. This phosphorylation did not affect the activation state of RhoA but increased its association with RhoGDI and its localization to the cytosol thus inhibiting its activity (Lang et al, 1996). Similarly, Cdc42 is phosphorylated at Ser64 by Src, which increases its association with RhoGDI (Forget et al, 2002). Rac1 is also the target of phosphorylation by Akt on Ser71, which is accompanied by a decrease in its activity (Kwon et al, 2000). Rac1 can also be phosphorylated by focal adhesion kinase (FAK) and Src at tyrosine 64, which decreases its activity and inhibits cell spreading. A phosphomutant form of Rac1 in which the tyrosine 64 is mutated to phenylalanine (Y64F) restores spreading of mouse embryonic fibroblasts (MEFs) by dissociating Rac from RhoGDI and increasing its association with GEFs (Chang et al, 2011).

The use of Rho GTPases FRET biosensors has increased our understanding on activation of Rho GTPases such as their activation should not be considered as global but rather very localized and spatiotemporally controlled. The diverse spatiotemporally restricted functions of the different GTPases are explained by the fact that GEFs and GAPs outnumber their GTPase targets, contributing to different outcomes (Pertz, 2010).

1.8.3 Downstream effectors and signaling pathways

Binding of cells to ECM substrates engages the activation of cell adhesion molecules such as integrins (Price et al, 1998, Ridley and Hall, 1992, Ridley et al, 1992), cadherins (Braga, 2002) and immunoglobulin superfamily members (Thompson et al, 2002) that induce activation of Rho GTPases mediating pronounced changes in the actin cytoskeleton. In particular, integrins play a prominent role in this process since cells plated on an uncoated surface are round and detached (Price et al, 1998).

Tension resulting from adhesion of the cells to the ECM activates Rho GTPases in time and space, leading to contractility. When RhoA is activated, stress fibers are formed. This is achieved through two distinct pathways: a myosin dependent pathway and an actin polymerization pathway. In fact, RhoA can induce these two pathways through distinct effectors. Activation of ROCK leads to the phosphorylation of myosin light chain (MLC) and inactivation of MLC phosphatase. This in turn enhances myosin activity resulting in myosin contractility. However, stress fibers can also result from actin polymerization. This is achieved through activation of another effector downstream of RhoA, mDia, a member of the formin family of proteins. mDia contributes to actin polymerization and formation of stress fibers. It is important to note that ROCK can also activate LIM kinase. LIM kinase phosphorylates and inactivates the

actin severing protein cofilin leading to stabilization of polymerized actin (Burridge and Wennerberg, 2004).

On the other hand, membrane ruffles and lamellipodia result from the activation of the actin polymerizing factor Arp2/3 (actin-related protein 2/3) downstream of Rac. This is achieved through Rac interaction with WAVE protein that belongs to the WASP family, that it turn activates Arp2/3. It was proposed that Rac activates WAVE through two distinct pathways. A first pathway involves the interaction of IRSp53 with both Rac and WAVE, linking these two proteins and leading to WAVE activation. Another pathway that has been described suggests that WAVE is present in an inactive complex with two Rac binding proteins, Nap125 and PIR121 as well as HSPC300 and Abi2. Rac activation disrupts this complex, freeing WAVE that can interact with and activate Arp2/3. Rac can also act through p21 activating kinase (PAK) that is a downstream target of Rac and Cdc42 and can bind these two GTPases through its Cdc42/Rac interactive binding domain (CRIB). PAK activates MLCK thus dephosphorylating and inactivating MLC that ultimately results in decreased contractility. Similar to ROCK, PAK can also activate LIM kinase and inactivate cofilin (Burridge and Wennerberg, 2004).

1.8.4 Rho GTPases: crosstalk and antagonism

Interestingly, it has been shown that the activation of Rho and Rac signaling are mutually exclusive and that the activation of one pathway antagonizes the activation of the other. In this section we discuss the different findings demonstrating antagonism of Rho GTPase signaling as well as crosstalk signaling that has been reported.

During cell adhesion, engagement of integrins inhibits RhoA and activates Rac1 and Cdc42 resulting in decreased contractility concomitant with an increase in the formation of protrusions. At later stages, a decrease in Rac1 and Cdc42 allows activation of RhoA that leads to maturation of focal adhesions (Huveneers and Danen, 2009). Interestingly, the differential regulation of cell adhesion by Rho GTPases affects many biological outcomes. For example, NGF-mediated PC12 differentiation depends on Rac1 activation and NGF leads to a rapid distribution of Rac1 to the membrane ruffles and its colocalization with F-actin where it induces the formation of protrusions (Yasui et al, 2001). This is blocked by RhoA activation in a Rho/ROCK dependent pathway (Yamaguchi et al, 2001). Interestingly, members of the same subfamily can also induce opposite biological outcomes. For example, Rac1 induces adhesion and differentiation of N1E-115 neuroblastoma cells whereas Rac3 inhibits this process (Hajdo-Milasinovic et al, 2007). Similarly, RhoA and RhoC are growth promoting whereas RhoB inhibits growth in many cases and is downregulated in cancer (Du et al, 1999, Chen et al, 2000).

Surprisingly, the activation state of Rho GTPases can also impact on cell fate. In fact, human mesenchymal stem cells, can differentiate into osteoblasts or adipocytes depending on RhoA activity that affects cell tension and shape. Activation of RhoA leads to cell spreading and differentiation to osteoblasts. However, inhibition of RhoA leads to cell rounding and commitment to the adipogenic fate (McBeath et al, 2004).

Despite the antagonistic functions that Rho GTPases exert, they can also all feed into the same pathway to facilitate certain biological functions. For example, Ras dependent formation of membrane ruffling depends on the activation of Rac, and this subsequently leads to stress fiber formation through activation of Rho (Ridley et al, 1992). It is also shown that anchorage dependent survival of fibroblasts is mediated by Ras dependent activation of Rac downstream of FAK (Almeida et al, 2000). In addition, Cdc42 is also reported to activate Rac (Kozma et al, 1995).

It is likely that antagonistic and collaborative signals between different GTPases permit fine tuning of spatiotemporal distribution and activation of these proteins. This tight regulation is likely necessary for establishing patterns of activation that impact on the biological functions.

The role of Rho GTPases in cell migration is very well documented where crosstalk and antagonism are very frequently observed. The role of Rho GTPases in cell migration will be discussed in details in the following section.

1.8.5 Role of Rho GTPases in cell migration

Rho GTPases contribute to different aspects of tumorigenesis and cancer progression ranging from migration and invasion, regulation of the cell cycle, survival, and apoptosis (Sahai and Marshall, 2002). Among these events, migration and invasion are prominent features of cancer aggressiveness and Rho GTPases are key regulators of the migratory events through the rearrangement of the cytoskeleton. In this section we will be focusing on the role of Rho GTPases in cell migration and in particular in cancer invasion.

Cell migration is a highly regulated process that controls many physiological events, such as embryogenesis, homeostasis, immune cell migration, and also during pathological conditions such as cancer. The mechanism of migration requires the rearrangement of the cytoskeleton that contributes to cell polarization in the direction of movement (Ridley et al, 2003). This is achieved through the formation of cell protrusions, lamellipodia and filopodia at the cell leading edge, and increased contractility at the cell rear. The lamellipodia direct the cells toward the direction of the movement and adhere to the substrate through the formation of focal contacts,

while filopodia act as sensors for exploring cues in the surrounding matrix. Protrusive forces at the leading edge are coupled by increased contractility at the cell rear that pushes the cell in the direction of the movement (Ridley et al, 2003, Yamazaki et al, 2005). Rho GTPases are key players in the migration event, and their polarized distribution during migration allows for the directionality of the movement (Georgiou and Baum, 2010). Rac and Cdc42 are localized at the lamellipodia and filopodia respectively inducing actin polymerization and integrin adhesion, whereas Rho is present at the trailing edge allowing for actomyosin contractility. On the other hand, it has been widely accepted that Rho and Rac signaling have opposite outcome on cell morphology and drive different types of migration: a mesenchymal elongated migration induced by Rac, and an amoeboid round migration induced by Rho, and that the inactivation of one pathway favors the other (Vial et al, 2003, Goldberg and Kloog, 2006, Sanz-Moreno et al, 2008, Yamazaki et al, 2009, Petrie et al, 2012). These two types of movement are interchangeable depending on the physical properties of the ECM (Yamazaki et al, 2009). The amoeboid movement constitutes a rapid form of migration that does not require proteolysis and in which the contractile force of the cells is the main driver of motility. On the other hand, the mesenchymal migration allows progression of cells on rigid ECM that requires matrix proteolysis (Wolf et al, 2003, Friedl, 2004). Mesenchymal movement is mediated through activation of the Rac-WAVE-Arp2/3 pathway, whereas the amoeboid movement requires activation of Rho/ROCK signaling (Sahai and Marshall, 2003, Sanz-Moreno et al, 2008).

Rac is known to promote migration through the promotion of membrane protrusions, whereas Rho inhibits migration through stabilization of focal adhesions (Vial et al, 2003, Goldberg and Kloog, 2006). Even GTPases of the same subfamily can drive different outcomes, depending on the cell context. For example, RhoA and RhoC differentially regulate migration in breast cancer as well as prostate cancer cell lines through acting via distinct effectors. RhoA/ROCK signaling inhibits migration by preventing Rac-dependent formation of protrusions, whereas RhoC, acting through the formin family member FMNL3, promotes migration through regulation of Rac activity at cell protrusions (Vega et al, 2011). Similarly in SNB19 glioma cells expressing both Rac1 and Rac3, only Rac1 is essential for lamellipodia formation and cell attachment, whereas Rac3 contributes to migration without affecting morphology (Chan et al, 2005), a differential role that was also observed in neuroblastoma cells differentiation (Hajdo-Milasinovic et al, 2007). It was recently reported that Rac1 is differentially expressed in brain tumors and in 3D spheroids showing high Rac1 expression in cells located at the tumor periphery, concomitant with their increased invasive properties, and low Rac1 expression at the center of the tumor mass (Hirata et al, 2012). This heterogeneity in

Rac1 levels is also accompanied with differential gene expression of these two cell populations in which high Rac expressing cells have increased transcription of genes involved in matrix proteolysis such as MMPs, whereas low Rac expressing cells express genes involved in cell cycle and mitosis (Yukinaga et al, 2014).

Although the mutually exclusive characteristic of Rho GTPases in cell movement is well described, crosstalk between the different types of migration exist and the role of specific GTPases on migration depends on the cell context. In fact, cooperative induction of Cdc42 and Rho signaling is essential for the maintenance of contractility in elongated cells (Wilkinson et al, 2005), and depletion of RhoA in round SW480 human colon adenocarcinoma cells, is not sufficient to switch the cells to an elongated morphology (Yamazaki et al, 2009), suggesting that RhoA and Rac/Cdc42 signaling play in concert to regulate the different modes of migration.

The development of FRET biosensors for Rho GTPases has helped us to follow in real time the activation of the different GTPases during cell migration and increased our understanding on their function. Interestingly, RhoA, Rac and Cdc42 were shown to act at the protruding edge of migrating MEFs, and RhoA is activated at the onset of the protrusion and is decreased as the protrusive edge retracts. The activation of Rac and Cdc42 follows that of RhoA and lasts longer even after retraction (Machacek et al, 2009). The cooperation of the different Rho GTPases is also observed during wound repair in *Drosophila*, where they differentially accumulate around the wound edge. In this situation Rho leads to actomyosin contractility important for wound closure whereas Rac and Cdc42 recruit actin to the wound site (Abreu-Blanco et al, 2014).

These findings demonstrate that Rho GTPase signaling play a key role in cell migration and cancer cell invasion. Even though the different GTPases could affect different types of migration by transducing different pathways, crosstalk exist, and are important for the correct patterning of Rho GTPase expression in order to regulate cell migration and inhibit the stochastic migration of the cells.

1.8.6 Rho GTPases and p75NTR

The link between Rho GTPases and p75NTR is not very well understood. Although many studies show the involvement of Rho GTPases downstream of p75NTR signaling, it is not very well established how these signal transduction mechanisms occur. In this section we will review available data in this field as well as regulatory events known to date.

1.8.7 Signaling of p75NTR to Rho GTPases

The relation between neurotrophin signaling and Rho GTPases has long been documented. Early studies in PC12 cells showed that NGF-mediated differentiation depends on activation and redistribution of Rac at the tips of neurites (Yamaguchi et al, 2001, Yasui et al, 2001, Kozma et al, 1997, Sebok et al, 1999). Interestingly, constitutive activation of RhoA, blocks NGF-mediated Rac activation, demonstrating an antagonizing mechanism between Rho and Rac that is activated by neurotrophin signaling (Yamaguchi et al, 2001). Although these studies implicated Rho GTPases in neurotrophin mediated differentiation, they do not show the involvement of neurotrophin receptors in this context. Further investigation of the mechanisms of neurite outgrowth gave more insight on neurotrophin regulation of Rho GTPases, and more specifically neurite growth inhibition by myelin inhibitory proteins.

Axonal extension and outgrowth inhibition during development or after injury mimics cytoskeletal events of actin polymerization and depolymerization observed in cell migration. Axonal outgrowth after injury in the CNS is blocked by the non-permissive environment of myelin inhibitory proteins. p75NTR plays a prominent role in this process through the formation of a complex involving the Nogo receptor (NgR) and LINGO-1 as coreceptors for myelin inhibitory proteins such as myelin associated glycoprotein (MAG), oligodendrocyte-derived myelin glycoprotein (OMgp), and Nogo66 (Wang et al, 2002, Domeniconi et al, 2005). Sensory and non-sensory neurons respond to myelin inhibitory proteins downstream of p75NTR/NgR/LINGO-1 receptor complex by activating RhoA. This process is seen in cerebellar granule neurons (CGNs), dorsal root ganglia neurons (DRGs), sympathetic neurons and septal cholinergic neurons upon exposure to myelin inhibitory proteins. Interestingly, activation of RhoA upon exposure to myelin inhibitory proteins was observed in neurons taken from normal mice but not from *p75ntr-/-* mice, denoting the importance of p75NTR in this signaling process (Yamashita et al, 2002, Domeniconi et al, 2005, Harrington et al, 2008, Park et al, 2010, Sun et al, 2012). A recent study also reported that a protein harboring a leucine rich repeat (LRR) motif, AMIGO-3, increases after injury in DRG neurons and retinal ganglion cells (RGCs). AMIGO-3 was shown to interact with NgR/p75NTR receptor complex and to induce neurite growth inhibition through activation of RhoA (Ahmed et al, 2013). Interestingly, this study shows that LINGO-1 levels are kept unchanged after injury and that AMIGO-3 is rather the mediator of axon growth inhibition.

The activation of RhoA downstream of p75NTR depends on ligand. Although Yamashita and colleagues show that MAG blocks neurite outgrowth of CGNs and DRG neurons through

p75NTR-RhoA signaling, they also note that this inhibition is reversed by NGF addition (Yamashita et al, 1999). Interestingly, tuning of the neurotrophin concentration highly impacts on Rho signaling. Sympathetic neurons plated on myelin undergo degeneration only when low NGF concentration was added (20 ng/ml) but not in the presence of high NGF levels (100 ng/ml). Low NGF-treated axons also have increased Rho activation and actin depolymerization (Park et al, 2010). Similarly, BDNF induces a p75NTR-dependent increase in filopodial length of growth cones of chick retinal neurons through inhibition of RhoA activation (Gehler et al, 2004a). Interestingly, recent findings indicate that neurotrophins and proneurotrophins induce opposite outcomes on neuronal outgrowth. In fact, proBDNF inhibits DRGs and neonatal cortical neurons axonal outgrowth, whereas BDNF promotes it (Sun et al, 2012). p75NTR-RhoA signaling is also involved in other models of axonal outgrowth. Isoflurane upregulates p75NTR in cortical and hippocampal neurons concomitant with an increase in RhoA activation leading to actin depolymerization and neurite retraction (Lemkuil et al, 2011). Similarly, ephrinB3-mediated neurite outgrowth inhibition of cortical neurons requires p75NTR-induced activation of RhoA, and *p75ntr-/-* neurons do not respond to ephrinB3 (Uesugi et al, 2013).

If the *in vitro* studies have agreed on the implication of p75NTR-RhoA signaling in neurite growth inhibition, the *in vivo* situation seems to be more complex, due to the lack of understanding of this pathway and the lack of information of the downstream players. Yamashita and colleagues show that *p75ntr-/-* mice embryos have delayed growth of sympathetic axons at E11 and E12, and they attributed this delay to the inability of neurotrophins to bind p75NTR, which leads to RhoA activation (Yamashita et al, 1999). However, work by Gehler and colleagues shows that *p75ntr-/-* DRG neurons of E14.5 mice have increased growth cone extension and filopodia formation, mimicking the wild type situation (Gehler et al, 2004a). They attribute this difference to the model used and age specificity of neurotrophins function. More studies are needed in order to understand the differential signaling of p75NTR to Rho GTPases.

Signaling of p75NTR through Rho GTPases has also been documented in other models, such as cell death during development or after injury although the mechanism is not well understood. In a mouse model of spinal cord injury, the levels of p75NTR and RhoA increase at the site of injury and RhoA activation leads to cell death at this site (Dubreuil et al, 2003). Similarly, proNGF-mediated cell death of RGCs occurs through RhoA activation (Al-Gayyar et al, 2013). On the other hand, oligodendrocytes plated on myelin binding proteins undergo apoptosis through a p75NTR-Rac dependent mechanism that is essential for activation of JNK (Harrington et al, 2002). It has also been reported that BDNF acting through p75NTR blocks Schwann cell migration and increase myelination through RhoA (Yamauchi et al, 2004).

p75NTR-Rho GTPases signaling does not only involve the nervous system. In fact, p75NTR-RhoA activation inhibits myoblasts differentiation and muscle regeneration after muscle dystrophy; interestingly, this process is reversed when p75NTR is bound to NGF (Deponti et al, 2009), further demonstrating the differential signaling of p75NTR that depends on ligand binding.

1.8.8 Mechanism of Rho GTPase regulation by p75NTR

Despite the diversity of the studies showing p75NTR signaling through Rho GTPases, little is known about the mechanisms and the downstream signaling through which p75NTR activates or regulates the activity of Rho proteins.

The preliminary work by Yamashita and colleagues first identified that p75NTR directly interacts with RhoA in a yeast two-hybrid screen and through coimmunoprecipitation in HEK 293 cells (Yamashita et al, 1999). The interaction between p75NTR and RhoA still did not explain how p75NTR activates RhoA, but nevertheless, it shed light on the potential existence of a complex containing Rho GTPase regulatory proteins together with p75NTR that could link p75NTR to Rho signaling. Indeed, in a later report, they identified RhoGDI as a p75NTR binding partner, rather than a direct interaction of p75NTR with RhoA (Yamashita and Tohyama, 2003).

In this work, Yamashita and colleagues suggest that p75NTR binding to RhoGDI serves as a displacement factor that separates RhoGDI from RhoA, which frees RhoA and activates it, thus leading to axonal growth inhibition in response to myelin inhibitors (Yamashita and Tohyama, 2003). Gehler and colleagues, suggest that in *p75ntr-/-* mice, RhoGDI is bound to RhoA and prevents its activation, which contributes to growth cone extension (Gehler et al, 2004a). In a recent report, residues K350/N353 and D410/E413 within the p75NTR death domain were identified as binding domains for RhoGDI. Surprisingly, residues D410/E413, also bind RIP2, suggesting a competitive interaction between RhoGDI and RIP2 for binding p75NTR. In RIP2 knockout MEFs overexpressing p75NTR, RhoGDI was readily immunoprecipitated with p75NTR and this interaction was lost in RIP2 wild type MEFs. Similarly, NGF treatment of wild type MEFs leads to displacement of RhoGDI from p75NTR, allowing RIP2 to bind the receptor. This suggests that RhoGDI-RhoA signaling could be interfering with NFκB signaling (Charalampopoulos et al, 2012) and that outgrowth observed after NGF treatment may involve NFκB signaling rather than inhibition of RhoA signaling by RhoGDI downstream of p75NTR.

Other TNFR members have also been shown to play a role in axon growth inhibition by myelin inhibitory proteins. TROY is an orphan member of the TNFR superfamily. It was identified as a potent binding partner for NgR and LINGO-1, and it can mediate axon growth inhibition through RhoA activation (Park et al, 2005). Interestingly, TROY not only mimics p75NTR in this context, but it also compensates for the absence of p75NTR in *p75ntr-/-* DRG neurons, where axon growth inhibition and activation of RhoA were still observed (Shao et al, 2005). Recently, it has been reported that TROY binds RhoGDI in CGNs independently of p75NTR, leading to RhoA activation and neurite growth inhibition by Nogo66, suggesting a possible redundant role for diverse TNFR members in Rho GTPases signaling (Lu et al, 2013). However, many questions remain to be answered. Is the displacement of RhoGDI from RhoA sufficient for RhoA activation? How does RhoGDI displacement translates into activation of RhoA by GEFs and how does p75NTR signaling convey into GEF activation?

Very little is known about regulation of GEFs and GAPs by p75NTR and only few studies identified an interaction between p75NTR and GEF signaling. Harrington and colleagues showed that p75NTR/NgR complex binds kalirin9, a dual Rho and Rac GEF. Kalirin9 competes with RhoGDI in binding p75NTR and subsequently leads to displacement of RhoGDI from p75NTR/NgR complex keeping RhoA in an inactive state. MAG binding to p75NTR/NgR leads to displacement of kalirin9 from this complex, which allows RhoGDI binding to p75NTR/NgR and releases kalirin9. Kalirin9 activates RhoA that subsequently drives axonal retraction (Harrington et al, 2008). This work is the first evidence linking p75NTR to RhoA activation through its association with a GEF. Subsequent studies aimed at finding other potential GEFs candidates interacting with p75NTR. It was later demonstrated that NGF-mediated differentiation of PC12 cells is effected through activation of Trio, a dual Rho and Rac GEF, that localizes at the tips of the neurites upon NGF stimulation (Estrach et al, 2002). Trio was later identified as a p75NTR interactor. Interestingly, Trio interacts with the p75NTR/SorCS2 complex and has been proposed to activate Rac1 at the tips of the growth cone leading to its extension. ProNGF binding to p75NTR/SorCS2 complex displaces Trio from this complex, leading to Rac inactivation and growth cone collapse through phosphorylation and inactivation of fascin, an actin bundling protein (Deinhardt et al, 2011). It appears that the effect of proNGF on actin polymerization depends on the cell context since other studies have shown that proNGF enhances the interaction of p75NTR with fascin in melanoma cells, leading to actin polymerization at the cell's leading edge and subsequent migration (Shonukan et al, 2003). This

study did not suggest any role for GEFs such as Trio in mediating the effect of p75NTR on fascin. It is possible that p75NTR regulates Rho GTPases through different GEFs to mediate outcomes that are context and model dependent.

Trio was first shown to bind TrkA upon NGF stimulation in PC12 cells and primary hippocampal neurons leading to neurite outgrowth through activation of Rac (Neubrand et al, 2010). Although the study by Neubrand and colleagues did not suggest a role for p75NTR in this context, the finding that Trio also interacts with p75NTR suggests a possible role for p75NTR in this process and it seems possible that a p75NTR/TrkA complex may function as a platform for Trio that facilitates Rac activation and neurite outgrowth.

The understanding of how p75NTR regulates the activity of Rho GTPases is increasing but there is still a long way before the mechanisms are fully deciphered. Many questions remain to be answered. How is the p75NTR-Rho GTPases signaling affected by ligand? How does the different p75NTR co-receptors affect this signaling? And importantly, how does p75NTR converge into GEF activation and RhoGDI?

1.9 THE MELANOMA ASSOCIATED ANTIGEN (MAGE) FAMILY OF ADAPTOR PROTEINS

Initial characterization of the MAGE family arose from a screening of melanoma cell markers in which they were identified as antigens bound to the major histocompatibility complex and later recognized on cytolytic T lymphocytes *in vitro* (van der Bruggen et al, 1991). Since then, all the members were identified and they are grouped in 2 major families.

Type I MAGE: this group comprises the MAGE-A, -B and -C family members that are encoded by a cluster of genes located on chromosome X. Their open reading frames are each encoded by single exons. The MAGE-A cluster is located on Xq28, MAGE-B on Xp21 and MAGE-C on Xp26-27. They are expressed mainly in germ cells and placenta and, not in adult tissues, but are overexpressed in a variety of tumor cells (Chomez et al, 2001, Barker and Salehi, 2002). Recent studies show that human melanomas and melanoma cell lines derived from patients harbor frequent *MAGE* mutations that are predominantly loss of function mutations (Caballero et al, 2010).

Type II MAGE: members of this family are encoded by genes located either on the X chromosome in regions outside of the type I gene cluster or on other chromosomes. Unlike the type I family, their open reading frames are encoded by multiple exons and some members also comprise alternative splice variants. Type II MAGE members are ubiquitously expressed in all normal tissues and some members of this family, including MAGE-D1-D4, MAGE-E1, and

MAGE-L2, constitute large proteins with extended N- or C-termini (Chomez et al, 2001, Barker and Salehi, 2002, Wang et al, 2011).

Sequence alignment of the different MAGE family genes shows little similarity between the type I and type II, suggesting that they are phylogenetically distinct branches of the MAGE family. However, all MAGE proteins contain a characteristic domain, known as the MAGE homology domain (MHD), conserved among many multicellular organisms such as mammals, *Drosophila, Aspergillus*, as well as in unicellular organisms such as yeast and protozoa (Chomez et al, 2001). In type I MAGE, the MHD is flanked by a small number of poorly conserved amino acids. The type II MAGE members also contain a second MHD of 200 amino acids, loosely conserved, termed MHD2. In MAGE-D1 the MHD1 and 2 are flanked by 25 tandem repeats of the WQxPxx hexapeptide that is conserved in mouse, rat, and human and constitutes a binding site for different proteins (Chomez et al, 2001).

1.9.1 The Neurotrophin Receptor interacting MAGE homolog (NRAGE)

NRAGE, also known as MAGE-D1 or Dlxin-1, belongs to the type II MAGE family proteins. As mentioned in the previous section, similarly to other MAGE members, NRAGE contains many different domains that enable this protein to fulfill many potential functions. In particular, NRAGE was identified in a yeast two-hybrid screen as a potent interactor of p75NTR, and this interaction defined the first characterized role for NRAGE (Salehi et al, 2000). Figure 1.3 represents a schematic of NRAGE protein structure with the putative binding partners.

1.9.2 NRAGE and p75NTR signaling

Screening of NRAGE expression during development shows that NRAGE mRNA expression is low throughout the rat embryo at E11.5 but enriched in the developing brain and the spinal cord. NRAGE protein expression increases in all tissues at E18 with a prominent expression in the cortex and cerebellum (Salehi et al, 2000). Examination of p75NTR and NRAGE expression in the nervous system in E14 rats shows that p75NTR and NRAGE colocalize in many regions such as the mantle zone of the medulla oblongata in which p75NTR is shown to mediate developmental apoptosis, in the trigeminal and dorsal root sensory ganglia as well as in facial motoneurons (Salehi et al, 2000). The pattern of expression changes in the adult nervous system in which p75NTR becomes more restricted whereas NRAGE is still widely expressed. However, all p75NTR positive neurons of the basal forebrain and hippocampus contain NRAGE, but NRAGE is also expressed in other regions devoid of p75NTR such as the

cerebral cortex, the striatum and motoneurons of the facial and trigeminal nuclei suggesting that NRAGE is involved in pathways distinct than those implicating p75NTR (Barrett et al, 2005).

NRAGE interacts with the juxtamembrane domain of p75NTR and this interaction transduces cell death in many different biological systems in the nervous system as well as in non nervous tissues (Salehi et al, 2002, Bertrand et al, 2008, Lebrun-Julien et al, 2010, Truzzi et al, 2011). In some cases, NRAGE antagonizes the interaction between p75NTR and TrkA, thus preventing the resulting survival signal and shifting the response of p75NTR to a death signal. NRAGE inhibits the interaction between p75NTR and TrkA in PC12 cells and in sympathetic neurons leading to death in response to NGF (Salehi et al, 2002, Bertrand et al, 2008). On the other hand, the death signal transduced by p75NTR in response to proneurotrophins also requires its association with NRAGE. Retinal ganglion cells (RGCs) exposed to proNGF undergo apoptosis through a p75NTR-sortilin pathway that signals via NRAGE to activate TNFα. Interestingly, in this case this pathway is activated in Müller glial cells and mediates killing of RGCs in a non cell autonomous mechanism, further showing that the function of NRAGE downstream of p75NTR is not only confined to nervous cells (Lebrun-Julien et al, 2009, Lebrun-Julien et al, 2010). It appears that NRAGE constitutes an important factor in p75NTR-mediated cell death since *NRAGE* knockout mice present a defect in developmental apoptosis of SCG sympathetic neurons (Bertrand et al, 2008). The apoptotic signal transduced by NRAGE involves the mitochondrial apoptotic machinery through the induction of JNK signaling and caspase activation (Salehi et al, 2002). The role of NRAGE in p75NTR-mediated killing is not only restricted to the nervous system. It has been shown that the stimulation of transit amplifying keratinocytes with β-amyloid increases the interaction between p75NTR and NRAGE and mediates their death downstream of p75NTR (Truzzi et al, 2011).

It is also established that p75NTR interacts with other members of the MAGE family such as necdin, MAGE-H1, and MAGE-G1. Necdin, MAGE-H1, and MAGE-G1 are type II MAGE family members that share extensive sequence homology with NRAGE (Tcherpakov et al, 2002, Kuwako et al, 2004). Necdin, and MAGE-H1 induce the differentiation of PC12 cells in response to NGF (Tcherpakov et al, 2002). Necdin and MAGE-G1 associate with the transcription factor E2F1 to suppress apoptosis. Overexpression of p75NTR or NGF stimulation promotes the translocation of necdin and MAGE-G1 to the cytoplasm where they interact with p75NTR and induce death of N1E-115 neuroblastoma cells. Thus, the interaction of p75NTR with necdin and MAGE-G1 potentiates the growth arrest signal of E2F1 by releasing the E2F1 inhibition by MAGE proteins (Kuwako et al, 2004). It is thus clear that NRAGE and other

MAGE family members can induce death downstream of p75NTR through multiple signaling pathways that rely on interaction with downstream signaling molecules and transcription factors depending on the cell type as well as on ligand stimulation.

1.9.3 NRAGE signaling mechanisms

The MHD domain of NRAGE constitutes a platform for interaction with different signaling molecules and cell surface receptors. The netrin-1 receptor, UNC5H1 has been described as a binding partner for NRAGE. UNC5H1 contains a ZU-5 domain as well as a PEST proapoptotic domain that is also present in p75NTR. NRAGE interacts with the juxtamembrane domain of UNC5H1, which is similar to p75NTR, and this interaction induces apoptosis in PC12 cells (Williams et al, 2003). The similarity of interaction between NRAGE and UNC5H1 and between NRAGE and p75NTR suggests that NRAGE could be signaling similar apoptotic pathways downstream of p75NTR and UNC5H1, but the precise mechanism of signaling downstream of NRAGE-UNC5H1 is still to be determined.

Cell death signals mediated by NRAGE cannot be solely explained by its interaction with death domain-containing receptors. Studies typically reported that NRAGE acts as an adaptor protein that does not support catalytic activitiy, but rather is required to bind signaling molecules that transduce signaling. Interestingly, NRAGE associates with the X linked inhibitor of apoptosis (XIAP) in 32D promyeloid leukemia cells and induces its degradation resulting in cell death in response to interleukin-3 (IL-3) withdrawal (Jordan et al, 2001). XIAP is part of the non-canonical bone morphogenic protein (BMP) pathway through which BMP mediates NFκB activation. NRAGE was identified as a member of this pathway that leads to NFκB nuclear translocation through TAK1-TAB1-XIAP and p38MAPK activation (Kendall et al, 2005, Matluk et al, 2010). This pathway is effective in mediating death of neural progenitor cells and P19 carcinoma cells (Kendall et al, 2005, Matluk et al, 2010), as well as the branching of the ureteric bud (Nikopoulos et al, 2009). It has recently been shown that NRAGE is ubiquitinated on lysine 63 by the F box protein, FBXO7 that enhances the interaction of NRAGE with TAK1 and TAB1 in response to BMP stimulation resulting in the activation of NFκB (Kang and Chung, 2014). Interestingly, p75NTR promotes apoptosis of bladder tumor cells through induction of the mitochondrial pathway and inhibition of cIAP1 (Tabassum et al, 2003). It is thus likely that p75NTR and NRAGE act through IAP proteins to induce cell death in tumor cells as well as in different systems.

NRAGE, similar to other type II MAGE family members, necdin and MAGE-G1, induces growth arrest by affecting the function of proteins implicated in cell cycle regulation (Wen et al, 2004, Di Certo et al, 2007, Reddy et al, 2010). In particular, NRAGE mediates p53-dependent growth arrest of HEK 293 cells by increasing phosphorylation of p53 and activating the $p21^{CIP/WAF}$ cell cycle inhibitor (Wen et al, 2004), a role also ascribed for necdin (Taniura et al, 1999). The regulation of $p21^{CIP/WAF}$ is also essential for NRAGE-mediated myoblast differentiation and muscle regeneration (Nguyen et al, 2010). Although, no direct interaction between NRAGE and p53 was described, it has been reported that NRAGE interacts with the apoptosis-antagonizing transcription factor AATF/Che-1 that plays an important role in cell cycle progression (Di Certo et al, 2007). Interestingly, a dual role for Che-1 has been attributed in cell proliferation as well as in growth arrest (Passananti et al, 2007, Bruno et al, 2008, Xu et al, 2013), and Che-1 affects the growth suppressing activity of retinoblastoma protein (Rb). It appears that NRAGE affects apoptosis and the regulation of the cell cycle through the modulation of Che-1 opposing functions. On one hand, NRAGE localizes with Che-1 in cortical neurons after traumatic brain injury, where Che-1 promotes the transcription of p53 after DNA damage and induces death of cortical neurons (Xu et al, 2013). On the other hand, Che-1 plays an antiapoptotic role through repressing Rb and interfering with the activation of E2F1 (Bruno et al, 2002). NRAGE sequesters Che-1 to the cytoplasm and promotes its ubiquitination and degradation thus releasing the inhibition of the cell cycle suppression machinery and inducing apoptosis of P19 mouse embryonal carcinoma cells (Di Certo et al, 2007). Che-1 also exerts its antiapoptotic effect through increasing XIAP levels in tumors (Bruno et al, 2008), a function that can also be counteracted by NRAGE interaction with Che-1.

In line with its role in regulating transcriptional events, NRAGE directly interacts with the homeodomain transcription factor family members, Dlx/Msx. NRAGE regulates Dlx5 transcriptional activity in response to BMP stimulation. Similarly, NRAGE interacts with Msx2 and both are found to be coexpressed in the interdigital soft tissue during embryogenesis where they regulate interdigital apoptosis (Sasaki et al, 2005a). The association of NRAGE with Msx2 is regulated by Ror2, a receptor tyrosine kinase, that binds Wnts. Ror2 interacts with NRAGE and inhibits nuclear localization of NRAGE and Msx2, thus regulating Msx2 dependent transcription (Sasaki et al, 2005a). In addition, NRAGE interacts with Ror2 in melanoma cells and inhibits Ror2-dependent migration of melanoma cells through inhibition of Src and FAK (Lai et al, 2012).

It has recently been shown that MAGE family members interact with the RING domain of specific E3 ubiquitin ligases, and act to increase ubiquitin ligase activity (Doyle et al, 2010).

Although, this has not been directly addressed, it is possible that NRAGE facilitates signaling pathways by regulating ubiquitination. In fact, NRAGE was found to colocalize with the ubiquitin ligase Praja1 and is itself ubiquitinated and degraded in a proteosomal dependent manner in PC12 cells leading to inhibition of neuronal differentiation (Teuber et al, 2013). It is also reported that NRAGE affects behavior through its ability to interact with the serotonin transporter (SERT). NRAGE targets SERT for ubiquitination and degradation resulting in increased expression of serotonin restoring a normal behavior in a model of depression in mice (Mouri et al, 2012).

NRAGE can also affect behavior and cognition through distinct mechanisms. Interestingly, NRAGE interacts with the nuclear receptors RORα and regulates the transcription of genes important for the circadian rhythm. NRAGE knockout mice exhibit impairment of the circadian rhythm (Wang et al, 2010b), as well as defects in social and sexual behavior (Dombret et al, 2012). Recently, it has been shown that NRAGE has an impact on learning and memory through maintenance of synapses and the induction of long term potentiation (LTP) in the hippocampus and increased BDNF release (Yang et al, 2014).

Besides the multiple roles of NRAGE in cell death, differentiation and cognition, NRAGE is also implicated in cancer and metastasis as well as in remodeling of the cytoskeleton. This will be described in detail in the section below.

1.9.4 NRAGE in cytoskeletal remodeling and tumorigenesis

As mentioned earlier, MAGE proteins were first identified as antigens presented at the surface of melanoma cells by the major histocompatibility complex and they were found to be overexpressed and mutated in melanoma and other tumors (Caballero et al, 2010). In particular, the mutation spectrum observed is reminiscent of the mutagenic impact of UV light where C>T – G>A alterations are predominant and represent inactivating rather that activating mutations (Caballero et al, 2010). The role of NRAGE in tumorigenesis is not well described and the data are still emerging. Studies available to date attribute an antitumorigenic role for NRAGE. In fact, NRAGE inhibits metastasis and tumor proliferation of pancreatic cells (Chu et al, 2007), melanoma cells (Lai et al, 2012), and brain tumor stem cells (Reddy et al, 2010) and its expression is downregulated in many tumor types such as breast cancer (Tian et al, 2005, Du et al, 2009), and colorectal cancer (Zheng et al, 2012). On the other hand, NRAGE is also reported to be upregulated in numerous cancers such as lung cancer, melanoma, colon cancer, breast

cancer, prostate cancer, and esophageal cancer (Yang et al, 2014), suggesting that NRAGE plays diverse and complex regulatory roles in cancer.

NRAGE promotes its antitumorigenic potential through regulating different signaling components important in cell adhesion and migration. It is proposed that NRAGE disrupts the E-cadherin/β-catenin complex leading to decreased association with the membrane, thus inhibiting cell adhesion (Xue et al, 2005). Disruption of E-cadherin/β-catenin complex negatively impacts cell migration and overexpression of NRAGE leads to decreased migration of pancreatic and melanoma cells (Chu et al, 2007). Similarly, NRAGE inhibits melanoma metastasis *in vitro* and *in vivo* induced by Ror2-Wnt5a by competing with the binding of Src to Ror2, thus decreasing activation of Src (Lai et al, 2012). Extracellular matrix proteolysis is essential for invasion of cancer cells and is achieved through secretion of MMPs that degrade the ECM, allowing for cell progression, and NRAGE has been proposed to inhibit cancer metastasis through the downregulation of MMPs activity (Chu et al, 2007, Reddy et al, 2010). Although the role of NRAGE in glioma is poorly studied, it was shown that NRAGE is downregulated in high grade glioma. Interestingly, overexpression of NRAGE in glioma stem cells decreases their proliferative as well as their invasive properties and reduces their tumorigenic effect *in vivo* (Reddy et al, 2010). It is proposed that NRAGE decreases the expression of pro-proliferative and prometastatic genes and increases tumor suppressor genes (Reddy et al, 2010). Studies also demonstrate that NRAGE expression is decreased in breast cancer cell lines compared to non-transformed cells (Tian et al, 2005, Du et al, 2009). This finding is interesting since it was shown that NRAGE interacts with BRCA2 and that this interaction stabilizes NRAGE expression, leading to the inhibition of cell cycle progression and decreased proliferation in normal tissue. In contrast, in breast cancer NRAGE is downregulated and this allows for cell cycle progression and cancer proliferation (Tian et al, 2005). In addition, overexpression of NRAGE in breast cancer cells inhibits their invasion and proliferation through a pathway involving p53, p21 and E-cadherin upregulation, and downregulation of β-catenin (Du et al, 2009). NRAGE was also reported to impair angiogenesis by inhibition of endothelial cell migration and vessel formation *in vitro* and *in vivo* through inhibition of HIF-1α expression under hypoxic conditions (Shen et al, 2007, Reddy et al, 2010).

Studies showing that NRAGE inhibits cell migration by disrupting the E-cadherin/β-catenin complex are intriguing, since reduction and relocalization of this complex enhances EMT and facilitates cell migration (Kumar et al, 2011). It is possible that the role of NRAGE on tumorigenesis is cell type and context dependent, and that it is highly affected by its binding

partners. In fact, in breast cancer cells NRAGE mediates the rapid degradation of β-catenin (Du et al, 2009), probably preventing its interaction with transcription factors essential for cell proliferation. In contrast to these findings, a recent study shows that, NRAGE enhances EMT through conferring resistance to anoikis in transformed mammary epithelial cells. It is suggested that NRAGE forms a complex with ankyrinG that binds E-cadherin and links the plasma membrane to the cytoskeleton and sequesters NRAGE to the cytoplasm. Upon EMT and E-cadherin loss, ankyrinG is downregulated, allowing translocation of NRAGE to the nucleus. NRAGE binds to the TBX2 transcriptional repressor that represses the transcription of p14ARF gene, an important mediator of anoikis. Thus, NRAGE overcomes anoikis through this mechanism leading to EMT and cancer progression (Kumar et al, 2011). Although studies have reported a downregulation of NRAGE in multiple cancers, an upregulation of NRAGE in many tumor types were also documented as mentioned above (Bhattacharjee et al, 2001, Boer et al, 2001, Ginos et al, 2004, Yang et al, 2014) suggesting that NRAGE could play a protumorigenic role. NRAGE mRNA levels were found to be increased in human lung carcinoma samples (Bhattacharjee et al, 2001) as well as in kidney cancer (Boer et al, 2001). It was recently shown that NRAGE is overexpressed in oesophagal carcinoma and that NRAGE depletion inhibits proliferation of the cells by inhibiting the cell cycle progression. Interestingly, NRAGE acts through increasing the expression of PCNA (proliferating cell nuclear antigen) that, upon NRAGE knockdown, is rapidly ubiquitinated and degraded in a proteosomal-dependent manner (Yang et al, 2014). Interestingly, MAGE-D2, another MAGE-D family member, has been documented as pro-proliferative in melanoma cells by its ability to reduce the resistance of melanoma cells for TRAIL-induced apoptosis (Tseng et al, 2012).

Altogether, these findings indicate that the role of NRAGE in cancer progression is controversial, implicating NRAGE in many different signaling pathways, ranging from apoptosis, cell cycle arrest, proliferation, and migration. The effect of NRAGE on these different signaling pathways could be mediated by the interaction of NRAGE with multiple players at the cell surface and in the cytosol, but also by its contribution to the regulation of transcriptional events.

1.10 NEDD9: NEURAL PRECURSOR CELL EXPRESSED DEVELOPMENTALLY DOWNREGULATED 9

The Cas (Crk associated substrate) family comprises a group of scaffolding proteins that were first identified as interacting partners for Crk and Src that are hyperphosphorylated in v-Crk and v-Src transformed cells (Reynolds et al, 1989, Kanner et al, 1991). The first member of this

family cloned was p130Cas/BCAR1 (p130 Crk-associated substrate/breast cancer antiestrogen resistance 1) that is phosphorylated upon binding to Src (Sakai et al, 1994). The human p130Cas gene is localized to chromosome 16q22-q23. Subsequently other members of this family were identified fulfilling similar functions to p130Cas namely in cancer. The second member identified was Efs/Sin (Embryonal Fyn-associated substrate) and was found to associate to the Src family kinases Fyn and Yes. Human Efs/Sin is localized on chromosome 14q11-q12 (Ishino et al, 1995, Alexandropoulos et al, 1996). NEDD9 (neural precursor cell expressed developmentally downregulated 9), also known as HEF1 (human enhancer of filamentation) and CasL (Crk-associated substrate-related protein, lymphocyte type), was identified in three separate screens. The first characterization of this protein was its identification in a screen for proteins involved in filamentous growth in yeast as a regulator of cell cycle and polarity (Law et al, 1996). Subsequently, the same protein was identified in T-lymphocytes where it was found to be hyperphosphorylated on tyrosine residues upon integrin β1 stimulation (Minegishi et al, 1996). Finally, a screening for genes expressed during development in the mouse brain identified NEDD9 that was shown to be downregulated in the adult (Kumar et al, 1992). Human NEDD9 localizes to chromosome 6p25-p24. Recently, a new member was identified. This member termed CASS4 (Cas scaffolding protein family member 4, or HEPL) was found in a screen of proteins that play similar roles than the other Cas family members (Singh et al, 2008). Human CASS4 localizes to chromosome 20q13.2-q13.31.

Members of this family are highly conserved from gnathostomes (jawed vertebrates) through mammals, and only one Cas family member is found in *Drosophila*, lower vertebrates and chordates. Whereas p130Cas is ubiquitously expressed in cultured cells and *in vivo*, NEDD9 abundance significantly varies in different cell types, tissues and under different growth conditions as well as in pathological conditions. NEDD9 mRNA levels are high in lung and kidney as well as in immature lymphoid cells and in the fetal brain, however it is downregulated in the adult brain. NEDD9 is highly expressed in many types of tumors. The expression of Efs/Sin is more confined to T-lymphocytes, the thymus, brain and skeletal tissue, whereas CASS4 is expressed in the lung and spleen with lower levels in other tissues. Despite their differential expression, the Cas family members have conserved structures and overlapping functions, and play important roles in cell migration, cell cycle control, differentiation, survival, as well as in many pathological conditions (Singh et al, 2007, Tikhmyanova et al, 2010a).

1.10.1 Structure of Cas family members

Cas family proteins are better recognized to act as scaffolds mediating signaling downstream from cell surface proteins. Although they do not contain a catalytic domain, they have multiple sites through which they interact with diverse molecules serving as a platform for downstream signaling. They are characterized by the presence of four conserved domain structure: an amino terminal Src homology 3 (SH3) domain that binds polyproline motifs containing proteins such as focal adhesion kinase (FAK), Pyk2, C3G, PTP-PEST, PTP1B among others; a large substrate domain that contains multiple tyrosine phosphorylation sites that upon phosphorylation serve as docking sites for Src homology 2 (SH2) domain-containing proteins, such as Crk, Crk-L, and CRKII. Further downstream of this region lies a loosely conserved domain of serine stretch organized as a four-helix bundle that is believed to act as a docking site for many p130Cas and NEDD9 members such as 14-3-3 proteins and GRB2. The carboxy terminal domain is highly conserved in its amino acid structure as well as secondary protein structure and has low similarity with members outside of this family. The C terminal domain is a binding domain for Src family of protein kinases and it also contains a second four-helix bundle through which NEDD9 can homodimerize or heterodimerize with p130Cas (Bouton et al, 2001, Singh et al, 2007). Figure 1.4 represents a schematic diagram showing the structure of the Cas family members and the different binding partners.

The Cas proteins are all shown to transduce signals downstream of integrins and to associate with focal adhesion kinases regulating functions such as adhesion, migration, differentiation, and cell survival and driving extensive signals to the cytoskeleton. But they are also involved in many pathological outcomes such as cancer as well as stroke and inflammation (reviewed in Bouton et al, 2001, Guerrero et al, 2012). In the last years, several studies have shown that NEDD9 plays a prominent role in cancer cell migration, as well as in the regulation of cell adhesion, mainly in relation to Rho GTPases. In this section we will discuss the functions of NEDD9 as well as its regulation, focusing particularly on the role of NEDD9 in cancer.

1.10.2 NEDD9 regulation

The function of NEDD9 is controlled by its extensive regulation at the transcriptional as well as at the posttranslational level. All these modifications are important for the correct function of NEDD9 and ensure a correct regulation of the different biological outcomes conveyed by this scaffolding protein.

1.10.2.1 Phosphorylation

Phosphorylation of NEDD9 is important for the recruitment of binding partners to the substrate domain to regulate downstream mechanisms. The subcellular localization of NEDD9 at focal adhesions makes this scaffolding protein an important mediator of cytoskeletal remodeling involved in cell adhesion and cell migration. Adhesion of cells to extracellular matrix substrates and changing of cell shape mediate integrin ligation and clustering that ultimately activate FAK and phosphorylate NEDD9 at tyrosine residues. Once phosphorylated, NEDD9 creates a binding domain for Src that further induces its tyrosine phosphorylation at the substrate domain. This promotes the recruitment of binding partners such as Crk to its substrate-binding domain (Manie et al, 1997, O'Neill and Golemis, 2001, Sawada et al, 2006, Baquiran et al, 2013).

The NEDD9/Crk association triggers a cascade of signaling events that converge on Rac activation and actin cytoskeleton rearrangement, promoting migration through Arp2/3 and PAK kinase activation (Smith and Li, 2004). It was shown that integrin engagement promotes phosphorylation of NEDD9 at discrete tyrosine sites, namely Y189, which is an essential process for the stabilization of focal adhesions (Manie et al, 1997, O'Neill and Golemis, 2001, Sawada et al, 2006, Baquiran et al, 2013). Consequently, MEFs lacking NEDD9, or in which Y189 is mutated to phenylalanine (Y189F), exhibit rapid focal adhesion disassembly accompanied by FAK phosphorylation and increased migration on 2D substrate (van Seventer et al, 2001, Zhong et al, 2012, Baqiran et al, 2013). Integrin signaling appears as an important mediator of NEDD9 regulation since MCF7 cells plated on substrates that do not favor integrin ligation such as poly-*L*-lysine or in serum free media, display a loss in NEDD9 phosphorylation accompanied by focal adhesion disassembly. This is reversed when cells are plated on laminin or fibronectin (O'Neill and Golemis, 2001). Interestingly, cell adhesion mediates phosphorylation of NEDD9 at tyrosine residues and at serine/threonine residues that result in the formation of a hyperphosphorylated form of NEDD9 migrating at 115 kDa (p115) as opposed to its normal apparent molecular mass of 105 kDa (p105) form (O'Neill and Golemis, 2001, Zheng and McKeown-Longo, 2002). The appearance of the hyperphosphorylated form of NEDD9 occurs when synchronized cells reenter the cell cycle (Law et al, 1998). Cell adhesion to ECM induces serine/threonine phosphorylation of NEDD9 and appearance of the p115 species. This phosphorylation stabilizes the protein and prevents its proteosomal degradation (Zheng and McKeown-Longo, 2006). In contrast, phosphorylation of NEDD9 at serine 369 enhances its proteosomal degradation (Hivert et al, 2009). Thus depending on its localization, phosphorylation of NEDD9 at serine/threonine residues could have different effects on the protein state and can regulate different functions. NEDD9 can also be phosphorylated by growth factors. In fact, TGF-β1 enhances tyrosine and

serine/threonine phosphorylation of NEDD9 independently of adhesion (Zheng and McKeown-Longo, 2002). Similarly, NEDD9 is also phosphorylated in response to G protein-coupled receptors (GPCRs) for calcitonin (Zhang et al, 1999), to PDGF signaling in glioblastoma cells (Natarajan et al, 2006), muscarinic receptor stimulation in neuroblastoma cells (Jope et al, 1999), and in response to global ischemia in the cerebral cortex and hippocampus of adult rats (Sasaki et al, 2005b).

In addition to its regulation through phosphorylation, NEDD9 is also modulated by dephosphorylation events, and it interacts with diverse protein tyrosine phosphatases. Interaction of NEDD9 with the SH2 domain-containing protein tyrosine phosphatase (SHP2) in A549 lung cancer cells dephosphorylates NEDD9 and inhibits migration of these cells (Yo et al, 2009).

NEDD9 phosphorylation is a key process that controls diverse functions of NEDD9 and it is important for the association of NEDD9 with downstream signaling partners, which ensure the progression of signaling pathways such as adhesion and migration. In addition, phosphorylation of NEDD9 controls the stability of the protein and prevents its degradation.

1.10.2.2 Transcription

Another level of NEDD9 regulation resides at its transcriptional level. This is observed in many situations and transcriptional regulation of NEDD9 plays key roles in NEDD9 functions. NEDD9 mRNA expression is increased when cells enter the cell cycle (Law et al, 1998), but transcriptional activation of NEDD9 is also observed in response to growth factors such as TGF-β1 in fibroblasts (Zheng and McKeown-Longo, 2005), and progesterone (Richer et al, 2002), whereas NEDD9 is downregulated in response to estrogen in MCF-7 cells and osteosarcoma cells (Monroe et al, 2003, Buterin et al, 2006). Interestingly, NEDD9 promoter contains a retinoic acid response element (RARE) that binds retinoic acid thus promoting NEDD9 expression in neuroblastoma cell lines (Merrill et al, 2004, Knutson and Clagett-Dame, 2008), and in migrating neural crest cells (Aquino et al, 2009). NEDD9 mRNA expression is also increased after global ischemia in the cerebral cortex and the hippocampus of adult rats (Sasaki et al, 2005b). Interestingly, the stem cell-associated transcription factors, SOX2 and NANOG, have been shown to co-occupy the NEDD9 promoter but the role of this interaction is still unexplored (Boyer et al, 2005).

1.10.2.3 Cleavage and proteolysis

NEDD9 is regulated by proteolytic cleavage. Products termed p115, p105, p65, and p55 are enriched at different phases of the cell cycle. As discussed above, the p115 species is a

serine/threonine hyperphosphorylated form of the p105 species, and both appear as cells enter the cell cycle. Some studies have suggested that they are important for cell cycle initiation (Law et al, 1998), and for focal adhesion dynamics (O'Neill and Golemis, 2001). However, the p55 species apparently results from cleavage of the full length protein by caspases at a putative DLVD site. The p55 species appears at mitosis and is localized to the mitotic spindle where it associates with Dim1p, an important mediator of spindle assembly (Law et al, 1998). Interestingly, MCF-7 cells stably expressing NEDD9 and treated with TNFα, generate two additional species, p65 and p28, that are caspase-dependent cleavage products. These species do not appear upon induction of the cell cycle but only during apoptosis, and it was shown that the p28 species is sufficient to induce apoptosis, through activation of the JNK signaling pathway (Law et al, 2000). NEDD9 turnover is ensured by proteosomal degradation that is also regulated by protein phosphorylation and actin reorganization (Zheng and McKeown-Longo, 2006). It was also suggested that TGF-β regulates NEDD9 proteosomal degradation through induction of its signal transducer, Smad3. In turn, Smad3 recruits the Anaphase Promoting Complex (APC) subunit, APC10 and NEDD9, which leads to ubiquitination and proteosomal degradation of NEDD9 (Liu et al, 2000, Nourry et al, 2004).

Hence, the regulation of NEDD9 is tightly controlled in the cell in order to ensure specific and localized functions of this multifunctional protein, essentially in cancer.

1.10.3 NEDD9 functions

1.10.3.1 Cell migration

NEDD9 plays a prominent role in cell adhesion and migration. Integrin ligation recruits NEDD9 to focal adhesions, which is subsequently phosphorylated by FAK. Tyrosine phosphorylation of NEDD9 constitutes a platform that recruits Src, further phosphorylating NEDD9 at its substrate domain forming a docking site for downstream signaling molecules such as Crk. Activation of downstream signaling converges on Rho GTPases that remodel the cytoskeleton and mediate adhesion and migration. NEDD9 is an important mediator of cell migration and is required for normal development of the nervous system. In fact, NEDD9 is implicated in the migration of neural crest cells from the neural tube to the underlying mesoderm where they start spreading in different regions and differentiate (Aquino et al, 2009). Interestingly, NEDD9 expression is regulated by retinoic acid that binds to the NEDD9 promoter (Knutson and Clagett-Dame, 2008), and is secreted at the time and site of neural crest cell migration (Aquino et al, 2009). Similarly, NEDD9 is required for the migration of T

lymphocytes, downstream of integrin β1 (van Seventer et al, 2001, Gu et al, 2012). NEDD9 is activated and phosphorylated by the Abl kinase in T cells upon inflammation and leads to actin reorganization and cell polarization that are important features for migration of T cells to the site of inflammation (Gu et al, 2012). Besides its role in normal cell migration during development or upon inflammation, NEDD9 plays a prominent role in cancer cell migration and was identified as a major metastatic factor. The role of NEDD9 in cancer will be discussed later.

1.10.3.2 Cell cycle progression

Besides its key role as a scaffolding protein downstream of integrins, a role in cell cycle regulation has also been attributed for NEDD9. As discussed earlier, NEDD9 is processed in the cell and different species of the protein appear during different phases of the cell cycle. The hyperphosphorylated form of NEDD9, the p115 species, appears at interphase, at the initiation of the cell cycle and is localized at focal adhesions together with the p105 species. However, an additional fragment of 55 kDa appears when cell enters the G2/M phase and localizes to the mitotic spindle, suggesting that NEDD9 could be playing a role in the regulation of the cell cycle (Law et al, 1998). Accumulating evidence now shows that NEDD9 is an important component of the cell cycle regulation machinery and importantly it interacts with AuroraA, a kinase that activates substrates necessary for cell cycle progression such as Cyclin B and Cdk1 (Pugacheva and Golemis, 2005, Pugacheva and Golemis, 2006). NEDD9 and AuroraA are colocalized at the centrosome and NEDD9 is necessary for the activation of AuroraA, which ultimately leads to centrosome duplication and formation of bipolar spindles (Pugacheva and Golemis, 2005). This pathway is in part regulated by Rho GTPases and inhibition of Rho GTPases prevents NEDD9 phosphorylation and activation of AuroraA (Dadke et al, 2006, Ando et al, 2007). NEDD9 levels are very important for the correct regulation of the cell cycle since depletion or overexpression of NEDD9 causes mitotic spindle abnormalities accompanied by defects in cytokinesis (Pugacheva and Golemis, 2005, Dadke et al, 2006). Levels of NEDD9 are tightly regulated through its association with Smurf2, an E3 ubiquitin ligase that stabilizes NEDD9 and prevents its proteosomal degradation, making NEDD9 available for AuroraA activation and progression to the cell cycle (Moore et al, 2010). In addition to its role in cell cycle progression, NEDD9 also plays a role in neuronal fate specification. Hippocampal progenitor cells treated with TGF-β have an increase in cell cycle inhibitor proteins as well as an upregulation of NEDD9. NEDD9 knockdown prevents TGF-β induced neuronal differentiation denoting that NEDD9 is an important factor for differentiation (Vogel et al, 2010), however, it is still unknown whether it promotes the exit from the cell cycle.

1.10.3.3 Apoptosis

Although the role of NEDD9 in apoptosis is not well defined and underestimated compared to its prominent role in cell migration, the initial work by Law and colleagues showed that MCF-7-NEDD9 inducible cells are rounded and detached from the plate after 48 hours induction and are accompanied with increased cell death. NEDD9 induced caspase 3 activation and JNK phosphorylation (Law et al, 2000). A role for p130Cas in JNK activation is described through a pathway involving Crk and Rac in COS7 and HeLa cells (Dolfi et al, 1998, Girardin and Yaniv, 2001). Interestingly, NEDD9 is also cleaved by caspases to generate three additional species, p55, p65, and p28. Surprisingly, the p28 fragment appears only during apoptosis and is sufficient to induce death in MCF-7 cells. The p28 fragment is rapidly degraded when cells are undergoing mitosis but is stabilized only during apoptosis (Law et al, 2000).

1.10.4 NEDD9 and cancer cell migration

NEDD9 has emerged during the last decades as a major driver of metastasis and invasion and data on the role of NEDD9 in metastasis is still growing, placing it as a major metastatic factor in various types of cancers. A wide oncogenomic screening of doxycycline inducible RAS dependent melanoma, showed that highly metastatic melanomas expressed high levels of NEDD9 mRNA and protein, compared to benign melanocytic neoplasia and non-transformed melanocytes and that it is a potential target of 6p gain mutations characteristic of metastatic melanoma. Knockdown of NEDD9 in transformed metastatic melanocytes derived from these mice or from human metastatic cell line *in vitro* reduced the invasive potential of these cells (Kim et al, 2006). Similarly, NEDD9 appeared as a key promoter of metastasis in breast cancer (Kong et al, 2011, McLaughlin et al, 2014, Stajduhar et al, 2014). *Nedd9*$^{-/-}$ mice that bear an MMTV-PyVmT transgene show delayed appearance of mammary tumors and have fewer lung metastases. Cells derived from primary *Nedd9*$^{+/-}$ and *Nedd9*$^{-/-}$ tumors showed persistently reduced FAK activation and had defects in both attachment and migration (Izumchenko et al, 2009). Not only NEDD9 plays a role in metastasis of breast cancer cells, but it was also shown to initiate tumors from mammary progenitor cells. In fact, MMTV-*neu*/*Nedd9*$^{-/-}$ mice show a delay in the appearance of mammary tumors, and progenitor cells derived from these tumors are unable to form intact mammospheres in culture with a net decrease in FAK and F-actin (Little et al, 2014). Together, these results show that NEDD9 plays an important role in tumor initiation as well as cancer progression and metastasis.

In addition to its role in melanoma and breast cancer, NEDD9 has emerged as a potent prometastatic factor in many tumor types such as glioblastoma (Natarajan et al, 2006), lung cancer (Miao et al, 2013, Kondo et al, 2012), colon cancer (Kim et al, 2010, Xia et al, 2010, Li et al, 2011), gastric cancer (Liu et al, 2014, Shi et al, 2014), and prostate cancer (Morimoto et al, 2014). The localization of NEDD9 in the integrin signaling pathway makes it a favorable candidate for driving metastatic behavior of cancer cells.

Interestingly, NEDD9 is activated by integrin β3 ligation leading to activation of Src. This in turn converts the amoeboid morphology of the cells to a mesenchymal migration through inhibition of the RhoA/ROCKII pathway (Ahn et al, 2012). In fact, NEDD9 has been documented as an inducer of EMT through Src-mediated lysosomal degradation of E-cadherin, thus decreasing E-cadherin localization at cell junctions (Kong et al, 2011, Tikhmyanova et al, 2011, Miao et al, 2013, Jin et al, 2014). Although NEDD9 does not act as a transcription factor itself, NEDD9 induces binding of the transcription factors Snail and Slug to the E-cadherin promoter and suppresses its activation (Kong et al, 2011). Thus, NEDD9 acts both at the transcriptional as well as at the posttranslational level to inhibit E-cadherin function, leading to EMT.

One important aspect of NEDD9-induced cell migration is the interaction with the Rho GTPase family proteins. Interestingly, NEDD9 interacts with DOCK3, a Rac GEF, and activates Rac in metastatic melanoma (Sanz-Moreno et al, 2008). NEDD9 and DOCK3 expression are regulated by the transcription factor Twist-1, a master regulator of EMT. Twist-1 acts by repressing the tumor suppressor miRNA *let-7i* in human head and neck squamous cell carcinoma (HNSCC), leading to a release of inhibition of NEDD9 and DOCK3, that in turn activates Rac1, and mediates EMT and increases invasion in 2D and 3D environments (Yang et al, 2012). Knockdown of NEDD9 deactivates Rac, releasing the inhibition on the Rho/ROCK pathway that promotes amoeboid movement (Sanz-Moreno et al, 2008, Kong et al, 2011).

The hypoxic environment of tumors also favors the activation of NEDD9. Colon carcinoma cells exposed to hypoxia, express high levels of the hypoxia-inducible factor, HIF-1α. Interestingly, HIF-1α binds to the NEDD9 promoter and promotes its transcription. This creates a positive feedback loop that further enhances HIF-1α production and promotes migration of cancer cells (Kim et al, 2010). The hypoxic environment of the tumors also favors induction of vascular endothelial growth factor (VEGF) that is required for angiogenesis and studies show that NEDD9 is required for VEGF upregulation (Kim et al, 2010, Lucas et al, 2010), concomitant with an increase in the migration of HNSCCs (Lucas et al, 2010).

Matrix degradation by matrix metalloproteases (MMPs) is an essential step for progression of cancer cells through the surrounding ECM. Migrating cells secrete MMPs at invadopodia that lead to matrix degradation creating spaces for the cells to migrate. Interestingly, NEDD9 increases the activation of MMPs at invadopodia through blocking the association of MMPs with their inhibitors, the tissue inhibitor of matrix metalloproteases (TIMPs) (McLaughlin et al, 2014).

Together, these findings define NEDD9 as a prominent tumorigenic factor. It is clear that NEDD9 is mainly observed as a prometastatic factor through the induction of diverse signaling pathways converging on cell migration. However it also plays important roles in other aspects of tumor progression such as proliferation, in part due to its role in cell cycle progression. Impaired NEDD9 expression can lead to uncontrolled cell cycle activation and defects in mitosis, contributing to increased proliferation. Thus, NEDD9 is a potent candidate for therapeutic strategies especially through targeting its regulatory functions such as phosphorylation state, proteolysis and transcriptional regulation.

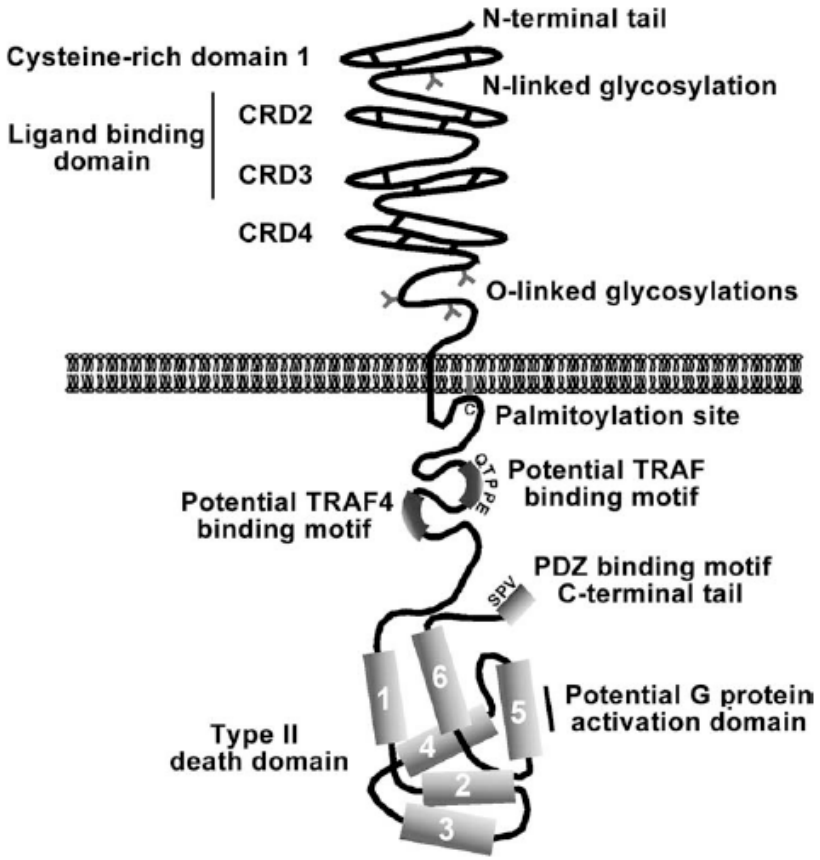

Figure 1.1. Schematic representation of the structure of the p75NTR protein. p75NTR is a Type I transmembrane receptor. The extracellular domain is *N*-glycosylated and contains four cysteine-rich domains (CRDs), the stalk region is *O*- glycosylated at several sites. The intracellular domain is palmitoylated at cysteine 279, and contains two potential TRAF-binding sites, a Type II death domain, a potential G protein activating domain, and a PDZ domain binding motif. (Adapted from Roux and Barker, 2002).

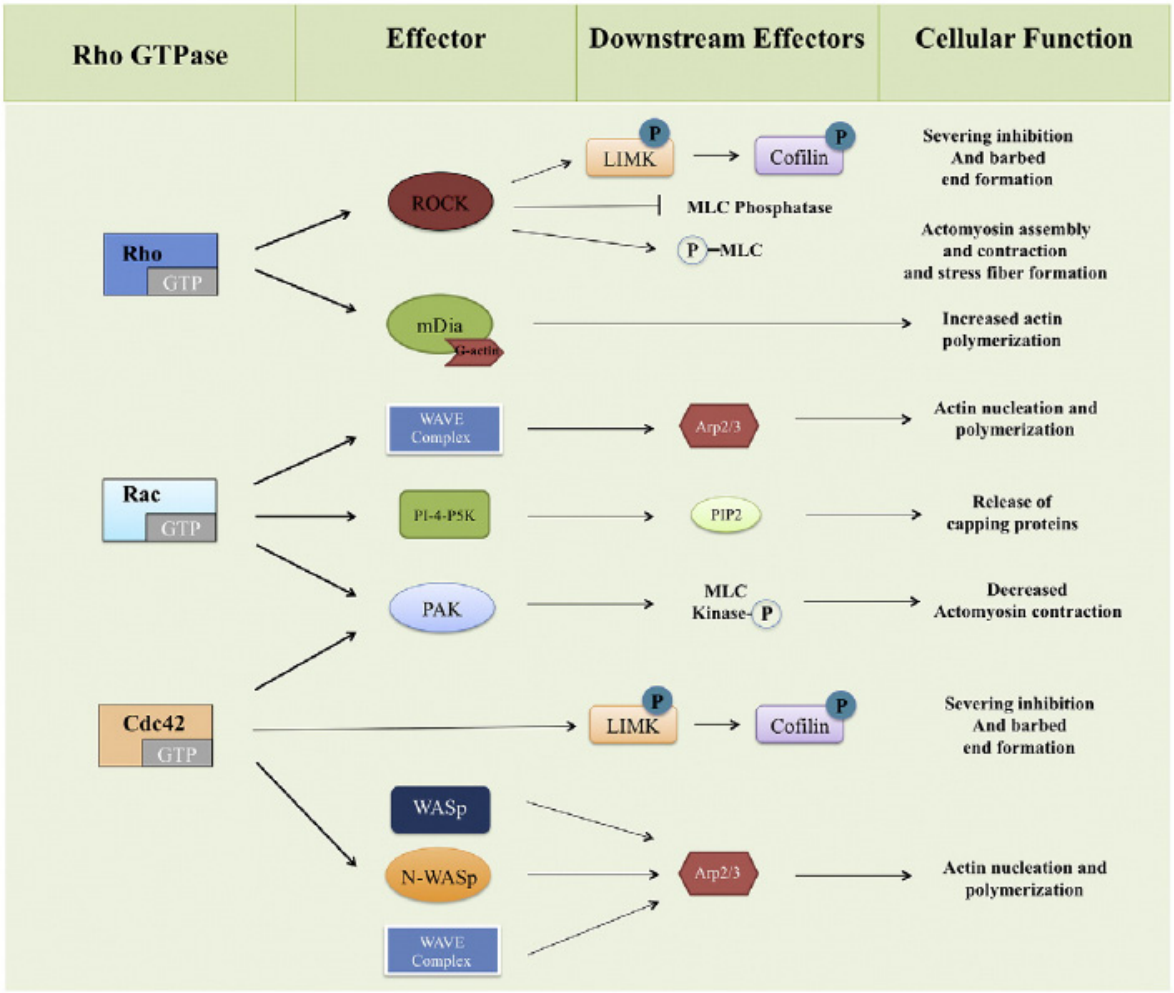

Figure 1.2. Rho GTPases and downstream effectors.
Rho downstream effectors involves Rho kinase (ROCK) and mDia. ROCK regulates stress fiber formation and focal adhesions through phosphorylation of myosin light chain (MLC). ROCK also phophorylates LIMK that in turn phosphorylates the actin severing protein cofilin and thus inhibits its severing activity. Rac and Cdc42 dowsntream effectors involve scaffold proteins belonging to the WASP/SCAR/WAVE family, key regulators of actin nucleation and polymerization. p21 activating kinase (PAK) is a common effector protein of both Rac and Cdc42. Active PAK prevents MLC phosphorylation by phosphorylating MLCK thereby inactivating it which inhibits contractiliy. PAK also phosphorylates and activates LIMK which potentially results in the phosphorylation of cofilin inhibiting its actin-severing function. (Adapted from Hanna and Sibai, 2013).

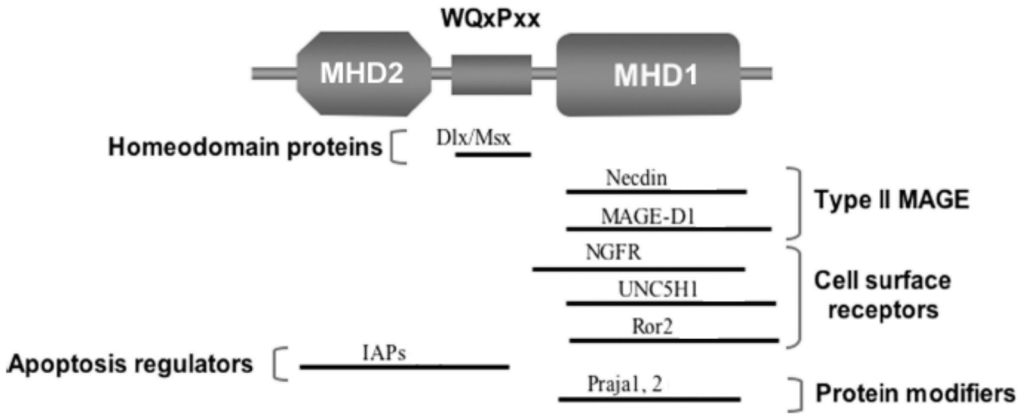

Figure 1.3. NRAGE structure and interacting proteins.
NRAGE is composed of two MAGE homology domains (MHD). MHD1 is highly conserved between other MAGE family members, whereas the MHD2 is loosely conserved. NRAGE is characterized by the presence of a hexapeptide repeat WQxPxx that is a putative binding site for multiple proteins. The different domains of NRAGE interact with multiple proteins as indicated. (Adapted from Sasaki et al, 2005a).

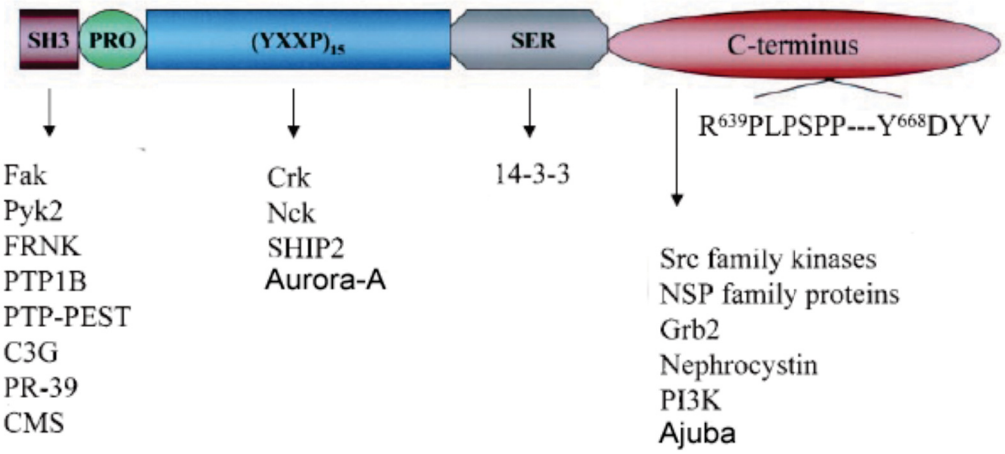

Figure 1.4. Cas structure and its binding partners.
Cas family members are composed of an SH3 domain, a proline rich region that is absent in NEDD9, 15 tyrosine rich SH2 substrate binding sites, a serine stretch, and an evolutionary conserved carboxy-terminus (C-terminus). Each region serves as a platform for interaction with multiple signaling partners as indicated. (Adapted from Bouton et al, 2001 and Guerrero et al, 2012).

RESEARCH RATIONALE AND OBJECTIVES

Rationale

p75NTR is a multifunctional receptor that conveys a myriad of different functions through transducing many signaling pathways. Signaling of p75NTR through Rho GTPases is implicated in many different cellular processes such as cell death, myelination, and neurite extension. The exact role of p75NTR in Rho GTPases regulation is still controversial and the downstream signaling leading to Rho GTPases activation is largely unexplored. The aim of this thesis is to study the p75NTR signaling leading to the regulation of Rho GTPases and in particular to study the role of p75NTR cleavage in this process. We identified novel signaling partners downstream of p75NTR that constitute an important link for Rho GTPases activation. We also introduced a FRET bioassay that enabled us to study the regulation of Rho GTPases downstream of p75NTR. Finally, we studied the role of p75NTR cleavage and the downstream signaling cascade in glioma migration.

Objectives

There are three main objectives to this thesis:

1- To identify the signaling cascade downstream of p75NTR leading to Rac1 activation using the COS7 spreading assay. In particular, we asked whether the cleavage of p75NTR is required for COS7 spreading and Rac1 activation. We also assessed the role of NRAGE in this process and identified NEDD9 as an NRAGE interactor through a yeast two-hybrid screen. This is discussed in chapter 2.

2- To establish a FRET bioassay using Rho GTPases biosensors that would allow us to study the regulation of Rho GTPases downstream of p75NTR. In particular, we asked whether p75NTR differentially regulates Rac1 and RhoA and whether this regulation is influenced by the presence of coreceptors such as NogoR1 and myelin inhibitory proteins such as Nogo66. This is discussed in chapter 3.

3- To determine the role of p75NTR in glioma migration. In particular, we asked whether cleavage of p75NTR is required for this process and whether NRAGE-NEDD9 act downstream of p75NTR to promote glioma migration. This is discussed in chapter 4.

PREFACE TO CHAPTER 2

The role of p75NTR in Rho GTPases signaling is not very well understood. This is mainly due to the absence of a robust assay that allows the study of p75NTR signaling pathway leading to Rho GTPases activation. In this work we used the COS7 spreading assay as a heterologous system to study Rho GTPases signaling. We show that p75NTR induces spreading of COS7 cells through the activation of Rac1. p75NTR undergoes a dual cleavage event known as regulated intramembrane proteolysis (RIP) that involves its first cleavage by ADAM17 and the release of the extracellular domain, and a second cleavage with γ-secretase that leads to the generation of the intracellular domain (ICD) with signaling capabilities. Here we show that cleavage of p75NTR and the release of the ICD are important for COS7 cell spreading. We also identify NRAGE as an important mediator of this process downstream of p75NTR. Using a yeast two-hybrid screen we identify NEDD9 as a NRAGE interactor that mediates the effects of p75NTR on cell spreading and Rac1 activation.

The work presented in this chapter defines a novel cascade downstream of p75NTR implicating NRAGE and NEDD9 in the activation of Rac1.

CHAPTER 2

p75NTR-DEPENDENT RAC1 ACTIVATION REQUIRES RECEPTOR CLEAVAGE AND ACTIVATION OF AN NRAGE AND NEDD9 SIGNALING CASCADE

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

[1]*Corresponding author* - *e*mail: phil.barker@mcgill.ca

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2.1 ABSTRACT

The p75 neurotrophin receptor (p75NTR) 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 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 demonstrate that the p75NTR adaptor protein NRAGE acts downstream of the $p75NTR^{ICD}$ in this cascade and, through a yeast two-hybrid screen, identify NEDD9, a Cas family adaptor protein, as an novel NRAGE binding partner, that mediates p75NTR-dependent 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.

2.2 INTRODUCTION

The p75 neurotrophin receptor (p75NTR), 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 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-GDI share a binding site on p75NTR and it has been proposed that MBIs shift the p75NTR binding preference from Kalirin-9 interaction to Rho-GDI interaction (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) 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 path 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 subset of p75NTR-dependent signaling events. Using this, 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 NRAGE-dependent pathway and, through yeast-two hybrid screening, 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.

2.3 MATERIAL AND METHODS

Reagents and plasmids: Polyclonal antibodies directed against p75NTR and NRAGE were described previously (Barker et al, 1994, Salehi et al, 2000), antibodies against ADAM17 were purchased from Cedarlane laboratories Ltd. (E8404, Burlington, ON, CA), antibodies against NEDD9 were obtained from Thermo Fisher Scientific (MA1-5784, Ottawa, ON, CA) and the M2 antibody (directed against the FLAG epitope) was obtained from Sigma (F3165, Oakville, ON, CA). The monoclonal antibody against β-actin was purchased from MP Biomedicals (691001, Irvine, CA, USA). The REX antibody was provided by Louis Reichardt. NGF and BDNF were obtained from Alomone laboratories (Jerusalem, Israel). Compound XXI, ilomastast (GM6001) and epoxomicin from Calbiochem (San Diego, CA, USA); batimastat (BB94) and NSC 23766 were from Tocris Bioscience (Ellisville, MO, USA). Laminin was purchased from BD Bioscience (Mississauga, ON, CA). Poly-*D*-lysine (PDL) was obtained from Sigma (Oakville, ON, CA). Rhodamine wheat germ agglutinin (WGA) was purchased from Vector laboratories (Burlington, ON, CA) and horseradish-peroxidase conjugated secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA, USA). All cell culture reagents were from Wisent Bioproducts (Saint Bruno, QC, CA). Dako anti-fading mounting medium was purchased from Cedarlane laboratories Ltd. (Burlington, ON, CA). The Rac biosensor was obtained from Addgene (Cambridge, MA, USA) and was described previously (Hodgson et al, 2010). Glutathione plasmids encoding full-length p75NTR, cleavage-resistant p75NTR and the $p75NTR^{ICD}$ were described previously (Kommaddi et al, 2011). Plasmids encoding RacN17 and RhoAN19 were kindly provided by Dr. Peter McPherson (McGill University).

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, in 5% CO2 at 37°C. COS7 cells were co-transfected with EGFP cDNA (0.5 ug) in the absence or presence of plasmids encoding the various p75NTR isoforms (2 ug) 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 ug) 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 lysed in Laemmli sample buffer for analysis by immunoblot.

NRAGE, NEDD9, and ADAM17 siRNA targeting sequences directed against the respective simian mRNAs were designed using the Invitrogen Stealth RNAi™ 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 media 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 immunoblot.

Cell spreading assay: Glass coverslips (12mm, Fisherbrand, Fisher Scientific, Ottawa, ON, CA) were coated for 30 minutes with PDL (0.5 ug/ml), washed with sterile water, coated with laminin (0.5 ug/ml) for two 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/5% CO2 for twenty four hours. Media was then removed and replaced with 4% paraformaldehyde in PBS for 15 minutes at 37°C. After washing with PBS (3 x 5 minutes), cells were incubated with rhodamine-tagged WGA (5 ug/ml) in PBS for 10 minutes and then washed with PBS (2 x 5 minutes) and with water (1 x 5 minutes). Coverslips were mounted in anti-fading mounting media (Dako), and kept at 4°C until imaging was performed. Imaging was performed using a 40X objective (N.A 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. 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 one hour prior to fixation and staining. For NGF or BDNF, cells were serum starved for two hours then treated with the different neurotrophins (25 ng/ml) for one hour or twenty four hours prior to fixation and staining. In the case of the REX treatment, cells were treated with the REX antibody (1:100) for twenty four hours prior to fixation and staining.

RNA extraction and rtPCR: mRNA was extracted from COS7 cells using Qiagen RNeasy Mini Kit according to the manufacturer's instructions (Valencia, CA, USA). cDNA was produced using the Ominscript RT kit (Qiagen) with random hexamers (GE Healthcare, Mississauga, ON, CA) as primers. PCR was then performed using the GoTaq green master

mix reagent (Fisher, Ottawa, ON, CA) with primers targeted against 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 55°C annealing temperature 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, USA). From 2.5 x 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, USA) 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 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 pH 8.0, 150 mM NaCl, 1% NP40, 10% glycerol) after 24

hours and then incubated with 20 ul glutathione-conjugated beads (GE Healthcare, Baie d'Urfe, QC, CA) for 1 hour at 4ºC. Beads were washed three times in NP40 buffer, re-suspended in Laemmli sample buffer, and incubated at 100ºC for 5 minutes. Levels of NRAGE or its deletion fragments that associated with the GST-NEDD9 fragment was determined by immunoblotting with M2.

Western blot analysis and immunoblotting: Cells were harvested in 2x Laemmli sample buffer and boiled for 5 minutes prior to loading on SDS-PAGE. In the case of ADAM17 detection, BB94 (0.2 uM) was added to the 2x Laemmli sample buffer before lysing the cells. For $p75NTR^{ICD}$ detection, cells were pre-treated with epoxomicin (1 uM) for 6 hours before lysis to block proteosomal degradation of the ICD, as previously described (Ceni et al, 2010). After SDS-PAGE and transfer to nitrocellulose, membranes were rinsed in PBS then blocked in TBST (10 mM Tris pH 8.0, 150 mM NaCl, 2% Tween 20) that was supplemented with 5% (w/v) dried skim milk powder. Primary and secondary antibody incubation was performed in TBST containing 2.5% (w/v) dried skim milk powder blocking solution, with primary incubations performed overnight at 4°C 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, USA), as per the manufacturer's instructions.

Rac activity using fluorescence resonance energy transfer (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 ug) using calcium phosphate transfection and incubated for 48 hours at 37°C/5% CO2. 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 using lipofectamine 2000 in antibiotic free media. Cells were kept for 48 hours at 37°C/5% CO2, then plated on glass coverslips precoated with PDL for 30 minutes and laminin for 2 hours at a density of 10000

cells/ml. Cells were kept for 24 hours at 37°C/5% CO2 then fixed with 4% paraformaldehyde for 15 minutes at 37°C. They were then washed with PBS (2 x 5 minutes each) and sterile water (1 x 5 minutes) and mounted on slides. Slides were kept at 4°C until imaging.

Imaging was performed using a 40X objective (N.A 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 (for excitation and emission, respectively): CFP: 430/24 (ex), 470/20 (em); FRET: 430/24 (ex), 535/30 (em); YFP: 500/20 (ex), 540/40 (em). 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) and substracts them from the FRET intensity and calculates the normalized FRET (NFRET) value according to the following formula:

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

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 mean ± s.e.m.

2.4 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 COS7 contractility assay previously used to study semaphorin-induced signaling events (Takahashi et al, 1999, Takahashi et al, 2001, Zanata et al, 2002, Mitsui et al, 2002). COS7 cells were transfected with EGFP together with p75NTR constructs (Figure 2.1A and B), then plated at low density on glass coverslips which had been pre-coated with laminin. Twenty four hours later, cells were fixed, left non-permeabilized, then exposed to wheat germ agglutinin conjugated to Cy3 to visualize cell surfaces. Figures 2.1C and D show that overexpression of full-length wild-type p75NTR or the p75NTR intracellular domain resulted in significant COS7 cell spreading, with 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 may provide a bioassay for p75NTR signaling activities that were dependent on receptor cleavage.

p75NTR acts through Rac1 to promote cell spreading

The Rho family of small GTPases play 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. Since 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. For this, COS7 cells were transfected with RacN17, a dominant-negative Rac1 isoform, and with full-length p75NTR or with the $p75NTR^{ICD}$. Figures 2.2A-B show 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 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 uM) for 6 hours. Figures 2.2C-D show that the Rac1 inhibitor effectively blocks cell spreading evoked by full-length p75NTR and by the p75NTR intracellular domain.

In parallel experiments, we tested whether dominant-negative form of RhoA (N19) altered spreading induced by full-length p75NTR or the $p75NTR^{ICD}$ but found it had no effect in this

assay (Figure 2.2E). Together, these data demonstrate that Rac1, but not RhoA, plays a critical role evoking p75NTR-induced cell spreading on COS7 cells.

ADAM17-mediated p75NTR cleavage is a prerequisite required for cell spreading

p75NTR undergoes a two step cleavage event, known as regulated intramembrane proteolysis (RIP) to generate the $p75NTR^{ICD}$. To determine if 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 ADAMs proteases, blocked the cell spreading induced by full-length p75NTR overexpression. 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 uM) and BB94 (200 nM) for one hour. Figures 2.3A-B show 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 extracellular cleavage of p75NTR (Kommaddi et al., 2011, Ceni et al., 2010) and therefore characterized its effect on cell spreading. Figure 2.3C 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 if ADAM17 is required for the effect of p75NTR on cell spreading, small interfering RNAs (siRNA) were used to reduce endogenous ADAM17 levels in COS7 cells overexpressing full-length p75NTR and $p75NTR^{ICD}$. Figure 2.3D shows that ADAM17 siRNA effectively reduced ADAM17 expression in COS7 cells and demonstrated that accumulation of the $p75NTR^{ICD}$ is blocked when ADAM17 levels are suppressed. 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. Figures 2.3E-F show that ADAM17 knockdown effectively blocked cell spreading induced by p75NTR overexpression but no effect on spreading induced by the $p75NTR^{ICD}$. Taken together, these data demonstrate that by initiating p75NTR cleavage and allowing generation of the $p75NTR^{ICD}$, ADAM17 plays a crucial role elaborating the cell spreading phenotype induced by p75NTR overexpression.

The p75NTR intracellular domain 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 (Figure 2.4A). Consistent with previous results, γ-secretase inhibition results in accumulation of a 25 kDa C-terminal fragment (CTF) of p75NTR (lane 2 of Figure 2.4A). 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 uM) or vehicle for one hour. Figures 2.4B and C show that compound XXI had no effect on cells expressing EGFP alone or expressing the $p75NTR^{ICD}$ yet it blocked spreading of cells expressing full-length p75NTR. Since 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 the four neurotrophins present in mammals and in our next experiments, we addressed whether these ligands have an impact on the COS7 spreading assay. Figure 2.4D shows that the addition of NGF or BDNF (each at 25 ng/ml), added either for one hour or for 24 hours, had no effect on p75NTR cell spreading. To determine if endogenous neurotrophins may play a role, we used rtPCR 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 (Figure 2.4E). The capacity of full-length p75NTR to drive cell spreading was not significantly changed when cells were maintained in REX (Fig 2.4F), 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 intracellular domain 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 if NRAGE is required for p75NTR-dependent COS7 cell spreading. After validating that NRAGE siRNAs suppress NRAGE protein expression in COS7 cells (Figure 2.5A), we asked whether NRAGE depletion altered cell spreading induced by overexpression of full-length p75NTR or the $p75NTR^{ICD}$. Figures 2.5B-C show that 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 $p75NTR^{ICD}$. NRAGE could mediate these effects by binding the $p75NTR^{ICD}$ and playing a direct role in downstream signaling or by facilitating generation or maintenance of the $p75NTR^{ICD}$. However, levels of full-length p75NTR or the $p75NTR^{ICD}$ were unchanged by NRAGE knockdown (data not shown), ruling out the latter and 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 (Figure 2.6A). 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 protein's C-terminus (amino acid 834) and Figure 2.6B 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 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 (Figure 2.6B). Examination of the primary sequence in 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). Figure 2.6C shows that the G722P substitution had no effect on the NRAGE-NEDD9 interaction whereas the G744P mutation abolished the interaction between the two proteins (Figure 2.6C). Together, these data indicate that the helix-loop-helix present in NEDD9 is required to bind NRAGE interaction and that the second helix of this domain plays a crucial role in their association.

To determine whether the NRAGE-NEDD9 interaction occurred in mammalian cells and to identify the domain in NRAGE that bound 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 Figure 2.7A), in HEK293T cells, and performed pull downs. Figure 2.7B shows that full-length NRAGE showed robust binding to the GST-NEDD9 fusion protein but not to GST alone. 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. 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 siRNA targeting NEDD9 to reduce its levels in COS7 cells (Figure 2.8A). 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 $p75NTR^{ICD}$ (Figure 2.8B and C). We conclude that NEDD9 is a downstream effector of a p75NTR-NRAGE pathway that mediates the cell spreading phenotype.

Finally, to determine if p75NTR activates Rac1 via a NRAGE- and NEDD9-dependent pathway, we assessed Rac1 activity in COS7 cells using the FRET biosensor described by Hodgson and colleagues (2010). Figure 8D shows that p75NTR overexpression produces a robust increase in Rac1 activity and significantly, the p75NTR-dependent increase was blocked when NRAGE or NEDD9 were depleted using siRNA (Figure 2.8D). Together, these findings demonstrate that NRAGE and NEDD9 function downstream of p75NTR to activate Rac1.

2.5 DISCUSSION

Here, we show that p75NTR induces cell spreading through activation of the small GTPase, Rac1. We demonstrate that p75NTR must be cleaved by a proteolytic process involving ADAM17 and γ-secretase for this effect to be manifest, 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.

Regulated intramembrane proteolysis (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 then releases the $p75NTR^{ICD}$ into the cytosol (Kanning et al, 2003, Zampieri et al, 2005). Our data demonstrates that ADAM17 is responsible for initial cleavage of p75NTR in COS7 cells and shows that this is a prerequisite for subsequent γ-secretase dependent proteolysis and release of the $p75NTR^{ICD}$. Since 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 NGF-dependent TrkA 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 MAG-induced growth cone collapse (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 may 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 p75NTR and induce its cleavage in this setting. However, we found 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 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/sin, and HEPL. 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, Baqiran 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 negative regulation of E-cadherin/β-catenin and in EMT. Since 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 if 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 guanine exchange factor 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 NEDD9 and activate Rac1-dependent melanoma migration (Sanz-Moreno et al, 2008).

In conclusion, we have used a novel cell-based assay to dissect signaling events lying downstream of p75NTR. We show that generation of the $p75NTR^{ICD}$ plays a critical 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.

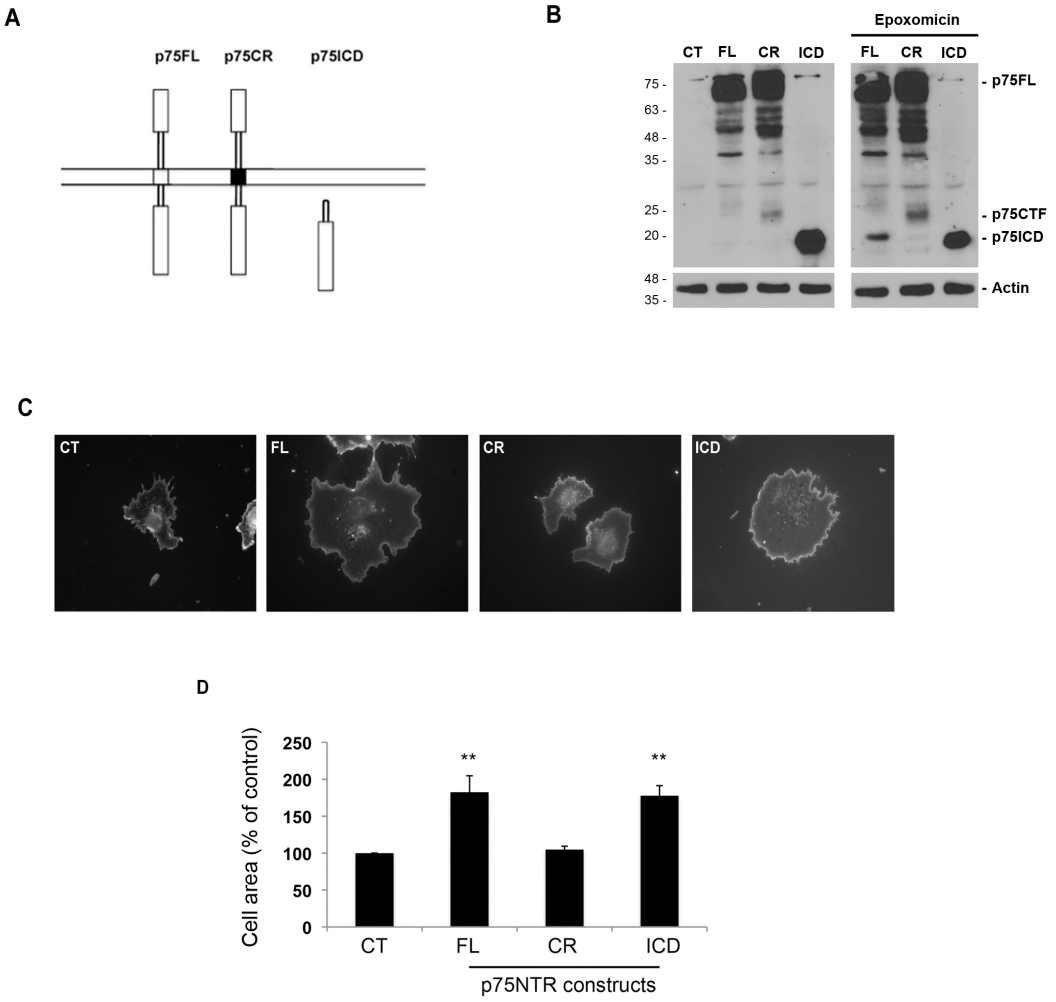

Figure 2.1. p75NTR^{ICD} mediates COS7 cell spreading.
A. Schematic showing the different p75NTR constructs overexpressed in COS7 cells: p75 full length (p75FL), p75 cleavage resistant (p75CR) in which the transmembrane domain (shown in black) is replaced by the transmembrane domain of Fas that is unable 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). D. Quantification of spreading assay showing a significant increase in cell area in p75FL and p75ICD expressing cells compared to control (CT) and p75CR cells. Values are expressed as average percent change in cell area compared to controls ± s.e.m for n=3 independent experiments. ** indicate $p<0.01$.

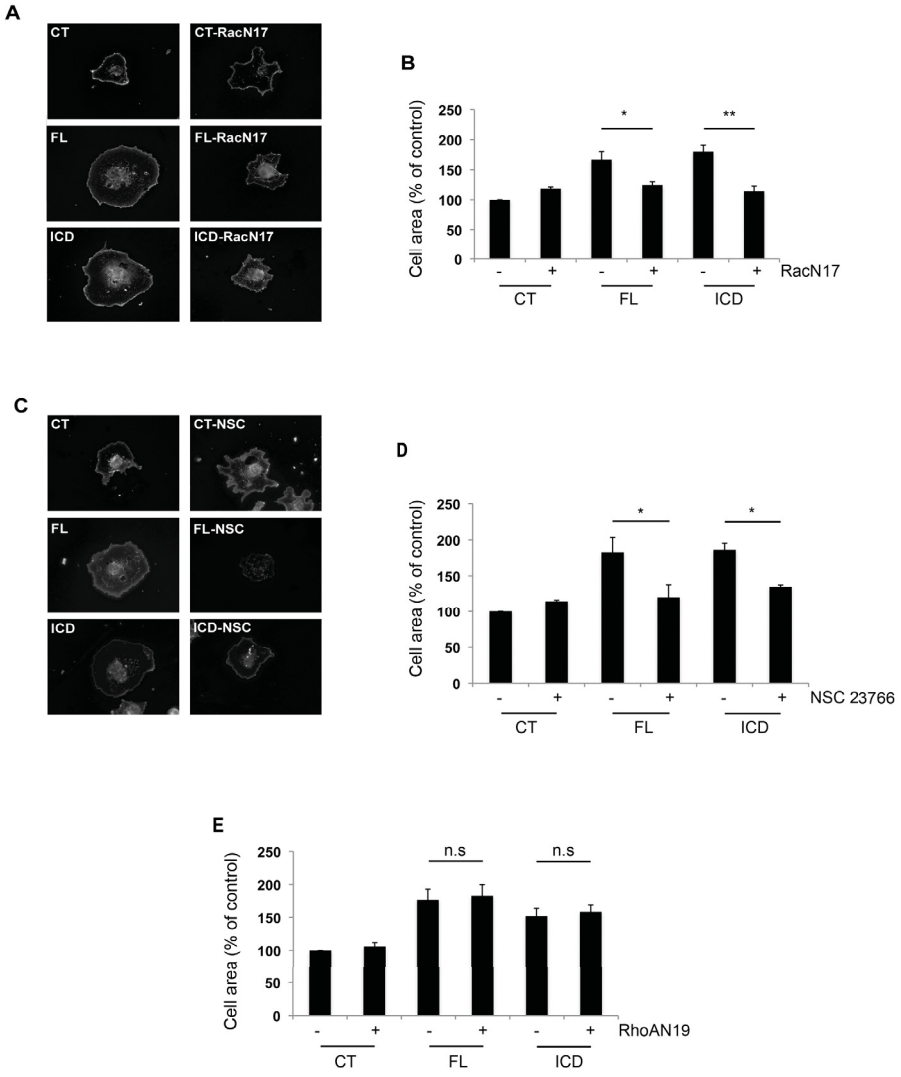

Figure 2.2. p75NTR acts through Rac1 to induce cell spreading.
A. Representative spreading assay of COS7 cells overexpressing EGFP (CT), p75FL, or p75ICD with or without dominant negative Rac (RacN17). B. Quantification of spreading assay showing a significant decrease in cell area of p75FL and p75ICD cells after overexpressing RacN17 but not in CT cells. Values are expressed as average percent change in cell area compared to controls ± s.e.m for n=5 independent experiments. C. Representative spreading assay of COS7 cells expressing EGFP (CT), p75FL, or p75ICD treated or not with the Rac inhibitor, NSC 23766, for 6 hours. D. Quantification of spreading assay showing a significant decrease in cell area of p75FL and p75ICD cells treated with NSC 23766 but not in CT cells. Values are expressed as average percent change in cell area compared to controls ± s.e.m for n=3 independent experiments. E. Quantification of spreading assay in cells overexpressing p75FL and ICD with or without dominant negative RhoA (RhoAN19) showing no effect on cell spreading. Values are expressed as average percent change in cell area compared to controls ± s.e.m for n=2 independent experiments. * and ** indicate $p<0.05$ and $p<0.01$ respectively.

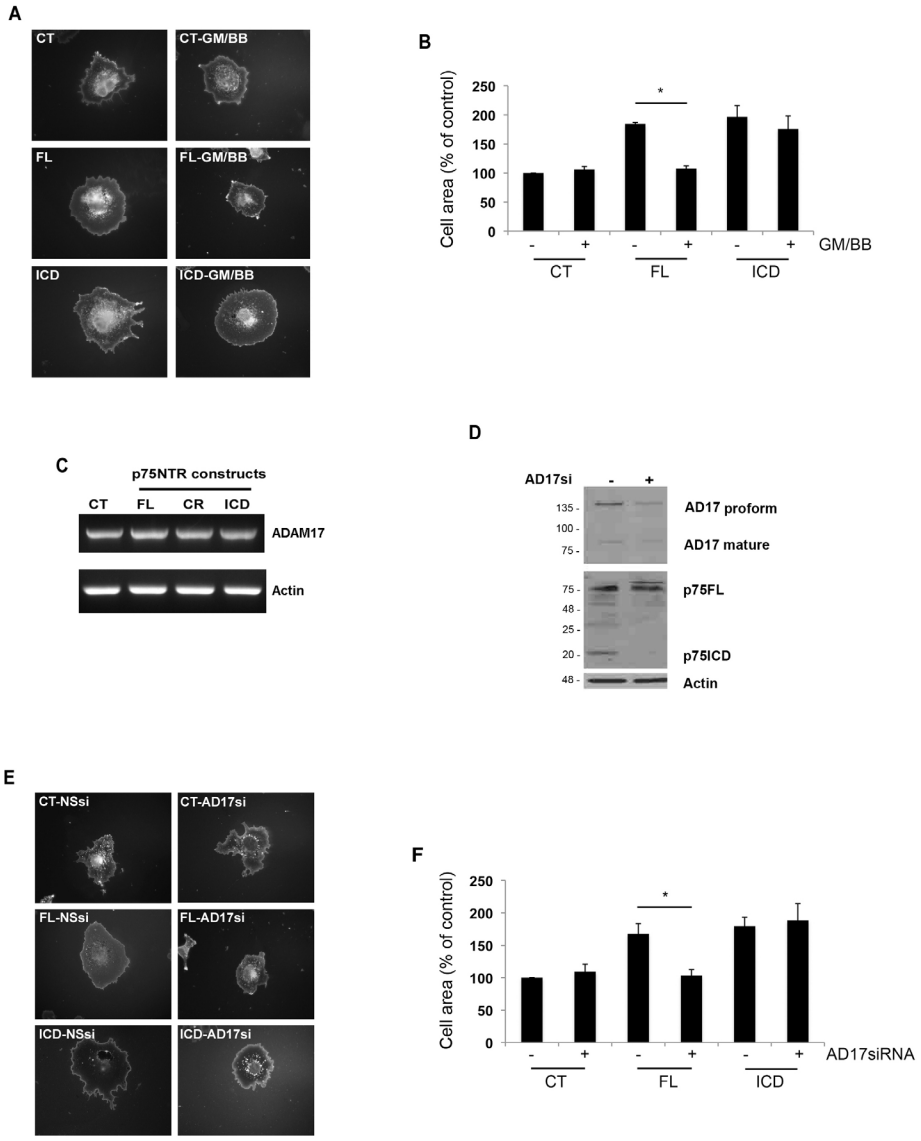

Figure 2.3. p75NTR cleavage by ADAM17 is necessary for cell spreading.
A. Representative spreading assay of COS7 cells overexpressing EGFP (CT), p75FL and p75ICD treated or not with the broad matrix metalloprotease inhibitors GM6001 and BB94 for one hour. B. Quantification of spreading assay showing a significant decrease in cell area of p75FL cells when treated with GM6001 and BB94, but not in p75ICD cells or CT cells. Values are expressed as average percent change in cell area compared to controls ± s.e.m for n=3 independent experiments. C. rtPCR of ADAM17 mRNA expression in control COS7 cells or cells overexpressing the different p75NTR constructs. D. Western blot showing a decrease in ADAM17 expression in COS7 cells expressing ADAM17 siRNA as well as a reduction in p75ICD generation upon ADAM17 knockdown. E. 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. F. Quantification of spreading assay showing a significant decrease in cell area of p75FL cells after ADAM17 knockdown, but not in p75ICD cells or CT cells. Values are expressed as average percent change in cell area compared to controls ± s.e.m for n=3 independent experiments. * indicates $p<0.05$.

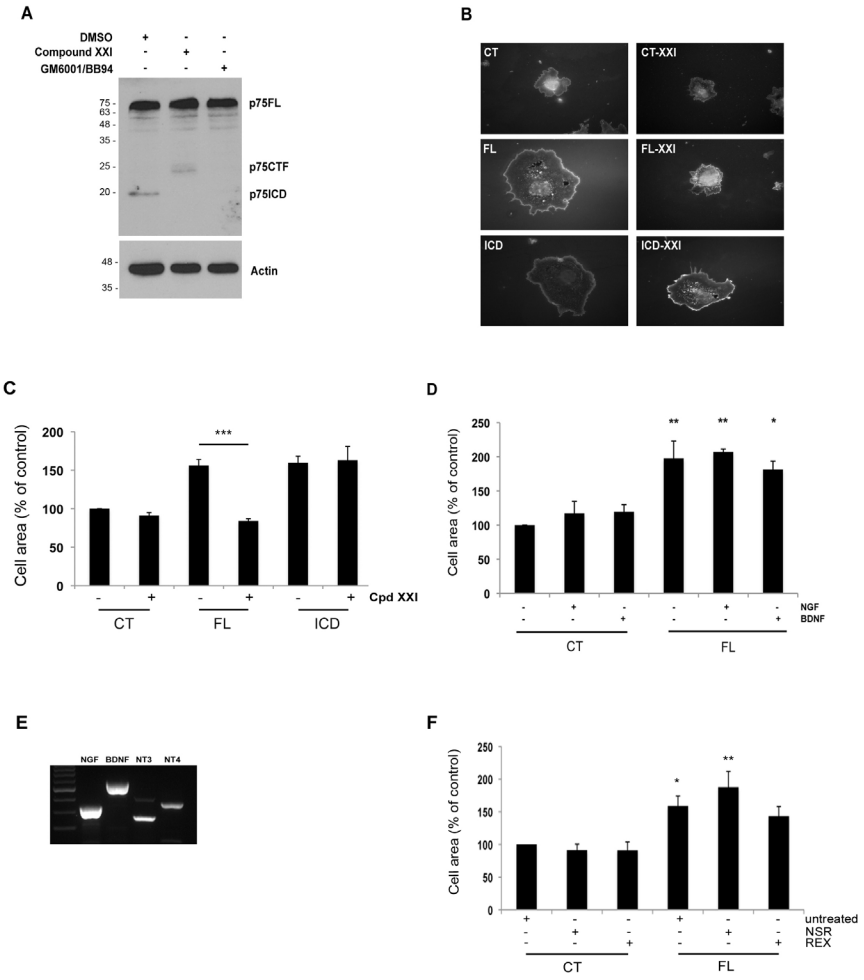

Figure 2.4. p75NTR is cleaved by γ-secretase to induce cell spreading.
A. Western blot showing the inhibition of $p75NTR^{ICD}$ generation but not CTF in COS7 cells when treated with compound XXI (lane 2) and inhibition of $p75NTR^{ICD}$ and CTF generation in cells treated with GM6001 and BB94 (lane 3). DMSO was used as vehicle control. B. Representative spreading assay of COS7 cells overexpressing EGFP (CT), p75FL, or p75ICD treated or not with the γ-secretase inhibitor, compound XXI, for one hour. C. Quantification of spreading assay showing a significant decrease in cell area of p75FL cells when treated with compound XXI, but not in p75ICD cells or CT cells. Values are expressed as average percent change in cell area compared to controls ± s.e.m for n=3 independent experiments. D. Representative spreading assay of COS7 cells overexpressing EGFP (CT), p75FL, with or without NGF or BDNF (25 ng/ml) for 24 hours. E. rtPCR 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. In C, D, and F values are expressed as average percent change in cell area compared to controls ± s.e.m for n=3 independent experiments. *, ** and *** indicate $p<0.05$, $p<0.01$ and $p<0.001$ respectively.

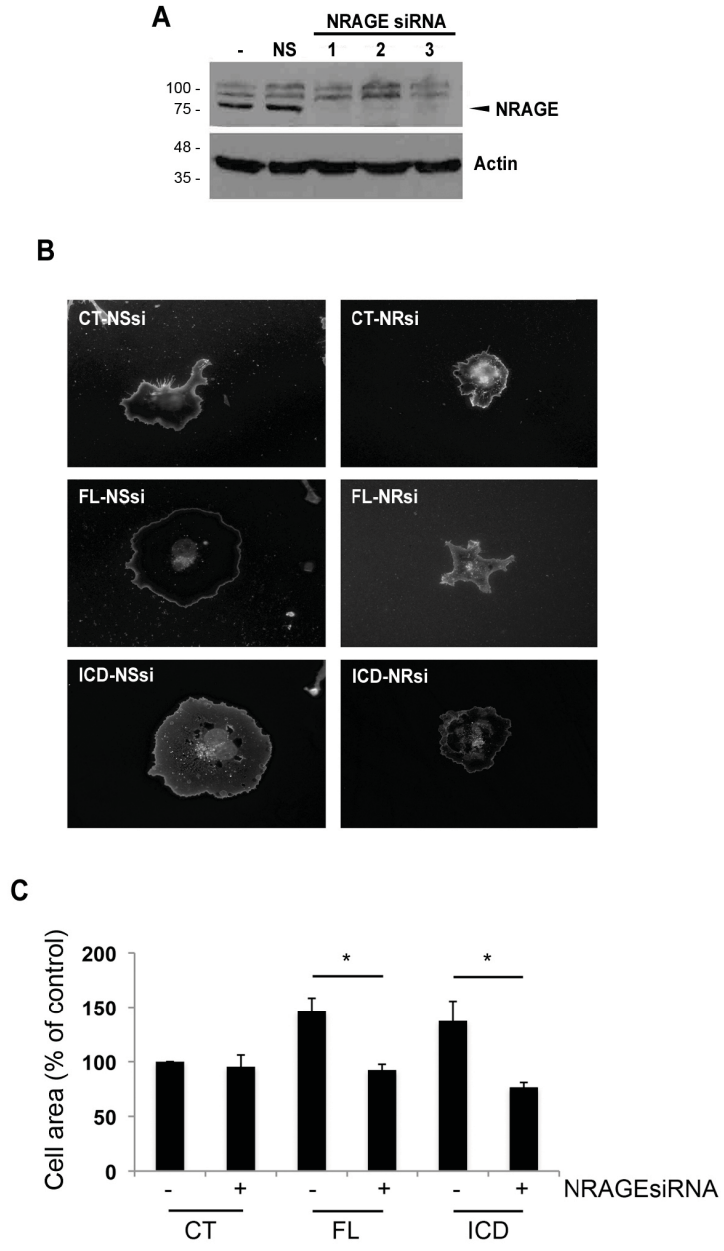

Figure 2.5. NRAGE acts downstream of p75NTR in cell spreading.
A. Western blot showing NRAGE knockdown in COS7 cells using three different siRNAs. Only NRAGE siRNA 1 was used in subsequent experiments. B. Representative spreading assay of COS7 cells overexpressing EGFP (CT), p75FL, or p75ICD with or without NRAGE siRNA. Non specific siRNA (NSsi) was used as control. C. Quantification of spreading assay showing a significant decrease in cell area of p75FL and p75ICD cells after NRAGE depletion but not in CT cells. Values are expressed as average percent change in cell area compared to controls ± s.e.m for n=3 independent experiments. * indicates $p<0.05$.

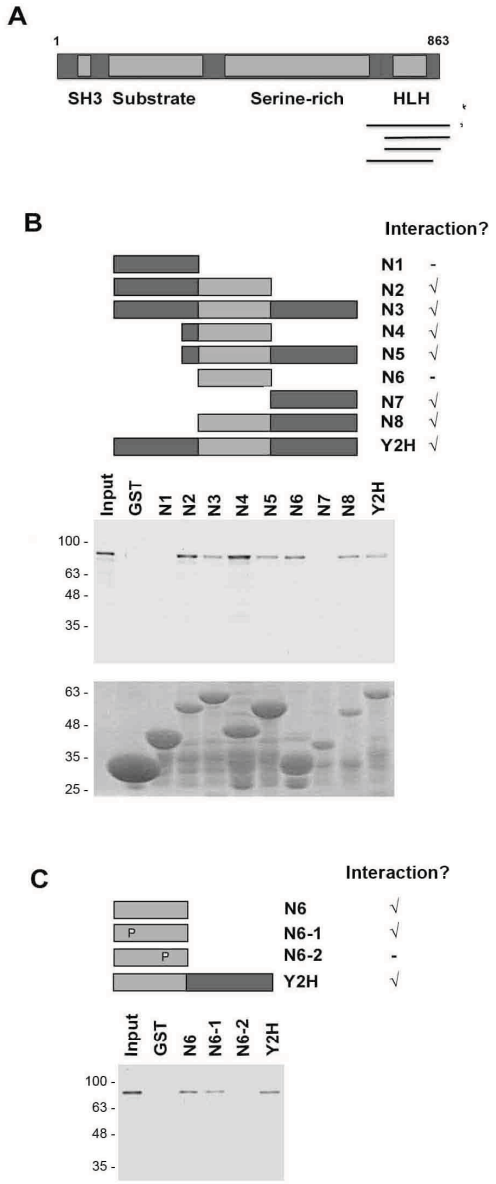

Figure 2.6. NRAGE interacts with the C-terminal domain of NEDD9.
A. Schematic showing the structure of NEDD9, which contains an SH3 domain, a substrate binding domain, a serine-rich domain, and a helix loop helix (HLH) domain. The 4 clones identified have overlapping regions of the HLH spanning from aa 637-834. B. Different GST-NEDD9 fusion proteins containing the 198 aa region were generated and their interaction with NRAGE produced in an *in vitro* translation assay 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 expression of the different GST-NEDD9 mutants. C. Introduction of two proline residues in helix 1 and helix 2 of the NEDD9 HLH identifies the helix2 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 NRAGE immunoblot.

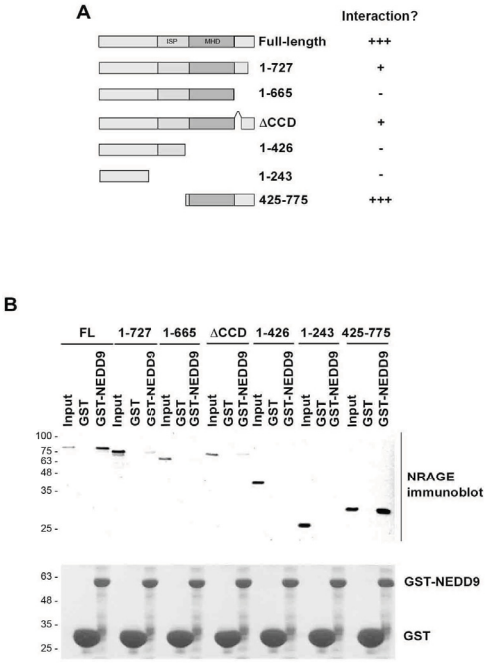

Figure 2.7. NEDD9 interacts with the C-terminal domain of NRAGE.
A. Schematic showing the different NRAGE deletion mutants that were generated. ISP = interspersed repeat domain; MHD = Mage homology domain. B. The interaction of NRAGE fragments with GST-NEDD9 fusions was detected using NRAGE immunoblot. The lower panel shows a Coomassie blue stained gel that confirms expression of GST and GST-NEDD9.

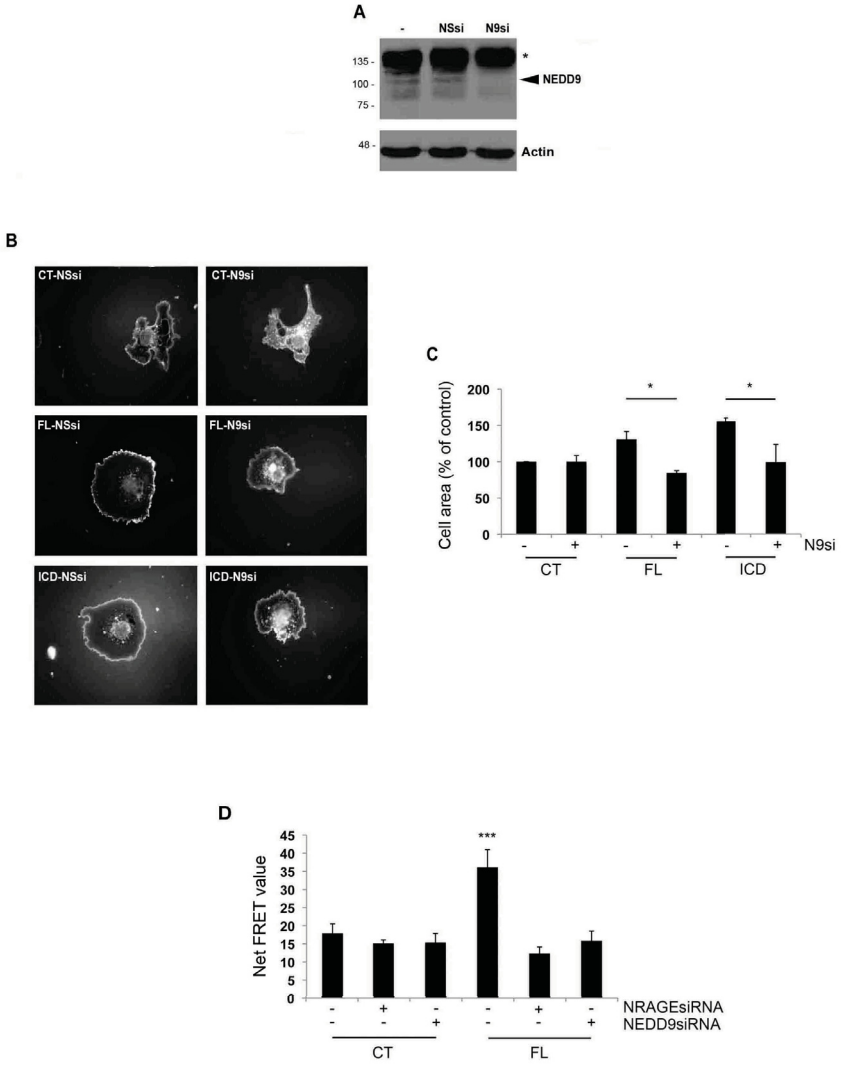

Figure 2.8. NEDD9 acts downstream of p75NTR in cell spreading.
A. Western blot showing knockdown of NEDD9 in COS7 cells with NEDD9 siRNA. The prominent band above NEDD9 and that is marked with an asterisk represents a background band. B. Representative spreading assay of COS7 cells overexpressing EGFP (CT), p75FL, or p75ICD with or without NEDD9 siRNA. Non specific siRNA (NSsi) was used as control. C. Quantification of spreading assay showing a significant decrease in cell area of p75FL and p75ICD cells after NEDD9 depletion but not in CT cells. Values are expressed as average percent change in cell area compared to controls ± s.e.m for n=3 independent experiments. D. Rac1 activation assay done by FRET analysis in COS7 cells overexpressing CT or p75FL with or without NRAGE or NEDD9 siRNA. Results show that NRAGE or NEDD9 depletion induce a significant decrease in Rac1 activity in cells overexpressing p75FL but not in CT cells. Values are represented as average of normalized FRET signal ± s.e.m for 3 independent experiments. * and ** indicate $p<0.05$ and $p<0.01$ respectively.

PREFACE TO CHAPTER 3

Rho GTPases are key regulators of cytoskeletal dynamics and contribute to many different physiological events. One of the major problems in measuring activity of Rho GTPases is their low dynamic range of activation as well as their subcellular distribution. In fact, even when activated with a physiological stimulus only 5% of the total pool is activated although this generates a robust phenotype. Methods available for measuring Rho GTPases activation do not take into account the subtle changes in activity of GTPases and underestimate the importance of their subcellular distribution. In particular, the lack of understanding of the regulation of Rho GTPases by p75NTR is mainly due to the absence of a robust assay that allows the study of this signaling pathway. In this chapter we describe a quantification method based on FRET using Rho biosensors designed by Klaus Hahn's lab (Hodgson et al, 2010). Using this method we show that p75NTR increases Rac1 activation. p75NTR does not significantly affect RhoA activity although there is a trend towards a decrease in RhoA activity in COS7 cells. We also examined the effect of NogoR1 and myelin inhibitory proteins on p75NTR-dependent small GTPase activation using this assay and found small shifts in activity that did not pass tests of statistical significance.

CHAPTER 3

A FRET BIOASSAY TO MEASURE THE ACTIVATION OF RHO GTPASES DOWSNTREAM OF P75NTR

3.1 ABSTRACT

The p75NTR fulfills an array of physiological functions ranging from cell death, to survival, differentiation, neurite outgrowth, and cell migration. Rho GTPases play an important role downstream of p75NTR in mediating different signaling pathways such as death of oligodendrocytes, neurite retraction in response to myelin inhibitory proteins (MIBs), and cell migration. However, it is not clear how p75NTR regulates Rho GTPases and how the presence of coreceptors switches this response. This is in part due to the lack of a robust assay that allows the measurement of slight changes in Rho GTPases activity as well as their subcellular distribution. In this work we used the recently designed Rho biosensors that permit the measurement of Rho GTPases activity through FRET. COS7 cells transfected with p75NTR and Rac1 or RhoA biosensors were plated on laminin coated glass coverslips. After fixation they were imaged and FRET activity of Rac1 and RhoA was assessed. Our results show that p75NTR leads to an increase in Rac1 activation whereas it does not induce a statistically significant change in RhoA activity.. Exogenous addition of neurotrophins did not change the response of Rho GTPases to p75NTR. However, in the presence of NgR1 and the MIB, Nogo66, RhoA and Rac1 levels are unaltered and are similar to control levels. We conclude that the role of p75NTR on Rho GTPases activation depends on the coreceptors expressed as well as the presence of ligands. This assay represents a useful method to investigate the mechanism of Rho GTPases activation by p75NTR.

3.2 INTRODUCTION

The p75 neurotrophin receptor (p75NTR) is the founding member of the tumor necrosis factor superfamily (TNFR). It is associated with a multitude of functions such as cell death, survival, neurite outgrowth, differentiation, and cell migration, and it also contributes to many pathological conditions. p75NTR binds all neurotrophins with equal low affinity. In addition, it can also bind neurotrophin precursors through which it induces cell death. Many of the functions of p75NTR have been attributed to its interaction with downstream players that link p75NTR to different signaling pathways (Roux and Barker, 2002, Schor, 2005, Reichardt, 2006).

Rho GTPases belong to the family of small GTPases that involve RhoA, Rac, and Cdc42. They switch between a GDP inactive form and a GTP active form. Their activation is controlled by guanine exchange factors (GEFs), GTPase activating proteins (GAPs), and guanine dissociation inhibitors (GDIs). Rho GTPases transduce signals downstream of growth factor receptors to the actin cytoskeleton affecting cytoskeletal dynamics (Burridge and Wennerberg, 2004). p75NTR has been shown to activate RhoA and Rac1 in a variety of contexts to produce a wide range of cellular outcomes. For example, a complex of p75NTR and the Nogo receptor (NgR) induces RhoA activation in response to myelin inhibitory proteins (MIBs) (Yamashita et al, 1999, Yamashita and Tohyama, 2003, Park et al, 2010), whereas p75NTR activated by proBDNF mediates axonal retraction through inhibition of Rac1 (Deinhardt et al, 2011). p75NTR activation decreases RhoA activity in glioma cells and thereby promotes cell migration (Johnston et al, 2007) and p75NTR-dependent Rac1 activation induces oligodendrocyte cell death (Harrington et al, 2002). We have recently shown that upon cleavage and release of its intracellular domain, p75NTR activates Rac1 in COS7 cells (Zeinieh et al, In Press, chapter 2). Thus, p75NTR can induce the activation or suppression of Rac1 or RhoA in different cellular contexts.

How p75NTR switches the balance from RhoA to Rac1 to mediate different signaling mechanisms is not very well understood. This is at least partially due to the lack of a robust technical assay that allows us to define subtle changes in the activation of Rho GTPases. Most techniques assess total levels of active GTPases, and hence underestimate small changes in their activation as well as the cell distribution of active GTPases. In order to better understand how p75NTR regulates Rho GTPases signaling in the presence or absence of MIBs, we took advantage of RhoA and Rac1 biosensors developed by Klaus Hahn's group that measure Rho GTPase activity *in vivo*, via Fluorescence Resonance Energy Transfer (FRET) (Hodgson et al,

2010). With this approach, we demonstrate that p75NTR activates Rac1 and that p75NTR overexpression produces a trend decrease in RhoA activity that did not achieve statistical significance. We also find that the regulation of Rho GTPases by p75NTR is not regulated by neurotrophins in COS7 cells. However, in the presence of NgR1 and MIBs such as Nogo66, RhoA and Rac1 levels are unchanged and are similar to control levels. Hence, the role of p75NTR on Rho GTPases activation depends on the coreceptors expressed as well as the presence of ligands.

3.3 MATERIAL AND METHODS

Reagents and plasmids: The polyclonal antibody directed against p75NTR was described previously (Barker et al, 1994, Salehi et al, 2000), antibody against the Nogo receptor was purchased from R&D systems (AF1440, Minneapolis, MN, USA). Anti-LGI1 antibody was obtained from Santa Cruz Biotechnology (SC-9583, Dallas, Texas, USA). The monoclonal antibody against β-actin was purchased from MP Biomedicals (691001, Irvine, CA, USA). Laminin was purchased from BD Bioscience (Mississauga, ON, CA). Poly-*D*-lysine (PDL) was obtained from Sigma (Oakville, ON, CA). Horseradish-peroxidase conjugated secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA, USA). All cell culture reagents were from Wisent Bioproducts (Saint Bruno, QC, CA). Dako anti-fading mounting medium was purchased from Cedarlane laboratories Ltd. (Burlington, ON, CA). Glutathione plasmids encoding full-length p75NTR and the $p75NTR^{ICD}$ were described previously (Kommaddi et al, 2011). Plasmid encoding the NogoR was described previously (Thomas et al, 2010). Plasmid encoding AP-Nogo66 was obtained from Juha Lauren's lab at Yale University and was described previously (Lauren et al, 2007). NGF and BDNF were obtained from Alomone laboratories (Jerusalem, Israel). RhoA biosensor (pTriEx-RhoA FLARE.sc Biosensor (WT)) and Rac1 biosensors composed of two different chains (pYPet-PBD and pCyPet-Rac1 (WT)) (Hodgson et al, 2010) were obtained from Addgene (Cambridge, USA).

Cell culture and transfection: COS7 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, in 5% CO2 at 37°C. COS7 cells were plated on six well plates at a density of 25000 cells/ml. Twenty four hours after plating cells were left untransfected or transfected with plasmid encoding p75 full length (p75NTR) (2 ug). RhoA or Rac1 biosensors (2 ug) were co-transfected in the cells using the calcium phosphate transfection method. In the case of Rac1 biosensors, each chain was also transfected independently to correct for potential fluoresence bleed-through in the analysis. For experiments involving NgR1 and Nogo66, COS7 cells were co-transfected with p75NTR and the biosensors with the different plasmids encoding NgR1 or AP-Nogo66 (2 ug of each plasmid). Cells transfected with the different constructs were maintained for 48 hours at 37°C before being replated on coverslips for FRET or being lysed in 2x Laemmli sample buffer for immunoblot analysis.

Plating of cells for FRET analysis: Glass coverslips (12mm, Fisherbrand, Fisher Scientific, Ottawa, ON, CA) were coated for 30 minutes with PDL (0.5 ug/ml), washed with sterile water, coated with laminin (0.5 ug/ml) for two hours at 37°C, and again washed with sterile water. COS7 cells transfected 48 hours earlier were plated on coverslips (10000 cells/slip) then incubated at 37°C/5% CO2 for twenty-four hours. Media was then removed and replaced with 4% paraformaldehyde in PBS for 15 minutes at 37°C. Cells were washed with PBS (2 x 5 minutes), and sterile water (1 x 5 minutes), coverslips were mounted in anti-fading mounting media (Dako), and kept at 4°C until imaging was performed. For some experiments, neurotrophins (NGF or BDNF (25 ng/ml)) were added to the media for one hour prior to COS7 cell fixation.

FRET imaging and analysis: Imaging was performed using a Zeiss Axioskop fluorescent inverted microscope equipped with Xenon illumination. Images were obtained using a 40X objective and captured using Zeiss Zen software. For emission ratio imaging, the following filter sets (Chroma Technology) were used (for excitation and emission, respectively): CFP: 430/24 (ex), 470/20 (em); FRET: 430/24 (ex), 535/30 (em); YFP: 500/20 (ex), 540/40 (em). In each case, images of the CFP and FRET channels were captured using the same exposure times. For the single chain RhoA biosensor, FRET intensity was calculated as FRET/CFP using a macro designed in the lab for the NIH ImageJ program. FRET analysis of the Rac biosensor was done using the PixFRET plugin in NIH ImageJ software. This routine subtracts bleed-through intensities from each chain (i.e the donor (CyPet-Rac1) and the acceptor (YPet-PBD)) and calculates the net FRET (NFRET) value according to the following formula:

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

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

Statistical analysis: Statistical analyses were performed using unpaired Student's t test or one-way ANOVA with Tukey post-hoc analysis. All values are expressed as mean +/- s.e.m.

3.4 RESULTS

p75NTR alters Rac1 activity

We have previously shown that p75NTR overexpression in COS7 cells increases Rac1 activity and that this requires p75NTR cleavage and the release of the p75 intracellular domain ($p75^{ICD}$) (Zeinieh et al, In Press, chapter 2). Previous data also demonstrate that p75NTR increases RhoA in response to MIBs (Yamashita et al, 2002, Domeniconi et al, 2005, Harrington et al, 2008, Park et al, 2010, Sun et al, 2012). To gain insight on how p75NTR differentially regulates Rac1 and RhoA under physiological conditions, we determined the activity of Rac1 and RhoA in COS7 cells overexpressing wild type p75, or left untransfected, using Rho GTPase biosensors (Hodgson et al, 2010). The Rac1 biosensor system contains two distinct peptides. One is comprised of Rac1 fused to CFP (Rac1-CyPet) and the other contains a Pak binding domain bound to YFP (PBD-YPet). The RhoA biosensor consists of a single chain that, from N-terminus to C-terminus, consists of i) the RhoA-binding domain of Rhotekin, ECFP, ii) an unstructured linker, iii) Citrine-YFP, and iv) RhoA. Cells transfected with p75NTR and Rac1 or RhoA biosensors were re-plated forty-eight hours after transfection on coverslips precoated with laminin then fixed twenty-four hours later. Cells were imaged and quantified through PixFRET to measure Rac1 activity or through calculation of FRET/CFP ratio for RhoA activity using ImageJ software. Our results show that p75NTR overexpression leads to a significant increase in Rac1 activity as shown in fig 3.1A and B. Our results for RhoA indicate that p75NTR does not change RhoA activity although our results show a trend towards a decrease in RhoA activity that did not achieve statistical significance as shown by the pool of five independent experiments (Fig 3.1E). As an example, one representative experiment is illustrated in fig 3.1D. Interestingly, we also observe a difference in the localization of the activated GTPases as shown by the gradient of activation using the heatmaps representation. Our results show that p75NTR increases the activation of Rac1 at the cell membrane where the intensity is maximal (Fig 3.1A). However, the RhoA activity is minimal in the p75NTR overexpressing cells compared to control cells and the maximum intensity of RhoA activity is detected at the cell membrane of control untransfected cells (Fig 3.1C).

Exogenously expressed neurotrophins do not alter the effect of p75NTR on Rac1 and RhoA activation

Previous studies showed that the effect of p75NTR on RhoA activity depends on neurotrophins and that NGF and BDNF decrease RhoA activity and promote axonal outgrowth in cerebellar granule neurons (CGNs), dorsal root ganglia neurons (DRGs) and retinal ganglion cells (RGCs), whereas proBDNF activates RhoA and inhibits outgrowth of DRGs and cortical neurons (Yamashita et al, 1999, Gehler et al, 2004a and b, Park et al, 2010, Sun et al, 2012). We recently showed that COS7 cells express high levels of all 4 neurotrophins (NGF, BDNF, NT-3, and NT-4) and that the role of p75NTR on COS7 cell spreading is not affected by the presence of exogenous neurotrophins, denoting that the role of p75NTR in cell spreading is ligand independent (Zeinieh et al, In Press, chapter 2). We asked whether activation of GTPases by p75NTR is affected by exogenous neurotrophins. For this purpose, COS7 cells overexpressing p75NTR with Rac1 or RhoA biosensors were plated forty-eight hours after transfection on laminin coated coverslips. Twenty-four hours after plating cells were treated with NGF or BDNF (25 ng/ml) one hour prior to fixation. Cells were imaged and Rac1 and RhoA FRET activities were measured using ImageJ software as described above. Our results show that addition of exogenous neurotrophins does not affect the response of Rho GTPases to p75NTR (Fig 3.2A-B).

Effects of Nogo receptor and Nogo66 on p75NTR-dependent Rac1 and RhoA activity

Previous work has attributed an essential role for the p75NTR/NgR complex in axonal retraction in the CNS in response to MIBs through RhoA activation (Yamashita et al, 2002, Domeniconi et al, 2005, Harrington et al, 2008, Park et al, 2010, Sun et al, 2012). In contrast, p75NTR can also mediate axonal retraction of hippocampal neurons through inactivation of Rac1 (Deinhardt et al, 2011). Thus we asked whether the regulation of RhoA and Rac1 by p75NTR is altered by coexpression of NgR1 and MIBs such as Nogo66, using Rac1 and RhoA biosensors and measured their activity using FRET. For this purpose, COS7 cells were transfected with p75NTR alone, NgR1 alone, p75NTR and NgR1 with or without Nogo66 (Fig 3.3A). In the case of RhoA activity, the RhoA biosensor was coexpressed with the abovementioned plasmids, whereas Rac1 biosensors were coexpressed for the analysis of Rac1 activity. Forty-eight hours after transfection, cells were plated on laminin coated glass coverslips and were fixed twenty-four hours later. Cells were then imaged and RhoA and Rac1 activity were measured using ImageJ software as described above. For each biosensor a representative example is shown as well as the pool of five independent experiments. Our

results demonstrate that in the presence of p75NTR there is a trend towards a decrease in RhoA activity although the results did not achieve significance for five independent experiments. In the presence of Nogo66 alone, RhoA activity is similar to controls. Similarly, NgR1 expressed alone does not affect RhoA activity (Fig 3.3B-C).

We also assessed whether the presence of NgR1 or Nogo66 affects p75NTR-induced Rac1 activation. Our results demonstrate that Rac1 shows a trend towards an increase in the presence of p75NTR; in the presence of NgR1, or NgR1 and Nogo66, Rac1 levels are not different from controls (Fig 3.3D-E).

3.5 DISCUSSION

This study was designed to develop tools that can be used to decipher signaling events that contribute to p75NTR-dependent Rho GTPases activation. Using Rac1 and RhoA biosensors, we demonstrate that when overexpressed in COS7 cells, p75NTR activates Rac1 but does not alter RhoA activation although there is a trend towards a decrease in RhoA activity; whereas RhoA and Rac1 levels are unchanged when p75NTR is coexpressed with NgR1 and Nogo66. Our study shows that p75NTR differentially regulates RhoA and Rac1 in the presence or absence of coreceptors such as NgR1 as well as MIBs.

The regulation of Rho GTPases by p75NTR is widely studied in many models. p75NTR decreases RhoA in U87MG cells allowing cell migration (Johnston et al, 2007), whereas p75NTR increases Rac1 in oligodendrocytes to mediate JNK dependent death (Harrington et al, 2002). We also recently demonstrated that p75NTR increases Rac1 activity and leads to spreading of COS7 cells (Zeinieh et al, In Press, chapter 2). Our results show that p75NTR decreases RhoA and activates Rac1. However, it appears that this regulation depends highly on the expression of coreceptors. In fact, association of p75NTR with the NgR allows the activation of RhoA in response to MIBs (Yamashita et al, 1999, Yamashita et al, 2002, Domeniconi et al, 2005, Park et al, 2010, Sun et al, 2012). Similarly, a recent study reported that EphrinB3-mediated axonal growth inhibition requires activation of RhoA through p75NTR and *p75ntr-/-* neurons do not respond to ephrinB3 (Uesugi et al, 2013). However, association of p75NTR with SorCS2 leads to inactivation of Rac1 and neurite retraction of hippocampal neurons in response to proNGF (Deinhardt et al, 2011).

In this work we show that coexpression of p75NTR and NgR1 does not alter RhoA levels in response to Nogo66 and levels are kept similar to controls. Similarly, Nogo66 does not affect Rac1 levels. It appears that the addition of Nogo66 abolishes the effect of p75NTR on Rho GTPases. Previous studies have shown that Nogo66 activates RhoA and inhibits Rac1 to induce neurite outgrowth inhibition (Niederöst et al, 2002, Mimura et al, 2006). In addition, it was demonstrated that MIBs act on a complex composed of p75NTR, NgR1 and LINGO-1, a Slit homolog present in the nervous system. Oligodendrocyte myelin glycoprotein (OMgp) increases RhoA in COS7 cells only in the presence of this tripartite complex but not when p75NTR and NgR1 are expressed alone (Mi et al, 2004), which is in agreement with our findings. Similarly, AMIGO-3, another LINGO-1 family member was shown to interact with p75NTR/NgR receptor complex and to induce neurite growth inhibition through activation of RhoA (Ahmed et al, 2013). Thus the lack of RhoA activation in our model is likely due to the absence of LINGO-1 or AMIGO-3, that are required for RhoA activation downstream of the

p75NTR/NgR1 complex. In addition, the difference between our findings and others could be attributed to the different model we used in our work compared to other studies that used neuronal cultures. Thus the response of these distinct culture systems could account for the divergence in the findings.

It was also shown that TROY, another TNFR member highly related to p75NTR, associates with the NgR and leads to axonal retraction through activation of RhoA in a RhoGDI dependent manner (Park et al, 2005, Shao et al, 2005, Lu et al, 2013). Using the Rho GTPases biosensors it is possible now to study the role of the different TNFRs in the regulation of Rho GTPases. This method would allow us to better understand how TNFRs affect Rho GTPases function in a spatiotemporal manner, such as the regulation of their GTPase activity as well as their subcellular localization in the cell.

Previous studies attributed an important role for neurotrophins in the regulation of Rho GTPases. In fact, NGF increases Rac1 activation in PC12 cells leading to its recruitment at the tip of differentiating neurites (Yasui et al, 2001). Binding of NGF to p75NTR in CGNs and DRG neurons blocks RhoA activation and induces axonal outgrowth (Yamashita et al, 1999). On the other hand, proBDNF inhibits DRGs and neonatal cortical neurons axonal outgrowth, whereas BDNF promotes it (Sun et al, 2012). BDNF inhibits RhoA in chick retinal neurons to promote growth cone extension through a p75NTR dependent mechanism (Gehler et al, 2004a). In our work, addition of neurotrophins did not affect RhoA and Rac1 activation compared to p75NTR alone. In fact, COS7 cells express all four neurotrophins (NGF, BDNF, NT-3, and NT-4) and we recently found that addition of neurotrophins does not affect spreading of COS7 cells, and we show that p75NTR-induced cell spreading is ligand-independent (Zeinieh et al, In Press, chapter 2). It is thus possible that the regulation of Rho GTPases by p75NTR is also ligand independent.

A major caveat in our work is that there is a discrepency between individual experiments compared to the pool of experiments in which the difference observed is not statistically significant. The lack of significance could be attributed to the variability between individual experiments that masks the difference observed in single runs. The variability could be attributed to the fact that the system used is not robust enough to detect small differences in GTPase activity that may be induced by p75NTR. We relied on transient transfection of COS7 cells and this could induce differences in the efficiency of transfection between runs. For this purpose it may be useful to establish stable cells lines that express the biosensors which would avoid variability in the transfection efficiency. Another problem in using this system is that COS7 cells do not express high basal levels of Rho GTPases, and this

results in very subtle changes that are hard to quantify. It would thus be essential to stimulate their expression by using stimulants such as EGF, LPA, bombesin, as was shown previously (Hall and Ridley, 1992, Ridley et al, 1992). This would trigger Rho GTPases activation and lead to a higher response that will be easy to quantify.

In conclusion, we applied in this work the method described by Hodgson and colleagues to study the regulation of Rho GTPases downstream of p75NTR. Our work confirms previously described data that p75NTR decreases RhoA activation and increases Rac1 activity under physiological conditions, and that this regulation is counterbalanced by the presence of MIBs. The results obtained for Rac1 activation in response to p75NTR are more robust than those for RhoA and confirm our previous findings (Zeinieh et al, In Press, chapter 2). Thus, this model serves as a good platform to study the signaling mechanisms downstream of p75NTR for the regulation of Rac1 activation. We and others have also shown the importance of the $p75^{ICD}$ in p75NTR signaling (Kenchappa et al, 2006, Kenchappa et al, 2010, Ceni et al, 2010), and essentially the role of the $p75^{ICD}$ in Rac1 activation (Zeinieh et al, In Press, chapter 2). Hence it will be interesting to use the Rho GTPases biosensors to study signaling downstream of the $p75^{ICD}$ and to look for potential interactors that link p75NTR to Rho GTPases activation. In contrast to Rac1, we were unable to find robust changes in RhoA activation using this model. Although the changes are small it will be hard for us to study mechanisms of RhoA activation downstream of p75NTR using this model. It will be essential to use another cell system that expresses high levels of RhoA or ameliorate our system as described above that would enable us to detect robust and significant changes in activity that will be easier to quantify.

The FRET bioassay we used in this work will provide us with a better understanding on how p75NTR regulates Rho GTPases and the implication of Rho GTPases regulators such as GEFs, GAPs and GDIs downstream of this receptor. It will also allow us to better understand the role of other TNFRs in Rho GTPases signaling. This method will give us more insight on the activation of these GTPases as well as on the changes in their distribution in the cell.

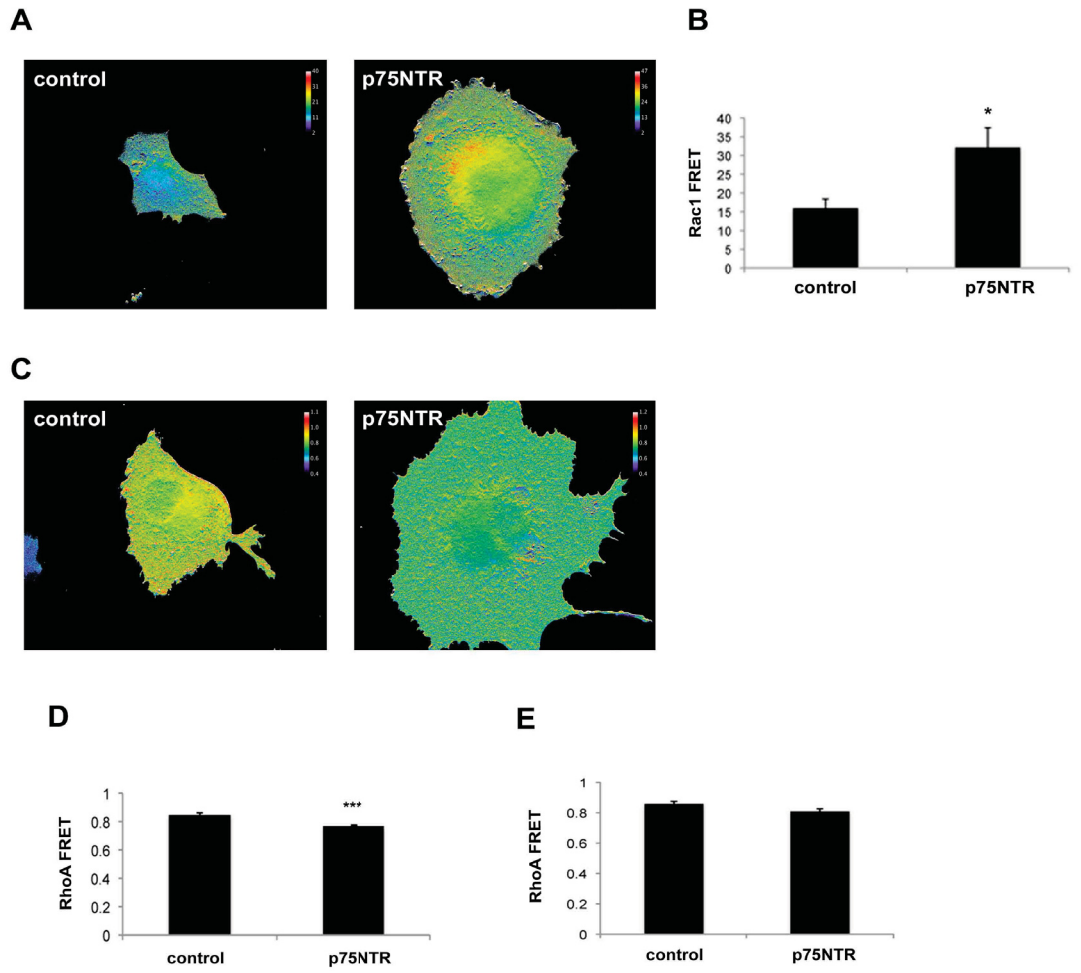

Figure 3.1. Effects of p75NTR on Rac1 and RhoA activity in COS7 cells.
A. Heatmaps showing the distribution of Rac1 activity in control and p75NTR overexpressing COS7 cells. B. Quantification of Rac1 FRET in control and p75NTR overexpressing cells showing a significant increase in Rac1 activation in p75NTR overexpressing cells. Results are expressed as means ± s.e.m for 5 different experiments. * indicates $p<0.05$. C. Heatmaps showing the distribution of RhoA activity in control and p75NTR overexpressing COS7 cells. D. Quantification of RhoA FRET in control and p75NTR overexpressing cells of one representative experiment. E. Quantification of RhoA FRET in control and p75NTR overexpressing cells of five independent experiments. Results are expressed as means ± s.e.m. *** indicates $p<0.001$. In each experiment an average of 25 cells were quantified.

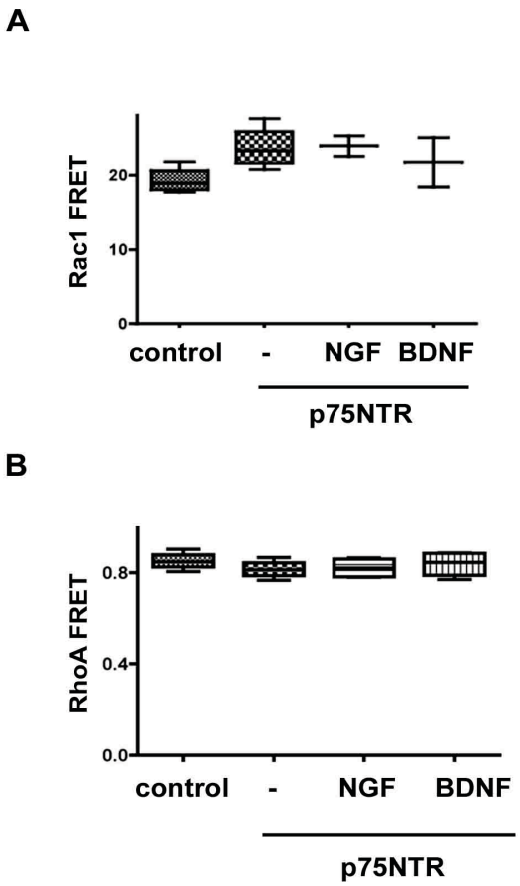

Figure 3.2. The effect of p75NTR on Rac1 and RhoA activation does not depend on neurotrophins.

A. Quantification of Rac1 FRET in control cells and p75NTR overexpressing cells with or without NGF or BDNF (25 ng/ml). NGF and BDNF were added one hour prior to fixation. B. Quantification of RhoA FRET in control cells and p75NTR overexpressing cells with or without NFG or BDNF. Results are expressed as means ± s.e.m for four independent experiments. In each experiment an average of 25 cells were quantified.

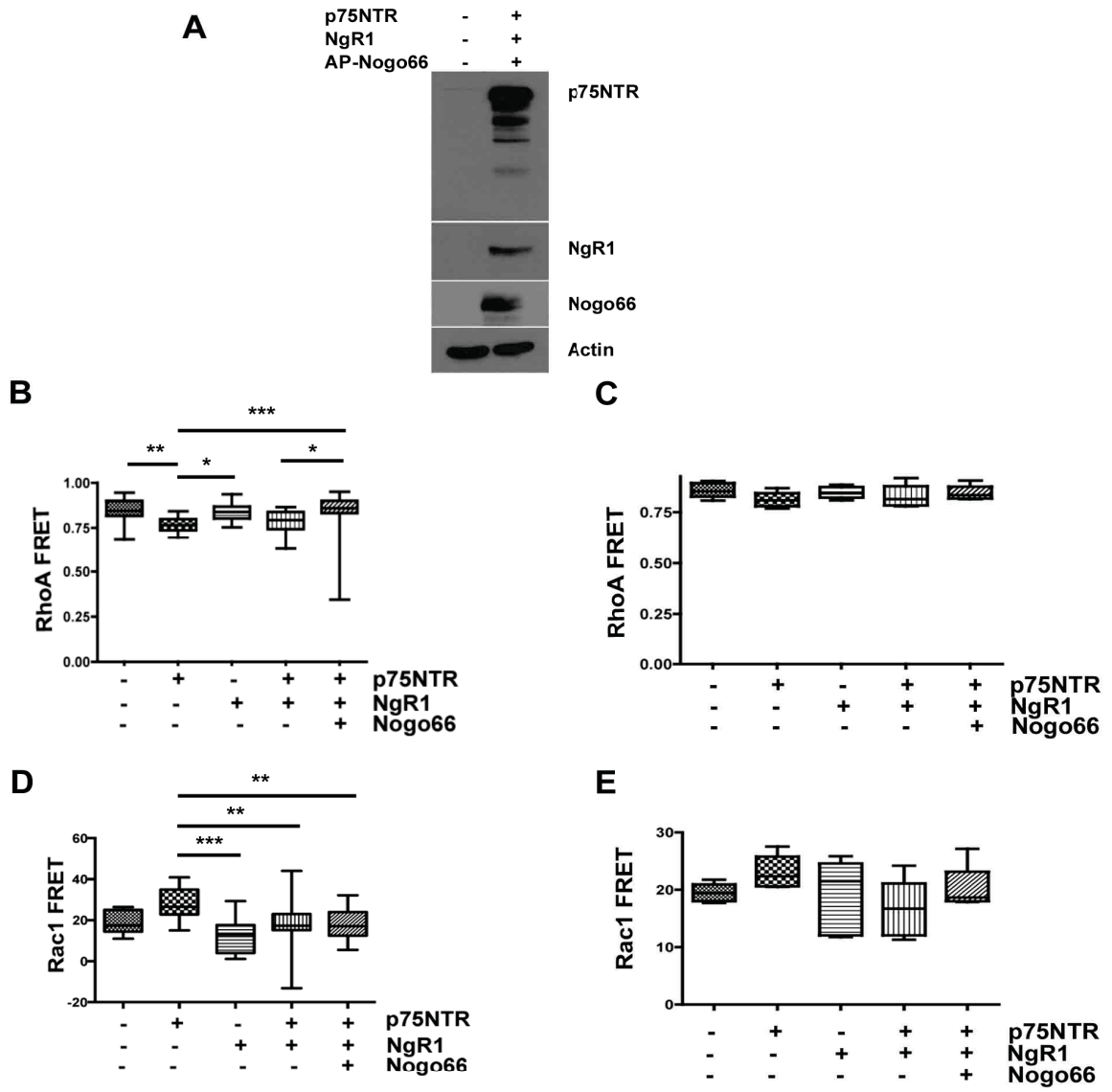

Figure 3.3. The effect of p75NTR on RhoA and Rac1 activity in the presence of NgR1 and Nogo66.

A. Western blot showing the expression of p75NTR, NgR1, and Nogo66 in COS7 cells. B. Quantification of RhoA FRET in control cells and cells overexpressing p75NTR, NgR1, p75NTR and NgR1, and p75NTR, NgR1 and Nogo66 for one representative experiment. C. Quantification of RhoA FRET in a pool of five independent experiments. D. Quantification of Rac1 FRET in control cells and cells overexpressing p75NTR, NgR1, p75NTR and NgR1, and p75NTR, NgR1 and Nogo66 for one representative experiment. E. Quantification of Rac1 FRET in a pool of five independent experiments. Results are expressed as means ± s.e.m.

PREFACE TO CHAPTER 4

Glioblastoma multiforme is the most common and one of the most severe malignant brain tumors. One of the obstacles for their treatment is their high invasiveness. Although they are not metastatic, they are highly locally invasive and single cells can escape the main tumor to form secondary tumor loci. Studies have focused on looking for potential candidates in glioma invasion. p75NTR appears to play a prominent role in glioma invasion *in vitro* and *in vivo*. However, the signaling mechanism through which p75NTR promotes migration is still unexplored. Here we show that p75NTR promotes migration of U87MG cells *in vitro* and that cleavage of p75NTR and the release of the ICD is important in this process. We looked for a potential role for NRAGE and NEDD9 dowsntream of p75NTR in this process. Our findings show that NRAGE and NEDD9 can mediate glioma migration independently of p75NTR through different signaling pathways that require further investigation.

CHAPTER 4

p75NTR SIGNALING IN GLIOMA MIGRATION

4.1 ABSTRACT

Glioblastoma multiforme is the most severe form of brain tumors with a survival rate of less than 5 years even after surgery and resection. This is mainly due to the high invasive properties of the tumor. p75NTR plays an important role in the migration of many types of cancer, in particular, it is shown to induce invasion of glioma cells *in vitro* and *in vivo*. In this work we investigated the signaling mechanism downstream of p75NTR in glioma migration. We first show that p75NTR is highly expressed in high grade human glioma samples. In order to study the role of p75NTR *in vitro*, U87MG cells that do not express p75NTR endogenously were infected with p75 wild type full length p75 (p75FL), p75 cleavage resistant (p75CR), and p75 intracellular domain (p75ICD). Migration of the cells was assessed through a 2D migration assay on laminin, the agarose drop assay. Our results show that p75NTR increases migration of U87MG cells and that cleavage of p75NTR is important in this process. p75NTR also increases random motility of U87MG cells. Our previous data report that NRAGE and NEDD9 act downstream of p75NTR to induce cell spreading and Rac1 activation. We asked whether p75NTR acts through NRAGE and NEDD9 to induce U87MG cell migration. Our results demonstrate that NRAGE and NEDD9 induce U87MG migration independently of p75NTR.

4.2 INTRODUCTION

Glioblastoma multiforme (GBM) is the most common type of brain tumor and the most severe one. GBM is highly resistant to both chemotherapy and radiotherapy, which contributes in part to their high aggressiveness. They are diffuse and highly invasive tumors and single cells can invade several centimeters away from the initial tumor mass. The highly invasive nature of GBM makes them refractory to surgery with five-year survival rate of only 3% (Giese et al, 2003, Senger et al, 2003). The increased invasive properties is due to genotypic and phenotypic alterations occurring in the tumor cells as well as their surrounding environment that favor invasion and migration of tumor cells (Mariani et al, 2001, Demuth and Berens, 2004). During the last decades efforts were made to find key players in glioma invasion that could serve as therapeutic targets.

The p75 neurotrophin receptor (p75NTR) belongs to the tumor necrosis factor superfamily (TNFR), and can bind all neurotrophins with equal low affinity. p75NTR is an important mediator of cell death, survival, differentiation, neurite outgrowth, and cell migration (Roux and Barker, 2002, Schor, 2005, Skeldal et al, 2011). In addition to its role during development p75NTR is also an important player in tumorigenesis. In fact, p75NTR promotes migration of melanoma (Hermann et al, 1993, Truzzi et al, 2008), glioma (Johnston et al, 2007, Wang et al, 2008), medulloblastoma (Wang et al, 2010), and pancreatic carcinoma (Zhu et al, 2002, Wang et al, 2009). Conversely, p75NTR is also shown to inhibit migration of gastric cancer cells (Jin et al, 2007a and b, Jin et al, 2010), and prostate cancer cells (Nalbandian and Djakiew, 2006, Wynne and Djakiew, 2010), suggesting that the role of p75NTR in tumorigenesis and cancer invasion depends on the cancer type. In particular, p75NTR is highly expressed in high grade glioma and it increases migration and invasion of U87 malignant glioma (U87MG) cells *in vitro* and *in vivo* (Jonhston et al, 2007, Wang et al 2008). Interestingly, p75NTR is also expressed in brain tumor stem cells and is associated with their increased proliferation (Forsyth et al, 2014). Little is known about p75NTR signaling in glioma invasion, but studies suggest that p75NTR cleavage is an essential mediator in this process (Wang et al, 2008, Wang et al, 2010). However, the downstream signaling mechanism that promotes migration is still unexplored.

The neurotrophin receptor interacting MAGE homolog (NRAGE) belongs to the MAGE family of adaptor proteins. It was identified as a p75NTR binding partner (Salehi et al 2000), through which it mediates cell death in PC12 cells as well as in sympathetic neurons of the superior cervical ganglia (Salehi et al, 2002, Bertrand et al, 2008). We have recently shown that p75NTR binding to NRAGE mediates COS7 cell spreading through the activation

of Rac1 (Zeinieh et al, In Press, chapter 2). The role of NRAGE in cancer migration is still controversial. It has been reported as an antimetastatic factor in pancreatic cancer (Chu et al, 2007), and melanoma (Lai et al, 2012). Similarly, NRAGE inhibits the migration of brain tumor stem cells (Reddy et al, 2010) and its expression is downregulated in many tumor types such as breast cancer (Tian et al, 2005, Du et al, 2009), and colorectal cancer (Zeng et al, 2012). On the other hand, NRAGE has been reported to be upregulated in lung cancer, melanoma, colon cancer, breast cancer, prostate cancer, and esophageal cancer (Yang et al, 2014), suggesting that NRAGE plays diverse and complex regulatory roles.

NEDD9 belongs to the Cas family of adaptor proteins, and it has been reported as a major metastatic factor in different types of cancer such as melanoma (Kim et al, 2006), glioblastoma (Natarajan et al, 2006), breast cancer (Izumchenko et al, 2009, Little et al, 2014), lung cancer (Kondo et al, 2012, Miao et al, 2013), colon cancer (Kim et al, 2010, Xia et al, 2010, Li et al, 2011), gastric cancer (Liu et al, 2014, Shi et al, 2014), and prostate cancer (Morimoto et al, 2014). NEDD9 transduces signals downstream of integrins to regulate the epithelial to mesenchymal transition (EMT) ultimately leading to increased cell migration (Ahn et al, 2012, Morimoto et al, 2014), through a process involving in part Rac1 activation (Sanz Moreno et al, 2008, Yang et al, 2012).

We have recently reported that NRAGE interacts with NEDD9, and that the formation of NRAGE-NEDD9 complex downstream of p75NTR is important for the activation of Rac1 and cell spreading (Zeinieh et al, In Press, chapter 2).

In this study we aimed at elucidating the downstream signaling linking p75NTR to glioma invasion. Most importantly we investigated whether p75NTR acts through NRAGE and NEDD9 to promote glioma migration. Using an *in vitro* migration model of U87MG cells we found that p75NTR promotes migration and that cleavage of p75NTR and the release of the p75 intracellular domain ($p75^{ICD}$) is required for this process. We also report that NRAGE and NEDD9 are essential for U87MG migration and act independently of p75NTR in this process.

4.3 MATERIAL AND METHODS

Reagents and plasmids: Polyclonal antibodies directed against p75NTR and NRAGE were described previously (Barker et al, 1994, Salehi et al, 2000), the antibody against NEDD9 was obtained from Thermo Fisher Scientific (MA1-5784, Ottawa, ON, CA). The monoclonal antibody against β-actin was purchased from MP Biomedicals (691001, Irvine, CA, USA). The p75NTR monoclonal antibody (ME20.4) was obtained from Millipore (05-446, ON, CA). Horseradish-peroxidase conjugated secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA, USA). Laminin was purchased from BD Bioscience (Mississauga, ON, CA). Poly-*D*-lysine (PDL) was obtained from Sigma (Oakville, ON, CA). SeaPlaque low melting temperature agarose was purchased from Lonza (Rockland, ME, USA). Ilomastast (GM6001) and epoxomicin were obtained from Calbiochem (San Diego, CA, USA); batimastat (BB94) was from Tocris Bioscience (Ellisville, MO, USA). All cell culture reagents were from Wisent Bioproducts (Saint Bruno, QC, CA).

Cell culture, transfections and infections: U87MG 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, in 5% CO2 at 37°C. For p75NTR overexpression, U87MG cells were infected with lentivirus expressing the open reading frame of the p75 full length (p75FL), the p75 cleavage resistant (p75CR), and the p75 intracellular domain (p75ICD). The open reading frames of the different p75NTR constructs were clones in the pRRLsinPPT mRFP stop-IRES-RfA plasmid containing a red fluorescent protein (RFP) sequence. For control, a non specific miRNA plasmid was used that harbors a eGFP or RFP sequence. Cells were kept for 5 days at 37°C then plated on 24 well plates precoated with laminin for agarose drop assay or on Lab-Tek II chambered coverglass (Nalge Nunc international, Rockaway, NJ, USA) for migration kinetics assay, or lysed in Laemmli sample buffer for analysis by immunoblot.

NRAGE and NEDD9 stealth siRNA targeting sequences directed against the respective human mRNAs were designed using the Invitrogen Stealth RNAi™ siRNAs prediction algorithm (specific sequences available on request). For knockdown experiments, cells were first infected with p75NTR overexpression lentiviruses constructs and 48 hours later, were transfected with the different siRNAs in antibiotic free media 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 for migration kinetics assay, or lysed in Laemmli sample buffer for analysis by immunoblot.

Agarose drop assay: 24 well plates were coated for 30 minutes with PDL (0.5 ug/ml), washed with sterile water, coated with laminin (0.5 ug/ml) for two hours at 37°C, and again washed with sterile water. U87MG cells were infected with a control lentivirus encoding an eGFP plasmid (CT-GFP) whereas p75FL, CR, and ICD were coexpressed with RFP. Cells were trypsinized and equal number of cells expressing CT-GFP or one of the p75NTR constructs were mixed for a total of 20.10^6 cells/ml. 0.1% (w/v) of low melting point agarose prepared in 1x phosphate buffered saline (PBS) were added to the cell mix at a 2:1 ratio cells:agarose. 2 ul drops of the mix were plated in the center of the each well. The plates were kept at 4°C for 15 minutes to allow solidification of the drops then 100ul of prechilled media was added around the drops and plates were kept for two hours at 37°C. An additional 200ul of prechilled media were then added to each well. Plates were kept at 37°C and were imaged 3 days later. Imaging was performed using a 10x objective on a Zeiss Axioskop fluorescent inverted microscope equipped with Xenon illumination, and images were captured using Zen software (Zeiss). The number of CT-GFP cells and p75-RFP cells that migrated away from the initital drop were counted using the Ring macro that we designed using NIH ImageJ software. This macro allows the drawing of concentric circles around the agarose drop and counts the number of cells in each circle.

Migration kinetics: U87MG cells were infected with p75NTR overexpressing lentiviruses or control miRNA lentivirus expressing RFP. Five days after infection, cells were plated at low density (10000 cells/ml) on Lab-Tek II chambered coverglass precoated with PDL and laminin as described above. Cells were incubated for 24 hours at 37°C then live imaged. Cells were imaged for 5 hours, at 10 minutes intervals. Four fields were taken for each well. The random migration of the cells was measured using the MTrackJ plugin in NIH ImageJ software and the total length of migration was assessed for each condition.

Collection of human brain tumor specimens: Brain tumors specimens of grades II, III, and IV were obtained from patients undergoing surgery at the Montreal Neurological Institute and Hospital. Specimens were collected and approved for the study under the McGill human ethics review board. Tumors were homogenized in RIPA buffer (10 mM Tris pH8, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 2% SDS). The homogenates were centrifuged

for 15 minutes at 10000 rpm at 4°C, and the supernatant was resuspended in 2x Laemmli sample buffer for immunoblotting. For immunohistochemistry, tumor specimens were snap frozen in liquid nitrogen and embedded in paraffin. 5 um sections were taken and processed for staining with p75NTR antibody.

Western blot analysis and immunoblotting: Cells were harvested in 2x Laemmli sample buffer and boiled for 5 minutes prior to loading on SDS-PAGE. After SDS-PAGE and transfer to nitrocellulose, membranes were rinsed in PBS then blocked in TBST (10 mM Tris pH 8.0, 150 mM NaCl, 2% Tween 20) that was supplemented with 5% (w/v) dried skim milk powder. Primary and secondary antibody incubation was performed in TBST containing 2.5% (w/v) dried skim milk powder blocking solution, with primary incubations performed overnight at 4°C 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, USA), as per the manufacturer's instructions.

Immunohistochemistry: Tumor sections were deparaffinized and incubated overnight with the p75NTR monoclonal antibody (1ug/ml) at 4°C. Slides were washed 3 times 5 minutes each in 1x PBS. Sections were incubated with the mouse secondary antibody tagged with DAB for one hour at room temperature. Sections were washed 3 times 5 minutes each in 1x PBS and counterstained with hematoxylin and eosin.

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

4.4 RESULTS

p75NTR is overexpressed in high grade tumors

It was reported that p75NTR protein is overexpressed in glioma (Wang et al, 2008). We tested whether the expression of p75NTR correlates with the tumor grade. p75NTR protein expression in grade II, III, and IV tumors were assessed by immunoblotting and the results show that p75NTR is highly expressed in high grade tumors (grade III and IV) compared to low grade tumors (grade II) ($p<0.01$) (Fig 4.1A and B). Similarly, p75NTR protein expression is detected in tumor tissues of high grade glioma compared to low grade samples (Fig 4.1C).

These results confirm previous findings and show that the expression of p75NTR positively correlates with the tumor grade.

p75NTR promotes the migration of U87MG cells through its ICD

The role of p75NTR in cancer migration is well documented. It promotes the invasion of many cancer types such as melanoma (Hermann et al, 1993, Truzzi et al, 2008), glioma (Johnston et al, 2007, Wang et al, 2008), medulloblastoma (Wang et al, 2010) and pancreatic carcinoma (Zhu et al, 2002, Wang et al, 2009). However, the downstream signaling mechanism is not well elucidated. We recently reported that p75NTR induces COS7 cell spreading and that this is achieved through cleavage of p75NTR and the release of its ICD (Zeinieh et al, In Press, chapter 2). In order to test whether p75NTR, and in particular p75NTR cleavage are involved in U87MG cell migration *in vitro,* we used the agarose drop assay, a 2D migration assay that allows us to measure the number of cells that migrated away from a concentrated drop of cells. U87MG cells do not express p75NTR endogenously, but p75NTR was shown to be upregulated in invasive U87MG cells when implanted in the brain of immunocompromised mice (Jonhston et al, 2007). In order to test the importance of p75NTR in U87MG migration, cells were infected with lentiviruses expressing the different p75NTR constructs coexpressing RFP, namely, wild type full-length p75NTR (p75FL), an uncleavable form of p75NTR (p75CR), and the p75 intracellular domain (p75ICD). For control cells, U87MG cells were infected with a control lentivirus expressing GFP (Fig 4.2A). A mix of cells containing equal number of control-GFP and p75-RFP expressing cells was mixed to low melting point agarose and 2 ul drops were plated on laminin coated plates and allowed to migrate for 3 days before imaging (Fig 4.2B). p75-RFP expressing cells and control-GFP cells were counted and the ratio between p75-RFP cells and control-GFP cells was calculated for each sphere using the Ring macro designed in our lab for ImageJ software

as described in fig 4.2C. Our results show that p75NTR overexpression correlates with an increased migration of U87MG cells. Cleavage of p75NTR seems to be required for this process since the CR form of p75NTR did not promote migration of U87MG cells ($p<0.001$) (Fig 4.2D and E).

To confirm the contribution of p75NTR and in particular the $p75^{ICD}$ in glioma migration, we tested the random migration of U87MG cells plated on laminin through monitoring their migration over 5 hours. The total length of the path traveled by the cells was measured using the MTrackJ plugin in ImageJ software. Our results show that p75NTR increases the migration of the cells on laminin and that cleavage of the ICD is important in this process since p75CR was unable to promote migration of cells (Fig 4.3A and B).

The results obtained with the p75CR constructs prompted us to determine whether cleavage of p75NTR is required for the migration process mediated by the receptor. Regulated intramembrane proteolysis (RIP) is a common event employed by many receptors that results in their cleavage through a dual processing event. p75NTR undergoes RIP and is characterized by a first proteolytic event by an α-secretase that results in its extracellular domain shedding, followed by its cleavage by γ-secretase that releases its ICD. We have previously shown that RIP is important for p75NTR signaling through which p75NTR mediates COS7 cell spreading (Zeinieh et al, In Press, chapter 2). We have also shown that cleavage of p75NTR by α-secretase, and in particular by ADAM17 is a prerequisite for the ICD release and p75NTR signaling (Zeinieh et al, In Press, chapter 2). In order to test whether cleavage of p75NTR is required for U87MG migration, cells expressing control lentivirus or p75FL lentivirus were plated on Lab-Tek II chambered coverglass and treated for 2 hours with a combination of two broad α-secretase inhibitors, GM6001 (10 uM) and BB94 (0.2 uM) prior to imaging, then live imaged for 5 hours every 10 minutes. The inhibitors were kept in the media during the imaging period. Our results demonstrate that inhibition of α-secretase decreases the migration of p75FL overexpressing cells (Fig 4.3C). Taken together these results suggest that cleavage of p75NTR and the release of the ICD are required for the promotion of U87MG cell migration.

p75NTR mediates U87MG cell migration independently of NRAGE and NEDD9

p75NTR is characterized by the lack of an intrinsic catalytic activity, a common feature of TNFRs members. For this purpose, it requires interaction with diverse downstream

adaptor proteins that mediate its many different effects. Previous work in the lab identified NRAGE as a p75NTR interactor through which it mediates cell death of sympathetic neurons as well as PC12 cells (Salehi et al, 2000, Salehi et al, 2002, Bertrand et al, 2008). The role of NRAGE in cancer migration is not very well understood but it is reported to have anti-invasive as well as proinvasive properties depending on the cancer type (Tian et al, 2005, Chu et al, 2007, Du et al, 2009, Reddy et al, 2010, Lai et al, 2012, Yang et al, 2014). It has also been reported as an important player in epithelial to mesenchymal transition (EMT) (Kumar et al, 2011). We have previously shown that NRAGE interacts with NEDD9 and that p75NTR acts through NRAGE and NEDD9 to mediate COS7 cell spreading through activation of Rac1 (Zeinieh et al, In Press, chapter 2). Hence, we asked whether p75NTR acts through this same pathway in glioma migration.

To test whether NRAGE is involved in glioma migration and in particular whether it acts downstream of p75NTR in this process we knocked down NRAGE using NRAGE siRNA in U87MG cells overexpressing p75FL or p75ICD, or in cells that were kept non-infected. A non-specific siRNA was used as control (Fig 4.4A). Cells were plated on laminin coated Lab-Tek II chambered coverglass and live imaged for 5 hours. The total length of the path traveled by the cells was measured using the MTrackJ plugin in ImageJ software. The results show that knockdown of NRAGE shows a trend towards a decrease in cell migration in all groups tested although the significance is lost when results from the four independent experiments are pooled (Fig 4.4 C-H). Thus, these results suggest that NRAGE contributes to U87MG cell migration through a p75NTR independent pathway.

NRAGE interacts with NEDD9 and NRAGE-NEDD9 act downstream of p75NTR in COS7 cells spreading (Zeinieh et al, In Press, chapter 2). NEDD9 is a major metastatic factor in many types of cancers (Kim et al, 2006, Izumchenko et al, 2009, Kim et al, 2010, Little et al, 2014, Liu et al, 2014, Morimoto et al, 2014), and it has also been shown to induce migration of glioma cells *in vitro* (Natarajan et al, 2006). In order to test whether NEDD9 is required downstream of p75NTR for glioma migration we knocked down NEDD9 in U87MG cells overexpressing or not p75NTR. A non specific siRNA was used as control (Fig 4.4B). Cells were plated on laminin coated Lab-Tek II chambered coverglass and live imaged for 5 hours. The total length of the path traveled by cells was measured using the MTrackJ plugin in ImageJ software. Similar to NRAGE, our results demonstrate that NEDD9 knockdown shows a trend towards a decrease in the migration of U87MG cells in all groups tested, however, these changes did not achieve significance when the four independent experiments were

pooled (Fig 4.4 C-H). Thus, these results suggest that NEDD9 induces cell migration of U87MG cells through a pathway that does not involve p75NTR.

Taken together these results show that p75NTR mediates migration of U87MG cells *in vitro* and that NRAGE and NEDD9 are not required for this process.

4.5 DISCUSSION

One of the major obstacles in the treatment of gliomas is their high invasive capacity. Individual cells can escape the main tumor mass and colonize long distances in the brain parenchyma. Many factors were described as important players in tumor invasion. The p75NTR has emerged as an essential player for glioma invasion. In this study we showed that p75NTR mediates the migration of glioma cells *in vitro* and that the cleavage of p75NTR is important in this process. We also show that p75NTR mediates migration independently of a NRAGE-NEDD9 complex.

Glioma invasion requires the interplay between paracrine factors arising from the tumor surroundings and the extracellular matrix (ECM), as well as autocrine factors secreted by the tumor itself (Hoelzinger et al, 2007). Moreover, genotypic alterations and gene reprogramming occur in tumor cells leading to upregulation of genes involved in migration and proliferation and downregulation of propapoptotic genes (Mariani et al, 2001, Hoelzinger et al, 2005, Formolo et al, 2011).

p75NTR has been reported as a proinvasive factor in many different types of cancers such as melanoma (Hermann et al, 1993, Truzzi et al, 2008), glioma (Johnston et al, 2007, Wang et al, 2008), medulloblastoma (Wang et al, 2010) and pancreatic carcinoma (Zhu et al, 2002, Wang et al, 2009). p75NTR has also been described as an antimetastatic factor in gastric cancer cells (Jin et al, 2007a and b, Jin et al, 2010), and prostate cancer cells (Nalbandian and Djakiew, 2006, Wynne and Djakiew, 2010). We found that p75NTR is highly expressed in high grade gliomas and that its expression correlates with the tumor grade, which confirms the findings of previous work (Staniszewska et al, 2008, Brown et al, 2008, Wang et al, 2008). Interestingly, it appears that p75NTR overexpression correlates with the invasive capacity of tumor cells *in vivo*. U87MG cells implanted in the brain of immunocompromised mice have an increase in p75NTR expression correlating with promotion of invasiveness (Johnston et al, 2007, Wang et al, 2008). Among the different genes that were upregulated in invading cells, p75NTR showed up to 8 fold increase (Jonhston et al, 2007), suggesting that this receptor plays an essential role in glioma invasion. Moreover, several studies have shown that p75NTR enhances migration through its ICD suggesting that cleavage of p75NTR is an important event in this process (Johnston et al, 2007, Wang et al, 2008, Wang et al, 2010). The $p75^{ICD}$ was also detected in glioma samples taken from patients, however, we were unable to detect the $p75^{ICD}$ in our samples, possibly due to the instability of the ICD and its rapid proteosomal degradation.

It is still unknown how p75NTR contributes to the invasion process. However, studies show that NGF and integrin α9β1 are both highly expressed in high grade glioma (Staniszewska et al, 2008, Brown et al, 2008), and their interaction increases the proliferation and migration of glioma cells *in vitro* and *in vivo* through an Erk1/2/MAPK dependent pathway (Brown et al, 2008). It is shown that p75NTR facilitates the trophic effect of NGF and integrin α9β1 on muscle cells during development and after ischemia through Erk1/2/MAPK pathway (Ettinger et al, 2012). Hence it is possible that p75NTR could be acting as a potentiator of NGF-integrin α9β1 signaling in glioma. On the other hand, p75NTR interacts with the polarization protein Par3 in Schwann cells to direct myelination (Tep et al, 2012). It is possible that p75NTR localizes and interacts with Par3 to drive the directional migration of glioma cells. On the other hand, TROY, another TNFR family member, highly homologous to p75NTR, mediates glioma invasion through a Pyk2/Rac mechanism (Paulino et al, 2010). TROY can mimic p75NTR function in some systems, such as neurite outgrowth inhibition by myelin inhibitory proteins (Lu et al, 2013). *p75ntr-/-* DRG neurons still respond to myelin inhibitory proteins presumably through TROY (Park et al, 2005). Thus it is possible that p75NTR is acting through Pyk2 and Rac to induce migration.

Previous work in our lab has shown that p75NTR interacts with NRAGE through which it mediates death of PC12 cells and sympathetic neurons of the superior cervical ganglia (Salehi et al, 2000, Salehi et al, 2002, Bertrand et al, 2008). We recently showed that p75NTR mediates cell spreading through NRAGE (Zeinieh et al, In Press, chapter 2), and we asked whether p75NTR mediates glioma invasion through this same pathway. Our results show that NRAGE knockdown impairs U87MG migration independently of p75NTR suggesting that NRAGE acts in a different pathway in glioma migration. The role of NRAGE in gliomagenesis is not well defined, but data available indicate a controversial role for NRAGE in tumorigenesis. It is reported that NRAGE inhibits metastasis and tumor proliferation of pancreatic cells (Chu et al, 2007), melanoma cells (Lai et al, 2012), and brain tumor stem cells (Reddy et al, 2010) and its expression is downregulated in many tumor types such as breast cancer (Tian et al, 2005, Du et al, 2009), and colorectal cancer (Zeng et al, 2012). On the other hand, NRAGE is also reported to be upregulated in numerous cancers such as lung cancer, melanoma, colon cancer, breast cancer, prostate cancer, and esophageal cancer (Yang et al, 2014). On one hand it is suggested that NRAGE inhibits migration through disrupting the E-cadherin/β-catenin complex (Xue et al, 2005, Chu et al, 2007); on the other hand, disruption of this complex by NRAGE enhances migration of transformed

mammary epithelial cells (Kumar et al, 2011). It is possible that these differences reflect distinct downstream signaling partners for NRAGE. It is also possible that NRAGE is inducing glioma migration downstream of p75NTR but it is also true that NRAGE regulates glioma migration through different pathways. We recently showed that NRAGE interacts with NEDD9 and mediates cell spreading downstream of p75NTR (Zeinieh et al, In Press, chapter 2). We asked whether NEDD9 acts downstream of p75NTR in glioma invasion. Our results demonstrate that NEDD9 mediates glioma invasion independently of p75NTR although it is possible that p75NTR also acts through NEDD9 in glioma invasion. In fact, NEDD9 has emerged as a prometastatic factor in multiple cancers such as breast cancer (Izumchenko et al, 2009, Little et al, 2014), melanoma (Kim et al, 2006), glioblastoma (Natarajan et al, 2006), lung cancer (Miao et al, 2013, Kondo et al, 2012), colon cancer (Kim et al, 2010, Xia et al, 2010, Li et al, 2011, Li et al, 2014), gastric cancer (Liu et al, 2014, Shi et al, 2014), and prostate cancer (Morimoto et al, 2014). NEDD9 regulates cell migration through modulation of Rho GTPases (Sanz-Moreno et al, 2008, Yang et al, 2012), activation of MMPs (McLaughlin et al, 2014), and regulation of EMT by affecting the cytoskeletal dynamics (Kong et al, 2011, Guerrero et al, 2012). The fact that NEDD9 induces glioma invasion independently of p75NTR does not rule out the role of NEDD9 downstream of p75NTR but shows the multitude of pathways this adaptor protein is involved in.

One caveat of this work is that the trend to differences observed in individual experiments did not pass tests of statistically significance when the data was pooled. This reflects variability between the different experiments. Sources of this variability may include changes in the knockdown efficacy of the siRNA used to achieve NRAGE and NEDD9 depletion, and heterogeneity within the U87MG cell population.

In conclusion, our study demonstrate an important role for p75NTR in glioma migration *in vitro*, in particular we demonstrate the importance of p75NTR cleavage and the release of the ICD in this process. Our findings also show that the signaling pathway conveyed by this recpetor in glioma migration does not involve NRAGE and NEDD9 and suggests that there are different players downstream of this receptor and in particular downstream of the ICD that contribute to glioma invasion. On the other hand, this study also shows an important role for NRAGE and NEDD9 in glioma migration independently of p75NTR. It will be interesting to understand i) how p75NTR regulates glioma migration and ii) what is the signaling pathway through which NRAGE and NEDD9 promote glioma migration.

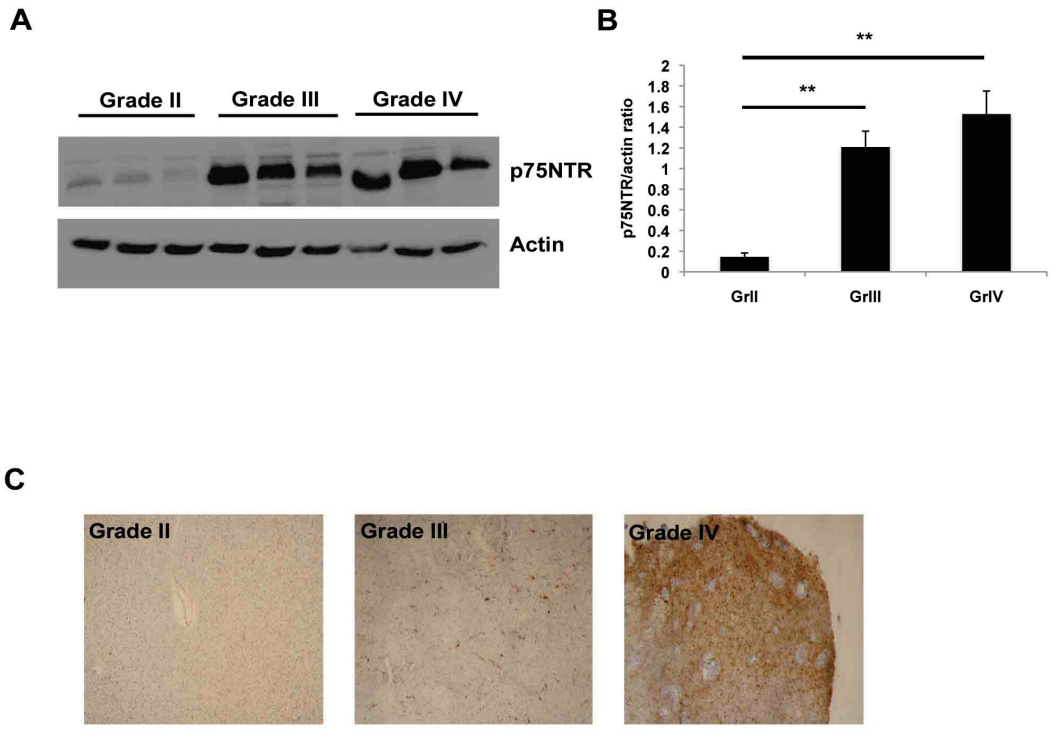

Figure 4.1. p75NTR protein expression is upregulated in high grade glioma.
A. p75NTR protein expression was examined by western blot in human glioma specimens of grades II, III, and IV. B. Quantification of bands intensity by ImageJ showing a significant increase in p75NTR expression in high grade glioma ($p<0.001$). Values are expressed as means ± s.e.m using one way ANOVA. C. Expression of p75NTR (brown) was examined by immunohistochemistry in human glioma specimens of grades II, III, and IV. Representative samples are shown. Slides were counterstained with hematoxylin.

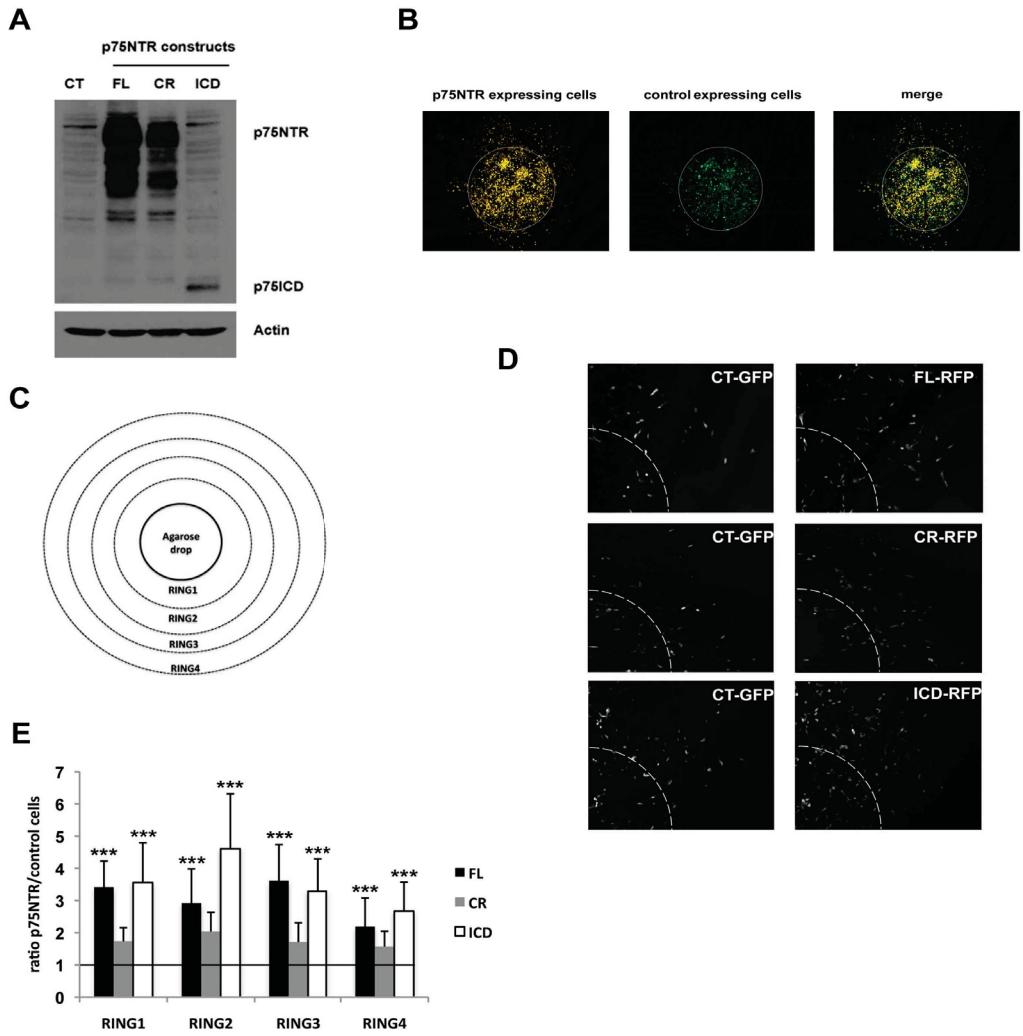

Figure 4.2. p75ICD increases migration of U87MG cells *in vitro* in an agarose drop assay. A. Western blot showing the overexpression of the different p75NTR constructs in U87MG cells. B. Representative image of the agarose drop assay showing U87MG cell spheres containing CT-GFP expressing cells and p75-RFP expressing cells mixed at equal density. C. Schematic diagram describing the quantification of the agarose assay using the RING macro. Rings are drawn around each sphere and the number of cells is counted in each ring. D. Representative micrographs of the agarose drop assay. Panels represent CT-GFP expressing cells and p75-RFP expressing cells of the same sphere. In each example one part of the sphere is magnified. E. Quantification of the agarose drop assay showing a significant increase in the number of p75FL and p75ICD overexpressing cells in each ring compared to controls ($p<0.001$). The values are expressed as ratios of p75-expressing cells to control cells. Values are expressed as means ± s.e.m for n=5 independent experiments. In each experiment 3-6 spheres were counted.

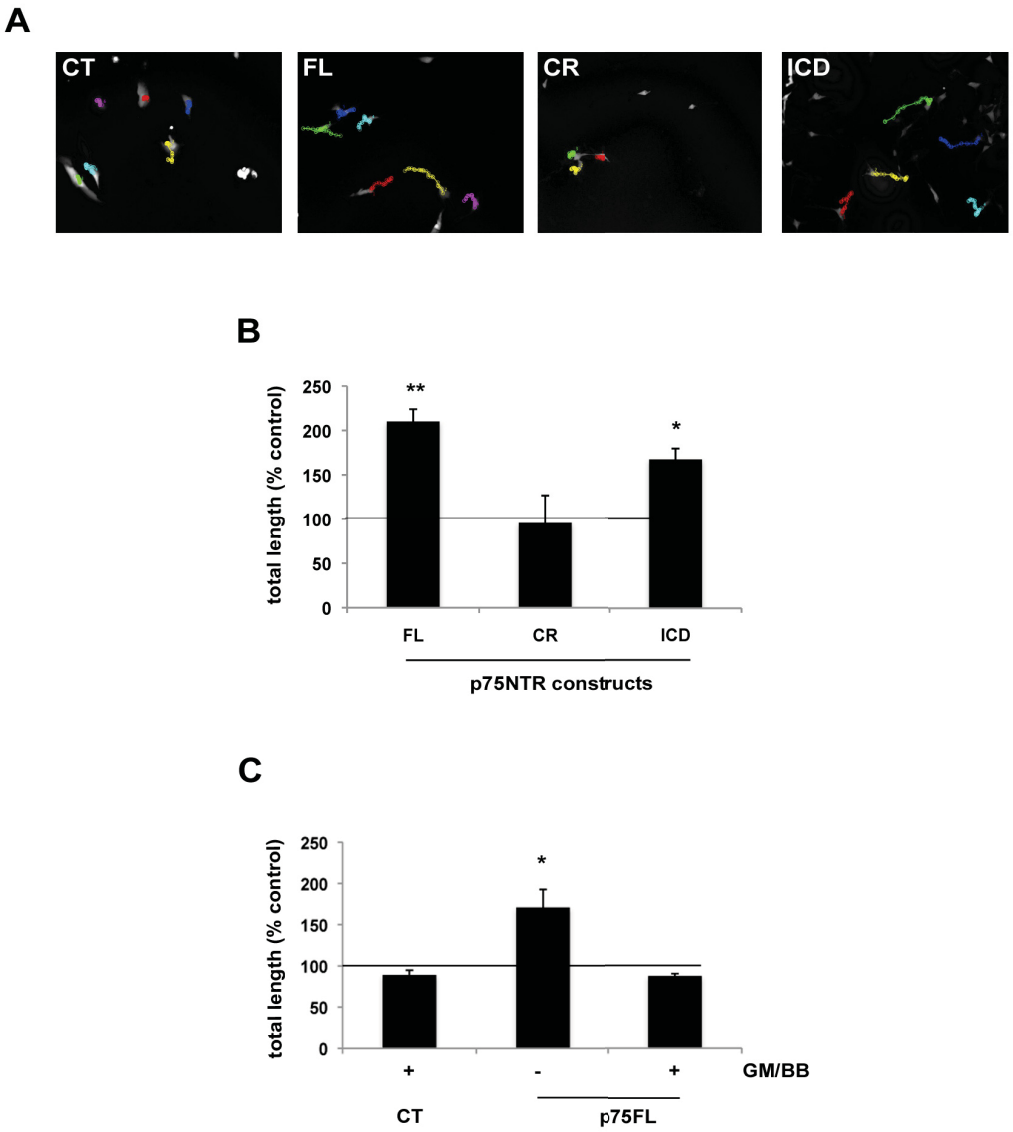

Figure 4.3. p75ICD increases the random migration of U87MG cells.
A. Representative micrographs of migration tracks for the different groups examined. B. Quantification of the total length path traveled by the cells expressed as percent of controls showing a significant increase in the migration of cells overexpressing p75FL and ICD but not in p75CR overexpressing cells in four independent experiments. C. Quantification of total length traveled by cells expressed as percent of control in control and p75FL cells with or without the α-secretase inhibitors GM6001 and BB94 for 2 independent experiments.

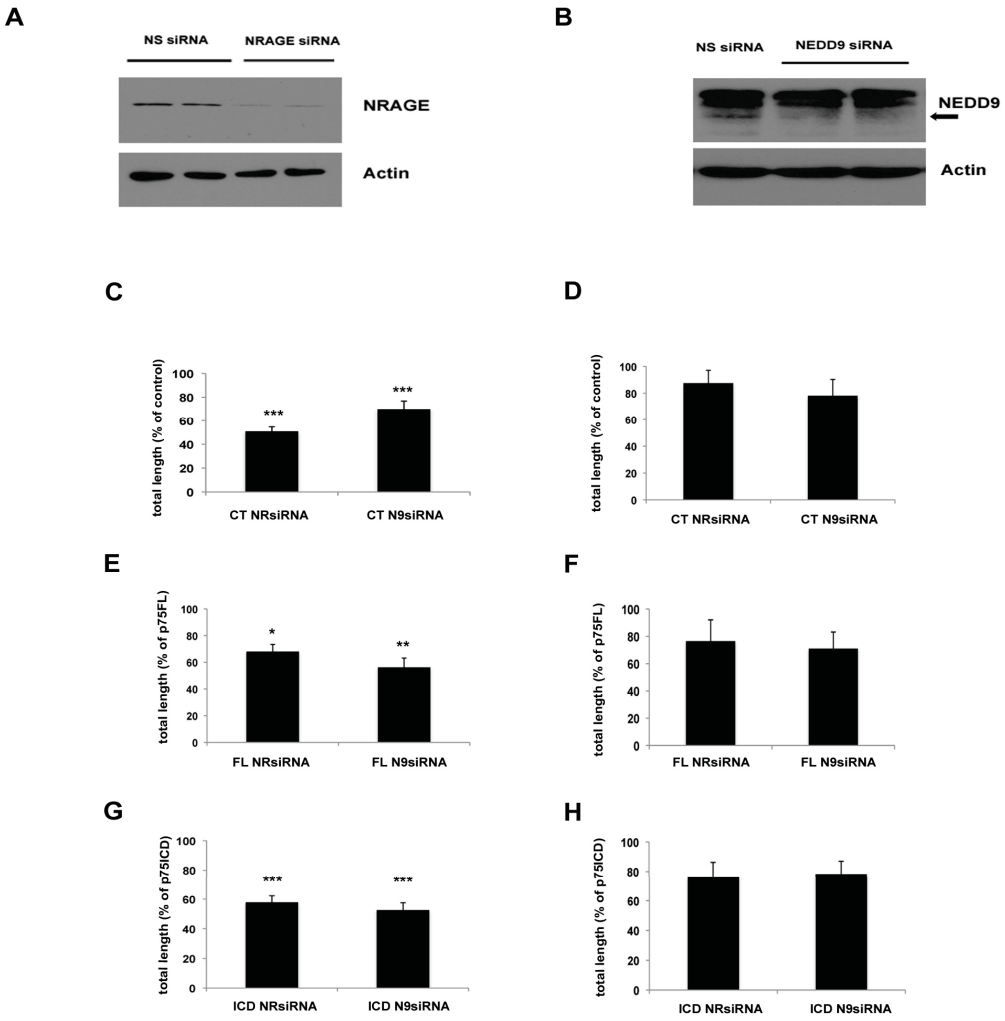

Figure 4.4. Effects of NRAGE and NEDD9 on U87MG migration in the absence and presence of p75NTR.

A. Western blot analysis showing knockdown of NRAGE in U87MG cells. B. Western blot analysis showing knockdown of NEDD9 in U87MG cells. C-D. Quantification of migration assay in control cells with and without NRAGE or NEDD9 siRNA represented as percent of controls in a single experiment (C) or in a pool of four independent experiments. E-F Quantification of migration assay in cells overexpressing p75FL with and without NRAGE or NEDD9 siRNA expressed as percent of p75FL in a single experiment (E) or in a pool of four independent experiments (F). G-H. Quantification of migration assay in cells overexpressing p75ICD with and without NRAGE or NEDD9 siRNA expressed as percent of p75ICD in a single experiment (G) or in a pool of four independent experiments (H). *, **, and *** indicate $p<0.05$, $p<0.01$, and $p<0.001$ respectively.

CHAPTER 5

GENERAL DISCUSSION OF THE THESIS

5.1 Major findings

The work proposed in this thesis has established a new pathway linking p75NTR to Rac1 activation. Our study characterized a novel interaction between the MAGE family member, NRAGE, and the Cas family member, NEDD9, and demonstrated the importance of this complex formation downstream of p75NTR, linking this receptor to Rac1 activation, using the COS7 spreading assay as a heterologous system to study p75NTR signaling. Importantly, we define an essential role for p75NTR cleavage in the regulation of this signaling pathway. We also show that p75NTR cleavage is important for glioma migration and that p75NTR acts independently of NRAGE and NEDD9 in this process. In order to better understand how p75NTR regulates Rho GTPases signaling we took advantage of the previously described Rho GTPases biosensors that allow the measurement of Rho GTPases activation by FRET. Using this method we were able to show that p75NTR constitutively activates Rac1 and modestly inhibits RhoA. Interestingly, we show that activation of these GTPases downstream of p75NTR depend largely on the coreceptors expressed such as the Nogo receptor and that the presence of myelin inhibitory proteins alters the regulatory mechanisms implied by p75NTR on these GTPases.

5.2 Cleavage of p75NTR: a prerequisite for p75NTR-mediated signaling

Regulated intramembrane proteolysis (RIP) is a conserved mechanism that has emerged as an important process in receptor signaling. Presenilin-dependent γ-secretase activity is responsible for the cleavage of a number of proteins such as Notch, APP, ErbB4, CD44, and low density lipoprotein (Brown et al, 2000, Ebinu and Yankner, 2002, Urban and Freeman, 2002). Cleavage of p75NTR through RIP has also proven to be an essential component of p75NTR-mediated signaling in cell survival (Ceni et al, 2010, Kommaddi et al, 2011, Matusica et al, 2013), cell death (Kenchappa et al, 2006, Kenchappa et al, 2010), neurite outgrowth (Yamashita and Tohyama, 2003, Domeniconi et al, 2005), cancer migration (Johnston et al, 2007, Wang et al, 2008), and cancer proliferation (Forsyth et al, 2014).

Cleavage of p75NTR occurs upon ligand binding to the receptor (Kenchappa et al, 2006, Urra et al, 2007, Skeldal et al, 2012), or by the association of p75NTR with coreceptors such as Trks (Urra et al, 2007, Ceni et al, 2010, Qu et al, 2013) or sortilin (Skeldal et al, 2012). Our work shows that the p75 intracellular domain ($p75^{ICD}$) is sufficient to mediate

signaling. Transgenic mice overexpressing $p75^{ICD}$ present death of peripheral and central neurons, without the requirement for neurotrophins and receptor cleavage (Majdan et al, 1997), suggesting that $p75^{ICD}$ can signal independently. NRH2, another TNFR family member, lacks the neurotrophin binding domain but shares substantial sequence homology with p75NTR at the transmembrane and the death domain regions. Studies of NRH2 signaling show that NRH2 is readily cleaved by γ-secretase generating an ICD with potent signaling capabilities (Kanning et al, 2003). Moreover, p75NTR mutant mice have provided interesting evidence for the function of p75NTR. Two p75NTR knockout mice have been generated: the p75 exon III knockout mouse that encodes a p75NTR splice variant lacking three of the four neurotrophin binding cysteine rich domains (Lee et al, 1992); and the p75 exon IV mutant that results from the insertion of a pGKneo cassette in exon IV leading to a disruption of the reading frame (von Schack et al, 2001). When these two mutants were originally described, von Schack and colleagues showed that the exon III mutant mouse still produced a splice variant of p75NTR whereas the exon IV mutant completely lacks p75NTR, although both mutants do not express the full length version of the receptor. The two knockout strains present defects in the nervous system manifested by a decrease in the number of sensory neurons and an increase in sympathetic neurons sprouting compared to the p75NTR wild type mice (Lee et al, 1994, von Schack et al, 2001, Dhanoa et al, 2006). In addition, the exon IV knockout mice have impaired vascularization whereas the exon III mice are not affected (von Schack et al, 2001). In contrast with these findings later studies showed that the exon III mouse completely lacks p75NTR whereas the exon IV mutant produces a splice variant of 26 kDa that corresponds to the transmembrane and intracellular domain of p75NTR and that is capable of transducing proapoptotic signaling (Paul et al, 2004). Although these mutants have provided important indications about p75NTR functions and in particular the role of the intracellular domain in transducing signals, other groups have also generated complete and tissue specific p75NTR knockout mice through Cre-mediated recombination of the p75NTR-flox conditional allele, that enables the study of p75NTR functions without the presence of the splice isoforms (Bogenmann et al, 2011).

Because of the similarities between p75NTR and NRH2 signaling, and of the findings that the exon III and IV null mice can exert signaling similar to the wild type (von Schack et al, 2001, Paul et al, 2004), it appears that factors other than neurotrophins are capable to induce p75NTR cleavage and activate downstream signaling. Although evidence for the role of adhesion molecules in p75NTR signaling is lacking, functional links between neurotrophin signaling and adhesion to the extracellular matrix (ECM) could exist. In fact, it was shown in

PC12 cells plated on laminin that NGF up-regulates integrin expression, leading to increased cell adhesion and differentiation (Rossino et al, 1990). NGF drives the accumulation of integrin β1 at the tips of PC12 filopodia, when cells are plated on laminin or fibronectin, ECM substrates known to bind and engage integrin β1 (Grabham and Goldberg, 1997, Grabham et al, 2000). ECM substrates such as laminin are known to be permissive for differentiation in response to NGF (Rossino et al, 1990, Liu et al, 2002, Tucker et al, 2005, Rankin et al, 2008). Although there is no evidence for the role of p75NTR in this process, its role in neuronal differentiation upon NGF treatment is well established and it is thus likely that p75NTR signaling upon NGF positively regulates adhesion (Nykjaer et al, 2005, Reichardt, 2006, Negrini et al, 2013). One study has suggested that NGF-induced neurite outgrowth of PC12 cells and hippocampal neurons on laminin is achieved through downregulation of p75NTR and that overexpression of p75NTR in PC12 cells plated on laminin inhibits their differentiation (Rankin et al, 2008). This effect might be attributed to the α and β integrin subunits involved in laminin binding. Laminin can bind the β1 as well as β4 subunits, and different integrins subunits could affect signaling differently. In fact, integrin α9β1 is identified as a receptor for NGF through which NGF enhances migration of cancer cells (Staniszewska et al, 2008, Brown et al, 2008), and glioma angiogenesis (Walsh et al, 2012). In view that NGF and integrin α9β1 (Brown et al, 2008), and p75NTR (Wang et al, 2008, Forsyth et al, 2014) are upregulated in high grade glioma, it is possible to envisage a crosstalk between the two pathways leading to enhanced migration of glioma cells. Similarly, TNF promotes apoptosis of endothelial cells when plated on collagen but not on laminin, suggesting that the modulation of the activity of distinct integrin receptors can exert different signaling mechanisms downstream of TNFRs (Huang et al, 2012). Other adhesion molecules have also been reported to enhance neurotrophin signaling. In fact, the activated leukocyte cell adhesion molecule (ALCAM) colocalizes with p75NTR in signaling endosomes in axons upon NGF treatment, and enhances neurite outgrowth of PC12 cells, reportedly by potentiating TrkA signaling (Wade et al, 2012). It is possible that adhesion to ECM engages adhesion molecules in specific compartments that bring neurotrophin receptors and signaling molecules in close proximity where they can potentiate signaling cascades.

The notion of signaling compartmentalization is particularly interesting for p75NTR. In fact, p75NTR was shown to be sequestered to lipid rafts where it induces different signaling pathways (Higuchi et al, 2003, Fujitani et al, 2005a and b). Interestingly, the γ-secretase complex is present in lipid rafts (Urano et al, 2005) and cleavage of APP by γ-

secretase occurs in these cholesterol rich microdomains (Wolozin, 2001, Ehehalt et al, 2003, Wada et al, 2003). ADAM17 undergoes maturation and cleaves target receptors such as TNFRs, CD30 and IL6 in cholesterol-rich microdomains (Tellier et al, 2006). Thus it is possible that p75NTR is targeted to lipid rafts microdomains where it undergoes RIP. Notably, integrin-based adhesions can stabilize lipid rafts and allow formation of integrin-containing complexes (Norambuena and Schwartz, 2011) that recruit Rho GTPases (del Pozo et al, 2004, Palazzo et al, 2004). Laminin also associates with the glycosphingolipid GM1 that allows the clustering of GM1 to lipid rafts. This in turn leads to the recruitment of integrins to lipid rafts and facilitates their interaction with laminin. Laminin recruitment to lipid rafts is essential for its binding to integrins and for induction of NGF-mediated neurite outgrowth of PC12 cells and DRG neurons (Ichikawa et al, 2009). In line with these findings, it was shown that signaling of neurotrophins to Rac1 requires lipid rafts (Fujitani et al, 2005a). Other studies have suggested that p75NTR is trafficked to lipid rafts only after phosphorylation by PKA; once present in the raft, p75NTR was proposed to inhibit Rho and facilitate neurite growth (Higuchi et al, 2003). Another study has suggested that p75NTR recruited to lipid rafts associates with NogoR and MAG and thereby induces cerebellar neuron growth cone retraction by activating RhoA (Fujitani et al, 2005b). Interestingly, FRET-based Rac biosensors have recently revealed that activated Rac translocates to lipid rafts where it associates with downstream signaling (Moissoglu et al, 2014).

Thus, p75NTR is activated by neurotrophin binding as well as integrin ligation that may lead to its recruitment to specified plasma membrane microdomains where it can be cleaved by ADAM17 and γ-secretase, culminating in downstream signal transduction events that converge on the activation of Rho GTPases at these specific membrane sites.

5.3 Role of ADAM17 in p75NTR cleavage

Our work defines an important role for ADAM17 in receptor cleavage and activation of the downstream signaling events that contribute to p75NTR-dependent Rac1 activation. ADAM17 is a transmembrane matrix metalloproteinase of the ADAMs family. Members of this family share common domains composed of a propeptide, metalloproteinase, disintegrin-cysteine-rich, epidermal growth factor-like, transmembrane and cytoplasmic domains (Mochizuki and Okada, 2007). Dissociation of the prodomain and generation of a mature active protein is believed to result from redox changes in sulfhydryl groups in the disintegrin-cysteine-rich domain referred to as the “cysteine switch” mechanism of activation (Seals and

Courtneidge, 2003). Hence, stress-induced ROS production could be an important inducer of ADAM17 activation.

ADAM17 is a sheddase for many cell surface proteins such as the proinflammatory cytokine TNFα, ligands of the EGFR, EGF, HB-EGF and TGFα, and growth factor receptors such as VEGFR2 and p75NTR (Mochizuki and Okada, 2007). Many members of the ADAM family can fulfill redundant activities; cleavage of Notch and APP can be achieved by either ADAM17 or ADAM10, that share similar specificities in their enzyme domain (Mochizuki and Okada, 2007). Similarly, ADAM17 and ADAM10 are overexpressed in glioma cells and in particular in brain tumor stem cells and they contribute to their proliferation, invasion, and self renewal (Bulstrode et al, 2012, Chen et al, 2013). ADAM17 activation is increased in tumor cells upon hypoxia through a mechanism involving the unfolded protein response (UPR) that is induced after ER stress (Rzymski et al, 2012). Interestingly, ADAM17 expression increases in glioma cells and breast cancer cells and promotes their invasion under normoxia and hypoxia conditions (Zheng et al, 2007, Zheng et al, 2009, Lu et al, 2011) through an EGFR-PI3K-Akt pathway.

The role of ADAM17 in p75NTR cleavage has been well documented. Early studies in p75NTR cleavage by α-secretase have identified ADAM17 as well as ADAM10 as p75NTR sheddases. In fact, p75NTR cleavage is not impaired in fibroblasts derived from *ADAM17-/-* animals after stimulation with PMA. Since these cells express high levels of ADAM10 this suggested that both ADAM10 and ADAM17 could serve as α-secretases for p75NTR (Kanning et al, 2003). In contrast, another study reported that p75NTR was unable to undergo cleavage in mouse embryonic fibroblasts (MEFs) lacking ADAM17, whereas MEFs lacking ADAM10 or ADAM9/12/15 have normal p75NTR cleavage upon PMA stimulation (Weskamp et al, 2004). Work from our laboratory further shows that ADAM17 is required for p75NTR cleavage and for potentiation of Trk mediated survival of PC12 cells and cerebellar granule neurons through activation of Erk and Akt pathways (Ceni et al, 2010, Kommaddi et al, 2011). These studies define ADAM17 as the only α-secretase for p75NTR. On the other hand cleavage of p75NTR by ADAM17 mediates death of sympathetic neurons (Kenchappa et al, 2010). It is also shown that ADAM17 mediates shedding of p75NTR and NgR and that it antagonizes the effect of myelin inhibitory proteins thus enhancing DRG neurite outgrowth (Ahmed et al, 2006).

Death of sympathetic neurons requires activation of p75NTR by BDNF (Bamji et al, 1998, Palmada et al, 2002, Linggi et al, 2005, Kenchappa et al, 2006, Kenchappa et al, 2010),

or proNGF (Lee et al, 2001). This mechanism involves p75NTR cleavage by ADAM17 and the activation of JNK (Linggi et al, 2005, Kenchappa et al, 2010). Interestingly, BDNF binding to p75NTR leads to an initial activation of JNK that results in ADAM17 transcriptional accumulation. This in turn mediates p75NTR cleavage that further increases JNK resulting in cell death (Kenchappa et al, 2010). This biphasic activation of JNK involves different players. In fact, the initial peak requires interaction of p75NTR with NRAGE since BDNF was unable to mediate JNK activation one hour after treatment of *nrage-/-* sympathetic neurons suggesting that NRAGE is essential for the pre-cleavage increase in JNK (Bertrand et al, 2008). The second peak and the induction of cell death require p75NTR interaction with NRIF and TRAF6 (Kenchappa et al, 2006, Linggi et al, 2005, Gentry et al, 2004). NRIF and TRAF6 are involved in the post cleavage increase in JNK. In addition to its role in ADAM17 activation, it is suggested that JNK is also necessary for γ-secretase function. In fact, JNK leads to phosphorylation of the C- terminal fragment (CTF) of APP and this promotes APP internalization and cleavage by γ-secretase (Small and Gandy, 2006). It has also been shown that $p75^{CTF}$ is internalized and it undergoes cleavage by γ-secretase in endosomes (Urra et al, 2007). Hence, it is possible that the second increase in JNK is required for the cleavage of p75NTR by γ-secretase and the release of the ICD. In line with these findings it has been shown that p75NTR-mediated death of oligodendrocytes requires the activation of JNK in a Rac1 dependent manner (Harrington et al, 2002). In addition, proBDNF induces death of cerebellar granule neurons (CGNs) through activation of a p75NTR-Rac1-JNK pathway (Koshimizu et al, 2010). However, it is not known whether Rac1 is required for ADAM17 activation, or whether Rac1 acts after p75NTR cleavage to induce the second peak of JNK that leads to cell death. Nevertheless, Rac1 is required for activation of ADAM17 downstream of TNFα in tubular epithelial cells, a pathway that enhances wound healing (Waheed et al, 2013). Similarly, activation of Rac1 is essential for ADAM10 activation and cleavage of CD44 upon EGF treatment of U251 glioma cells (Murai et al, 2006). This is also observed for PDGF-mediated APP cleavage by β- and γ-secretases (Gianni et al, 2003). Hence, it seems reasonable to speculate that Rac1 activation plays a role in the initial increase in ADAM17 activation and that cleavage of p75NTR induces additional Rac1 activity that drives further JNK activation. This second increase would be responsible for cytoskeletal changes that translate into cell spreading or cell migration.

5.4 p75NTR and Rho GTPases

Our work shows that p75NTR activates Rac1 through a NRAGE-NEDD9 signaling cascade. Using RhoA and Rac1 biosensors we also demonstrate that p75NTR increases Rac1 activity and that it induces a slight decrease in RhoA activity. Interestingly, p75NTR differentially regulates Rho GTPases in the presence of the coreceptors such as NgR1 coexpression as well as myelin inihibitory proteins such as Nogo66.

The link between p75NTR and Rho GTPases activation is not very well understood. Our appreciation of a regulatory role between p75NTR and Rho GTPases comes from studies in neurite outgrowth inhibition by myelin inhibitory proteins that bind to p75NTR and NgR and lead to outgrowth inhibition by activating RhoA downstream of p75NTR (Yamashita et al, 1999, Wang et al, 2002, Yamashita and Tohyama, 2003, Domeniconi et al, 2005, Park et al, 2010). p75NTR directly interacts with RhoGDI sequestering it away from RhoA and allowing activation of RhoA and the consecutive inhibition of neurite outgrowth (Yamashita and Tohyama, 2003). Further work aiming at deciphering the signaling mechanism through which p75NTR activates RhoA identified kalirin9, a dual Rho and Rac GEF that competes with RhoGDI for p75NTR binding. RhoGDI displaces kalirin9 from p75NTR upon activation with myelin inhibitory proteins and this allows kalirin9 to activate RhoA (Harrington et al, 2008). On the other hand, studies investigating the role of NGF on neuronal differentiation describe the requirement of different GEFs downstream of NGF in the differentiation process. It was shown that NGF mediates PC12 differentiation by antagonistically activating Rac1 and inhibiting RhoA signaling (Yamaguchi et al, 2001, Yasui et al, 2001). Trio, a dual Rho and Rac GEF, acts through Rac to induce neurite outgrowth via activation of another GTPase member, RhoG (Katoh et al, 2000, Estrach et al, 2002). Tiam1, a specific Rac GEF, is also involved in NGF-mediated neurite outgrowth of PC12 and SCG neurons in a pathway involving TrkA (Shirazi Fard et al, 2010). Similarly, kalirin5, a Rac GEF, induces PC12 cells and DRG neurons differentiation through NGF-TrkA signaling pathway (Chakrabarti et al, 2005). Although these studies do not examine the role of p75NTR, it is possible that p75NTR acts as a Trk coreceptor in this context and activates Rac through these different pathways.

Thus, it appears that activation of GTPases downstream of p75NTR highly depends on the context as well as the coreceptor it is associated with. On one hand, association of p75NTR with NgR activates RhoA possibly via kalirin9 (Harrington et al, 2008). On the other hand, association of p75NTR with TrkA would mediate Rac1 activation through Trio, Tiam1 or other kalirins. In addition, association of p75NTR with SorCS2 displaces Trio from the complex in response to proNGF and inactivates Rac1 leading to neurite retraction (Deinhardt et al, 2011). Similarly, the association of p75NTR with ephrinB3 prevents elongation of

embryonic cortical neurons through activation of RhoA (Uesugi et al, 2013). This regulation is further complicated by the finding that TROY, a p75NTR homolog, binds RhoGDI and inhibits the outgrowth of cerebellar granule neurons, and thus can compensate for p75NTR in this process (Lu et al, 2013). It is then possible that p75NTR and TROY can work independently or in conjunction to activate RhoA through the activation of different arms of the signal transduction pathway by activating different GEF or GAPs ultimately converging on activation of Rho GTPases.

Our results also show that p75NTR overexpression in COS7 cells activates Rac1 and leads to cell spreading, and we describe NRAGE and NEDD9 as new players downstream of p75NTR leading to Rac1 activation (Zeinieh et al, In Press, chapter 2). Interestingly, NEDD9 interacts with DOCK3, a Rac1 GEF, and promotes melanoma migration through activation of Rac1 (Sanz-Moreno et al, 2008, Yang et al, 2012). It is thus possible that NEDD9 is playing a pivotal role in the process of Rac1 activation through the association with specific GEFs depending on the context described. Interestingly, a recent finding shows that EphB receptors control synaptogenesis through the regulation of a complex composed of a Rac-GEF, Tiam1, and a Rac-GAP, Bcr. Tiam1 mediates EphB-induced Rac1 activation and spine formation whereas Bcr restricts Rac1 activation. This allows an optimal activation of Rac1 for correct spine formation and synaptogenesis (Um et al, 2014). p75NTR associates with ephrinA (Lim et al, 2008) and ephrinB3 (Uesugi et al, 2013) and regulate axonal outgrowth. On the other hand, ephrinA1 mediates NIH3T3 fibroblasts spreading and actin cytoskeletal rearrangement through the activation of FAK and p130Cas, the closely related NEDD9 family member (Carter et al, 2002). Interestingly, a recent finding reports that p75NTR is required for axonal polarization and specification (Zuccaro et al, 2014). p75NTR associates with the Par3 polarity complex in Schwann cells to regulate myelination (Chan et al, 2006), and it is also shown that Bcr associates with the Par complex to control cell polarization through regulating the Par-Tiam complex in astrocytes (Narayanan et al, 2013). It is thus possible that p75NTR also associates with the Tiam1/Bcr complex to regulate neurite outgrowth by controlling the fine tuning of Rho GTPase activation and maintaining a well regulated balance of Rho GTPase activity. This pathway likely involves activation of NEDD9 or other Cas family members, and depends on the coreceptor and the cell context.

Another role for p75NTR through Rho GTPases resides in myelination in the peripheral nervous system. It is proposed that different neurotrophins have distinct effects on myelination and Schwann cell migration and differentiation through activation of different receptors and signaling pathways. BDNF binding to p75NTR induces Schwann cells

differentiation and enhances myelination, whereas NT3 binding to TrkC inhibits Schwann cell differentiation and promotes their migration and proliferation (Chan et al, 2001, Cosgaya et al, 2002, Yamauchi et al, 2004). The activation of these signal transduction pathways involves a differential pattern of Rho GTPases activation. BDNF-p75NTR signaling activates RhoA through Vav2, a Rho GEF, whereas NT3-TrkC activates Rac/Cdc42/JNK pathway (Yamauchi et al, 2004) probably through activation of DOCK7, a Rac/Cdc42 GEF (Yamauchi et al, 2011). In addition to these findings, p75NTR regulates myelination through its localization with the polarity protein Par3 at the Schwann cell-axon junction (Chan et al, 2006), and this complex leads to the polarized activation of Rac1 at the onset of the myelination process (Tep et al, 2012). Interestingly, NEDD9 plays an important role in polarization. Dcas, the only Cas family homolog in *Drosophila*, is essential for correct cell polarity, and Dcas mutants have defects in E-cadherin localization together with impaired integrin signaling and cell adhesion (Tikhmyanova et al, 2010b). NEDD9 is also required for T lymphocytes polarization and migration downstream of chemokine activation (Gu et al, 2012). Although there is no evidence for NRAGE in myelination and cell polarization, studies demonstrate that necdin, a closely related MAGE family member, plays a crucial role in cell polarization and migration as well as directional extension of axons of hippocampal neurons. Necdin is essential for Cdc42 activation and myosin light chain phosphorylation that promote cell polarization (Bush and Wevrick, 2010). It is possible that NRAGE plays a similar role in cell polarization but this aspect of NRAGE signaling is still unexplored. Hence, NRAGE-NEDD9 complex appears as a potent candidate in myelination downstream of p75NTR, possibly through the association with the polarity complex including Par3, and the subsequent activation of Rho GTPases required for this process.

All these studies show the complexity of the signaling of p75NTR through Rho GTPases and suggest that p75NTR differentially activates Rho GTPases depending on the cell context as well as its upstream signaling partners such as neurotrophin binding and cognate receptors as well downstream adaptors.

5.5 p75NTR and NEDD9: a link to Rho GTPase activation?

Our work provides for the first time an association between p75NTR and NEDD9 signaling in the activation of Rho GTPases. NEDD9, a Cas family member, is associated with cell migration and regulation of cytoskeletal dynamics, transducing signals downstream of integrin ligation and focal adhesions. A growing body of evidence shows the implication of NEDD9 in Rho GTPase signaling. NEDD9 is known to associate with many GEFs and

regulates activation of Rho GTPases. Importantly, NEDD9 associates with DOCK3, a Rac GEF, and promotes the mesenchymal movement of melanoma cells by activating Rac and inhibiting Rho signaling (Sanz-Moreno et al, 2008, Yang et al, 2012). In addition, NEDD9 associates with AND-34, a Cdc42 GEF, in murine splenic B cells, and decreases polarization of B cells (Cai et al, 2003). Additionally, p130Cas, another Cas family member, highly homologous to NEDD9, interacts with many GEFs upon integrin ligation such as C3G, a Rap1 GEF, Sos, a protein with both Cdc25 and Dbl GEF domains, and DOCK180, a Rac GEF (Vuori et al, 1996, Kiyokawa et al, 1998a, Kiyokawa et al, 1998b). Interestingly, p130Cas associates with the adaptor protein CrkII upon tyrosine phosphorylation on SH2 domains and induces activation of Rac through association with DOCK180, resulting in increased cell migration and spreading (Kiyokawa et al, 1998b, Gu et al, 2001), a pathway that highly depends on the ECM substrate. In fact, p130Cas-CrkII-DOCK180-Rac is preferentially activated on laminin substrates in A549 human lung carcinoma cells expressing integrin $\alpha 3\beta 1$, whereas cells plated on fibronectin exert Rho activation with decreased cell spreading (Gu et al, 2001). Interestingly, it has been reported that NGF and EGF induce differentiation of PC12 cells through a p130Cas-CrkII pathway and CrkII mutants completely abrogate NGF dependent PC12 differentiation (Ribon and Saltiel, 1996). These results propose a mechanism associating neurotrophin and Cas signaling, and suggest that neurotrophin signaling can induce integrin engagement and cell adhesion by binding to their receptors, Trk or p75NTR. Subsequently, neurotrophin signaling would induce a signaling cascade downstream of integrins involving Cas family proteins ultimately converging on Rac activation through regulation of multiple GEFs. The work we present here is in accordance with this model, where neurotrophins secreted by COS7 cells on one hand engage integrin signaling that are activated by laminin, and on the other hand, act in an autocrine manner on p75NTR leading to recruitment of NRAGE and NEDD9 complex. NEDD9 is activated by FAK and Src downstream of integrin engagement and recruits CrkII leading to Rac1 activation possibly through activation of DOCK3 that associates with NEDD9. Activation of Rac1 promotes cytoskeletal remodeling and cell spreading in the case of COS7 cells, but could also be involved in migration of U87MG cells downstream of p75NTR.

5.6 A new role for NRAGE in cytoskeletal remodeling and cell migration

Our work shows a new role for NRAGE downstream of p75NTR in cytoskeletal remodeling as well as a role in cancer cell migration independently of p75NTR, and identifies

a novel interaction with the Cas family member, NEDD9. The interaction of NRAGE with NEDD9 links NRAGE to Rho GTPases signaling.

NRAGE is predominantly regarded as a death mediator by interacting with p75NTR in neuronal as well as non neuronal tissues (Salehi et al, 2002, Bertrand et al, 2008, Truzzi et al, 2011) but several studies have indicated that it plays a role in cell migration and cytoskeletal remodeling. NRAGE inhibits metastasis of pancreatic and melanoma cells in part through disruption of E-cadherin/β-catenin cell-cell adhesions (Xue et al, 2005, Chu et al, 2007), and NRAGE has been reported to interfere with Ror2-Src association and thereby inhibit melanoma metastasis (Lai et al, 2012). Similarly, NRAGE inhibits the tumorigenic potential of glioma stem cells by decreasing the expression of genes implicated in proliferation as well as decreasing MMP levels that are important for tumor invasion (Reddy et al, 2010). NRAGE has also been identified as an inducer of epithelial-mesenchymal transition of transformed mammary epithelial cells, apparently by downregulating E-cadherin and thereby disrupting cell-cell adhesions (Kumar et al, 2011). Interestingly, there is evidence showing that E-cadherin could modulate Rho GTPase expression. In fact, in non small cell lung carcinoma (NSCLC), E-cadherin expression is downregulated and this permits anchorage-independent growth and migration. Downregulation of E-cadherin is accompanied by an increase in RhoA-GTP or Cdc42-GTP depending on the cell line studied, and overexpression of E-cadherin abrogates Rho GTPases activation leading to decreased migration and growth (Asnaghi et al, 2010). It is thus possible that E-cadherin regulates Rho GTPases through a pathway involving NRAGE. The Asnaghi study also demonstrated that E-cadherin decreases RhoA activation by increasing its association with the Rho GAP, p190 (Asnaghi et al, 2010). It is conceivable that NRAGE could regulate this interaction, possibly through a NEDD9-dependent route. To date, there is no evidence that NRAGE interacts with, or regulates, GAPs or GEFs. However, the novel interaction with NEDD9 that we report may link these signaling pathways.

The induction of cell death by p75NTR-NRAGE interaction involves JNK and caspase3 activation (Salehi et al, 2002, Bertrand et al, 2008) and p75NTR-mediated death of oligodendrocytes requires the activation of JNK in a Rac1-dependent pathway (Harrington et al, 1998). Thus p75NTR-NRAGE mediated death also certainly involves a Rac1-JNK pathway. However, one report shows that Rac1-JNK signaling can promote the survival of glioma cells *in vitro* and *in vivo* (Senger et al, 2002) and many studies have shown a role for JNK in cell migration, and cytoskeletal remodeling events (reviewed in Sun et al, 2007, You

et al, 2013, Ebelt et al, 2013, Zdrojewska and Coffey, 2014). For example, JNK induces PC12 neurite sprouting in response to Fas signaling (Waetzig et al, 2008), as well as neuronal migration (Zdrojewska and Coffey, 2014), and JNK appears to be a potent mediator of tumorigenesis (Senger et al, 2002, Desai et al, 2013, Wei et al, 2013, Chuang et al, 2014, Min et al, 2014). It is proposed that Wnt5a signaling activates JNK in pancreatic cancer tissues and that it induces invasiveness through downregulation of E-cadherin and upregulation of MMPs (Wei et al, 2013). Moreover, in a *Drosophila* model of tumor invasion, JNK was associated with the remodeling of the cytoskeleton and was activated by Src-Rho1 (Rudrapatna et al, 2014), as well as effector caspases (Rudrapatna et al, 2013). JNK has also been shown to localize to focal adhesions and to mediate cell survival signaling downstream of FAK in a cascade involving p130Cas and Rac1 (Almeida et al, 2000). Given the wide array of cellular effects mediated by JNK signaling, it seems likely that the cell will use a variety of tightly controlled mechanisms to control this pathway. A p75NTR-NRAGE-NEDD9-Rac-JNK cascade would be expected to play different roles in cytoskeleton remodeling and cancer cell migration, probably depending on the p75NTR-activating signal, such as adhesion or ligand binding. Cell death mediated by p75NTR-Rac1-JNK could also be involving different pathways downstream of p75NTR, such as the involvement of different adaptor proteins such as NRAGE, NRIF, or TRAF6 that were all shown to induce cell death through JNK. It is also possible that the identification of NEDD9 as an important player downstream of p75NTR switches the function of the p75NTR-Rac1-JNK cascade from a death signal to a migration and cell adhesion signal. It will be very important to investigate the role of this cascade in a more physiological context such as neurite extension or cancer cell migration and to study the influence of the cell context on this signaling pathway.

5.7 Conclusions and future directions

In this work we established a signaling pathway downstream of p75NTR linking this receptor to the activation of Rho GTPases. Using the COS7 cell spreading model as well as FRET bioassay, we were able to show that p75NTR induces increased cell spreading concomitant with an increase in Rac1 activation. We demonstrated that p75NTR cleavage is an important aspect in this process. Importantly we show that p75NTR-mediated Rac1 activation requires the formation of a NRAGE-NEDD9 complex. To our knowledge, our work is the first to show an interaction between NRAGE and a Cas family member namely NEDD9, and we propose that NEDD9 links p75NTR to the activation of Rac1. We also showed that a p75NTR-NRAGE-NEDD9 cascade plays a prominent role in glioma migration.

We also provide evidence that p75NTR constitutively activates Rac1 and slightly decreases RhoA and that the presence of NgR1 and Nogo66 can shift this regulation.

The link between p75NTR signaling and Rho GTPases is still largely unexplored. The work by Yamashita and colleagues provided the first evidence that p75NTR could modulate the activation of Rho GTPases and in particular RhoA through associating with RhoGDI (Yamashita et al, 1999, Yamashita and Tohyama, 2003) and further work by other groups showed an association between p75NTR and GEFs, in particular kalirin9 (Harrington et al, 2008) and Trio (Deinhardt et al, 2011). Although, these findings shed light on a link between p75NTR and Rho GTPases, many questions still need to be answered. It will be crucial to understand how NEDD9 links p75NTR-NRAGE complex to Rho GTPase signaling. In particular what are the GEFs that are involved downstream of this complex. Is there a role for Dock3 in this pathway through its association with NEDD9? Does NEDD9 also associate with kalirin family members such as kalirin9? Moreover, signaling downstream of p75NTR-NgR1 needs further investigation and it will be important to know whether NEDD9 also acts in this pathway, linking this complex to Rho GTPases through other GEFs or GAPs. A central question remains as to understand how neurotrophins modulate the activation of the p75NTR-NRAGE-NEDD9-Rac complex and what is the role of myelin inhibitory proteins on p75NTR-NgR1 regulation of Rho GTPases. It will also be essential to investigate the role of the $p75^{ICD}$ in the regulation of Rho GTPases essentially through the identification of downstream signaling partners. In addition, it will be interesting to study the role of other p75NTR partners such as Trks, sortilin, SorCS2, and ephrins in the regulation of Rho GTPases. On the other hand, the Rho bioassay we described in this work will also be useful to study the role of other TNFRs in the regulation of Rho GTPases.

Our work defines an important role for p75NTR in cell adhesion and spreading. We did not investigate the role of integrins, in this process but available evidence indicates that p75NTR signaling is likely to be modulated by integrins. It will be of paramount importance to understand whether there is a convergence between p75NTR and integrin signaling and how ECM ligands affect p75NTR cleavage and subsequent downstream signaling events.

Understanding how p75NTR is affected by integrin ligation and how signals downstream of this receptor are transduced to Rho GTPases, will also help us to understand p75NTR signaling in cell migration, especially glioma invasion. Further investigation are needed to better explore the pathway linking p75NTR to glioma migration, a prerequisite in the development of therapies targeting key components in the evolution of the disease, and also to better understand how p75NTR downstream signaling is regulated.

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