The Netrin/ Neogenin-1 complex promotes cell migration by activating Integrin β1 through FAK, in human neuroblastoma cells

(El complejo Netrina/ Neogenina-1 promueve la migración celular al activar a Integrina β1 a través de FAK, en células de neuroblastoma humano)

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“Caminante, no hay camino, se hace camino al andar”
A mi mamá y mis abuelos que ya no están conmigo en vida,
sino que viven en mi corazón

BIOGRAPHY

I was born in Santiago, on January 7th, 1989. I had a very quiet childhood, in the company of my mother and my grandparents, where I was always instilled with a taste for science. At age 16, I decided to study Biotechnology and from there I did not stop until I got a doctorate. My life has been full of vicissitudes, trips, experiences and happy moments. I am a very restless person and until 2018 I lived in Santiago and I was a lucky one in Valdivia, where I currently reside (not for long). I love cats, cooking, reading and dreaming, enthusiastic about doing new things.

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LIST OF SYMBOLS, ABBREVIATIONS OR NOMENCLATURE

cDNA	: coding DNA
DAPI	: 4′,6-diamidino-2-phenylindole
DCC	: Deleted in Colorectal Cancer
DEPC	: Di-ethyl pyro carbonate
DNA	: deoxyribonucleic acid
DRG	: dorsal root ganglion
ECM	: extracellular matrix
FAK	: Focal adhesion kinase
FBS	: Fetal Bovine Serum
FN	: Fibronectin
FNIII	: Fibronectin type 3
GFP	: Green fluorescent protein
Ig	: immunoglobulin
IgG	: immunoglobulin isotype G
ITGB1	: integrin β1
Kb	: Kilobase
kDa	: Kilodalton
LM γ 1	: laminin γ 1
mLM1	: mouse laminin-1
mRNA	: messenger RNA
NB	: neuroblastoma
NEO1	: Neogenin-1
NEO1ICD	: Neogenin-1 intercellular fragment
NTN1	: Netrin-1
NTN4	: Netrin-4
PBS	: Saline phosphate buffer
PFA	: Paraformaldehyde
RGM	: repulsive guidance molecule
rhNTN1	: recombinant human NTN1
rhNTN4	: recombinant human NTN4
RNA	: ribonucleic acid
rpm	: revolutions per minute
Q-PCR	: Quantitative polymerase chain reaction
shRNA	: small hairpin RNA
shSCR	: scramble shRNA (control)
UNC5	: Uncoordinated 5

ABSTRACT

Cell adhesion to the extracellular matrix (ECM) is key to the regulation of cellular processes such as proliferation, survival, and migration. Mesenchymal cell adhesion requires the formation of focal adhesions, defined as multiprotein complexes that allow cell binding to the ECM. Focal adhesions involve the congregation of more than 150 proteins, which include integrins, the main ECM receptors, and the Focal Adhesion Kinase (FAK), which has as a fundamental role in the turnover of focal adhesions.

Formation and disassembly of focal adhesions are dynamically regulated during cell migration, and numerous studies show that increased expression or activity of focal adhesion proteins is associated with many human diseases. For example, increased levels and/or activities of FAK have been associated with oncogenic transformation in a variety of tissues and organs. Aside the roles commonly described for FAK in activating integrins, such as integrin β_1 (ITGB1), and modulating integrin downstream signaling, this kinase has been shown to behave as an intracellular effector of multiple signaling pathways, including Neogenin-1 (NEO1). NEO1 is a transmembrane receptor involved in tissue growth during embryogenesis, although it is also broadly expressed in adulthood. More recently, overexpression of NEO1 has been observed in a wide variety of human cancers including those of the breast, pancreas, cervix, colon and medulloblastoma. Despite the latter, its role in tumorigenesis is still controversial and remains to be elucidated.

In the context of neural development NEO1 promotes neuronal migration and axonal guidance through interaction with the extracellular Netrin (NTN) ligand family. Interestingly, NEO1 is strongly expressed in neuroblastoma (NB), a cancer derived from neural progenitors of the sympathoadrenal lineage. Therefore, it will be relevant

determining if NEO1, by interacting with NTNs, could promote the migration and metastasis of NB cells, and if this phenomenon is mediated through the activation of FAK, thus promoting the intracellular activation of the ITGB1.

It was shown that both the NEO1 receptor and its main NTN ligands family members, Netrin-1 (NTN1) and Netrin-4 (NTN4), are expressed in NB patient samples. We reveal that NEO1, in addition to promoting chemotactic migration in association with NTN4 *in vitro*, promotes metastasis *in vivo*, as demonstrated in the chicken embryo model. Recent data from the literature supports our results and allow us to suggest that the interaction between NTN4 and NEO1 might not be direct, but rather mediated by a signaling complex also made up by laminin γ1 and ITGB1. NTN1, on the other hand, acts as a chemoattractant molecule and binds directly to NEO1. In addition, we show that FAK is an downstream effector of NEO1, and that the NTN1/ NEO1 signaling complex interacts with ITGB1, in the SK-N-SH NB cells. Thus, our results suggest that NTN1/ NEO1 promotes the activation of ITGB1 through FAK, most likely through the formation of a macromolecular complex, driving cell migration. All these results are consistent with our *in vivo* observations, which show that NEO1 promotes the metastasis of SK-N-SH cells in an immunodeficient mouse model.

In summary, this thesis shows that NEO1 promotes the migration of NB cells and that its mechanism of action is via interaction with ITGB1, with FAK being an intracellular mediator of this signaling pathway. NEO1, by promoting cell migration, could play a key role in the process of NB metastasis. Therefore, the signaling complex

conformed by NTNs / NEO1 is proposed as a possible prognostic marker of the progression of NB.

INTRODUCTION

Neuroblastoma: a highly metastatic pediatric cancer

Neuroblastoma (NB) is one of the most common malignant solid tumors in children, where 95% of cases are diagnosed before 10 years old and 90% occur in children under 5 years old (Rostión *et al.*, 2005). According to the Children's Antineoplastic Drug Program (PINDA), NB represents 4.2% of childhood neoplasms in Chile (Rostión *et al.*, 2005). NB develops in the peripheral sympathetic nervous system and derives from the neural crest, which is a cell lineage transiently formed during embryogenesis that develops into a variety of tissues, including spinal and autonomic ganglia (Cheung and Dyer, 2013). Neuroblasts from the sympathoadrenal lineage migrate and differentiate into adrenal chromaffin cells and dorsal root ganglia (DRG) (Cheung and Dyer, 2013). Under pathological conditions, undifferentiated neuroblasts mature into a benign ganglioneuroma or a metastatic tumor (Choudhury *et al.*, 2012). Malignant NB is characterized by being highly vascularized, with high rates of growth, invasion, and metastasis (Choudhury *et al.*, 2012).

Metastasis, a fundamental characteristic of aggressive NB, is the ability of tumor cells to disseminate and colonize distal organs to those where tumors originate, which is highly dependent on the capacity of tumor cells to migrate and invade the extracellular matrix (Reymond *et al.*, 2013). Mechanisms involved in NB metastasis remain poorly understood, and hence, research is required in this field, in order to understand the development and progression of this malignancy.

Cell migration as a key process in metastasis

Cell migration involves the movement of cells from their origin site to a new site in response to physical and biochemical signals. It has roles in tissue formation during development, regeneration, immune responses, angiogenesis and in pathological processes such as tumor metastasis (Ridley *et al.*, 2003).

In the process of cell migration, cells extend a membrane and cytoskeleton-derived protuberance (*filopodium*) to establish the first contact with the Extracellular matrix (ECM). These extensions are stabilized by small adhesive foci, that depend on extracellular interactions of integrins with the ECM, and intracellular formation of macromolecular complexes involving proteins, such as paxilin and α-actinin (Wozniak *et al.*, 2004). These initial foci induce the formation of stress fibers, which establish focal complexes and involve a larger amount of proteins, such as talin, vinculin and the focal adhesion kinase (FAK). Subsequently, focal complexes mature and stabilize to form the so-called focal adhesions, which involve the additional recruitment of zyxin and tensin, allowing the stabilization of cellular protrusions (Figure 1). Focal adhesions are fundamental during the adhesion process and are characterized by the presence of large stress fibers (Wozniak *et al.*., 2004). On the other hand, focal adhesion disassembly, which is critical to achieve the cell migration process, involves proteolysis (mediated by calpain) of focal adhesion proteins, such as FAK, paxillin and talin (Young *et al.*, 2013). The dynamics of focal complex and focal adhesion formation are controlled by small GTPases of the Rho family, where Rac is critical to the early protrusion events, while RhoA is

required for the maturation of focal adhesions to increase the cell stability during cell migration (Wozniak *et al.*, 2004).

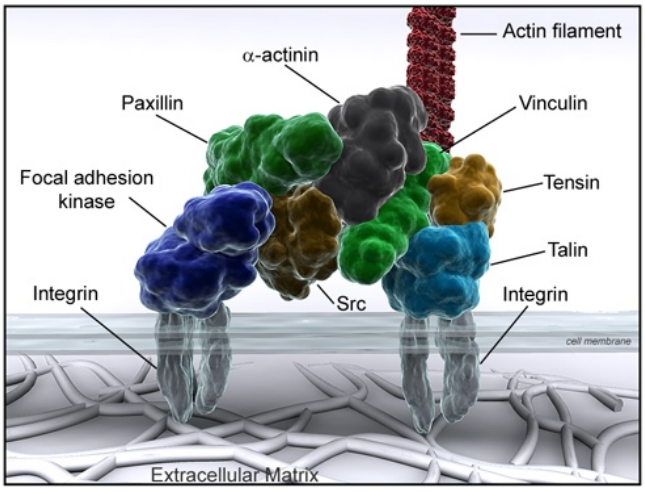

Figure 1: Illustrative diagram showing a focal adhesion complex, and the most important proteins that are involved in its formation. Adapted from http://www.reading.ac.uk/cellmigration/adhesion.htm

The actin cytoskeleton dynamics has an important role in cell migration and adhesion, as it is involved in *filopodia* and *lamelipodia* formation (Friedl and Wolf, 2003). Filamentous actin (F-actin) can be found in two possible conformations, cortical actin or forming fibrillar adhesions. Cortical actin corresponds to an actin mesh that branches and forms along the inner face of the plasma membrane (Friedl and Wolf, 2003). It also provides rigidity, associating with integrins and can be rapidly remodeled, which correlates with the dynamics of cell migration. On the other hand, fibrillary adhesions are thick and highly organized bundles in parallel (Friedl and Wolf, 2003). These structures extend from the focal adhesions to the cytoplasm and their formation is related to a lower dynamic of cell anchoring (Friedl and Wolf, 2003).

Focal adhesions form with the activation of integrins, transmembrane proteins that bind to the ECM, triggering the phosphorylation of two major components of focal adhesions, FAK and Src (Wozniak *et al.*, 2004). FAK is a critical factor that promotes both the assembly and disassembly of focal adhesions and hence is a master regulator of cell migration (Schaller, 2010). FAK is activated via autophosphorylation on tyrosine 397 (Y397), which is initiated by the interaction with ligated integrins. FAK recruitment to focal adhesions promotes its autophosphorylation, a process assisted by positive regulators residing within focal adhesions. FAK autophosphorylation generates a binding site for the tyrosine kinase Src, which phosphorylates FAK at Y576 and Y577 to fully activate its kinase activity (Schaller, 2010). FAK is important for the phosphorylation of a variety of focal adhesion components, such as paxilin and p130Cas, which are phosphorylated after integrin-mediated stimulation (Schaller, 2010).

The expression of FAK is increased in many neoplasms, such as thyroid, prostate, oral, colon, rectum, ovarian and NB cancers (Gillory and Beierle, 2010). Consequently, FAK upregulation has been frequently correlated with poor prognosis and increased metastasis (McLean *et al.*, 2005).

In several types of tumors, integrin expression correlates with an increased progression of the disease and the decrease in patient survival (Mizejewski, 1999). Although integrins alone are not oncogenic, recent data have reported that some oncogenes, such as members of the Ras family, may require integrin signaling for their ability to initiate tumor growth and invasion (Desgrosellier and Cheresh, 2010). In addition to tumor cells, integrins are also found in host cells within the tumor microenvironment, such as vascular endothelium, perivascular cells, fibroblasts and

marrow-derived platelets (Desgrosellier and Cheresh, 2010). Integrin signaling mainly regulates the contribution of these cell types to the progression of cancer, and thus, integrin antagonists have been designed and proved to inhibit tumor progression by blocking crucial signaling events both in the tumor microenvironment and in the tumor cells themselves (Desgrosellier and Cheresh, 2010). In order to elucidate the particular contribution that integrins make to the metastatic process, it is important to know how these surface receptors are activated in the context of cellular migration.

Integrin regulation and activation

Integrins, like other cell adhesion molecules, differ from cell surface receptors commonly described for soluble hormones and factors, in that they bind to their ligand with a lower affinity and are generally present at a concentration up to a hundred times higher on the cell surface (Srichai *et al.*, 2010). These glycoproteins are heterodimers formed by α and β subunits, which are non-covalently bound, and they function as adhesion molecules playing key roles in many biological processes, by participating in the organization of the actin cytoskeleton and intracellular signal transduction (Giancotti and Ruoslahti, 1999). Integrins bind to a variety of extracellular matrices and the specificity of the integrins to bind components of the ECM, including laminins, collagen and fibronectin, depends on the extracellular domains of the α and β subunits (Srichai *et al.*. 2010). Laminin receptors include the integrins $\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_6\beta_4$ and $\alpha_7\beta_1$ (Ramovs *et al.*. 2017). Integrin β_1 (ITGB1) is ubiquitously expressed and can bind multiple α pairs, playing roles in cell migration, as well as tissue remodeling and organization since, depending on the α subunit, it can bind to laminin, collagen and fibronectin (Srichai *et al.*.

2010). Once activated, integrins can also bind to soluble ligands such as Netrins and the soluble form of fibronectin, stimulating chemotactic migration, which is defined as the directional motility towards gradients of chemoattractant molecules in the ECM (Bradford *et al.*, 2009). These gradients are important for axonal guidance and angiogenesis (Bradford *et al.,* 2009).

The mechanisms of regulation and activation of integrins differ between individual integrins and according to the cell type. Integrins have a bimodal signaling: on the one hand, integrins undergo extracellular activation signaling ("outside -in") that is closely related to their role in signal transduction. The binding of integrins to extracellular ligands leads to changes the conformation of integrins and thus contributes to their clustering (Shattil *et al.*, 2010). Conversely, the intracellular activation signaling of the integrins ("inside-out"), begins when an intracellular activator, such as talin, binds to the intracellular domain of integrins (β1 or β3), allowing conformational changes that result in an increased affinity for extracellular ligands (activation of integrins). The binding of talin to β-integrin generates second messengers of signaling pathways of growth factors, such as Ca^{+2} and diacylglycerol (DAG) (Anthis, 2011). This signaling controls the strength of the adhesion and allows sufficient interactions between integrins and the ECM, where integrins transduce the force required for cell migration and remodeling of the ECM (Shattil *et al.*, 2010). Both types of signaling are linked and the combination of these two events generates intracellular signals that control cell polarity, cytoskeletal structure, gene expression and cell survival (Figure 2) (Shattil *et al.*, 2010). It has been shown that intracellular signaling precedes extracellular signaling in the cell migration process (Shattil *et al.*, 2010).

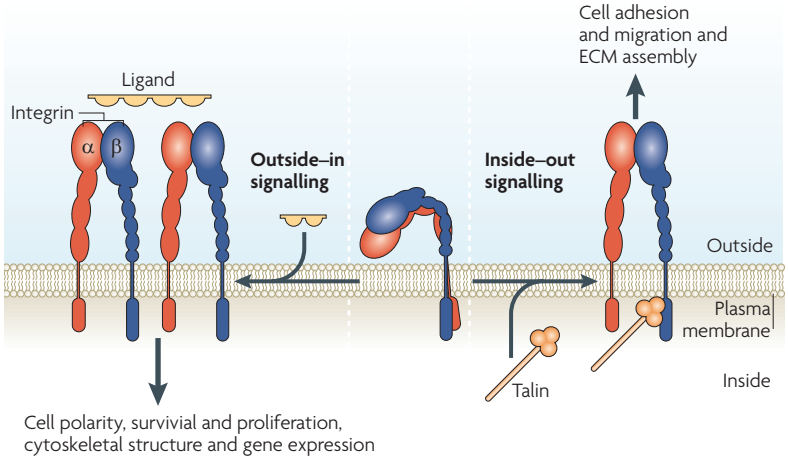

Figure 2: Bimodal signaling of integrins. The signaling of intracellular activation is produced by the binding of talin to the intracellular domain of the β integrin and changes the conformation of the dimer to a state of high affinity with the ECM and thus the union with this is produced. In extracellular activation signaling, the integrin binds to an extracellular ligand that produces changes in cell polarity, survival and proliferation, through processes that involve gene expression (Adapted from Shattil *et al.*, 2010).

Many cellular responses to soluble growth factors, such as epidermal growth factor (EGF), platelet derived growth factor (PDGF), lipoprotein A (LPA), Vascular Endothelial Growth Factor (VEGF) and thrombin, depend on the adhesion of the cell to a substrate through integrins (Hynes, 2002). Growth factors and integrins act cooperatively through this activation (Ross, 2004). For example, intracellular activation signaling has been observed during T cell lymphocyte migration via activation of its TCR receptor (Kinashi, 2005), as well as in interaction with insuling growth factor 1 (IGF-1) and other factors such as brain-derived neurotrophic factor (BDNF) (Carlstrom *et al.*. 2011). It has also been shown that the repellent axonal guide molecule Efrin, through its EphA4 receptor, inhibits Rap1 and Rap2 GTPases and, consequently, the activity of integrins (Pasquale, 2008). The latter accounts for a close relationship between the axonal guidance molecules and the activation of integrins.

Axonal guidance and neuronal migration

Axonal guidance is a process that is closely related to the machinery of focal adhesion formation (Nakamoto *et al.*. 2004). During axonal guidance, developing neurons use a combination of orientation signals to mount a functional neuronal network (Nakamoto *et al.*. 2004). Immobilized proteins from the ECM provide specific binding sites for integrin in the neurons. The integrins in the growth cones are associated with several cytosolic and signaling proteins that regulate the dynamics of the cytoskeleton and cell adhesion forming focal complexes (Nakamoto *et al.*. 2004). Evidence suggests that the ECM and classical guidance signals can direct targeting and axon growth by controlling adhesion via integrins (Myers *et al.*, 2011). The ECM can bind secreted proteins and act cooperatively with soluble axonal guide ligands to influence axon growth, and, in the growth cone, focal adhesion molecules, such as ITGB1, vinculin and paxilin, are located (Myers *et al.*, 2011). In addition, *filopodia* and *lamellipodia* are actively assembled in the growth cones, resembling the dynamics involved in cell migration, since critical activators, such as FAK and Src are also involved (Myers *et al.*, 2011).

The axonal guidance is controlled by chemotactic processes (Suter and Forscher, 2000). In chemoattraction, movement along a given trajectory is achieved by the extension of actin filaments, forming a *filopodium* towards the source of the orientation signal (Suter and Forscher, 2000). In contrast, chemo-repulsion promotes actin depolymerization and subsequent retraction of the filopodium, resulting in the collapse of the growth cone and ultimately migration away from the source of ligand (Suter and Forscher, 2000). A point of convergence of signal transduction pathways triggered by axonal guidance receptors,

is observed at the level of Rho small GTPases, which govern the directionality of cell motility by the direct binding of proteins to actin (De Vries and Cooper, 2008). Cdc42 and Rac1 mediate migration of the growth cone through an attractive signal promoting the extension of *filopodia* and *lamelipodia*, respectively, whereas the collapse of the growth cone and its retraction is mediated by RhoA (De Vries and Cooper, 2008).

Intriguingly, a relationship between axonal guidance ligands and integrins has been also established. For example, cortical GABAergic interneurons that express the integrin $\alpha_3\beta_1$ bind directly to NTN1, which is necessary for the correct migration of these neurons in the cortex (Myers *et al.*, 2011).

Axonal guidance molecules: Neogenin-1 and Netrins

Initially discovered as an axonal guidance receptor, Neogenin-1 (NEO1) is a member of the immunoglobulin superfamily of transmembrane receptors (Vielmetter *et al.*. 1997). It is a multifunctional receptor involved in axonal guidance, neuronal differentiation, morphogenesis and cell death. The expression of NEO1 is ubiquitous during embryonic development, particularly in regions where there is robust cell proliferation, differentiation and migration (Cole *et al.*. 2007). NEO1 was described as a homologue of DCC (Deleted in colorectal cancer), as these proteins share about 50% amino acid identity and possess the same secondary structure, consisting of an extracellular domain that contains four loops type Immunoglobulin and six repeated regions type Fibronectin-III (FnIII), followed by a single transmembrane region and a cytoplasmic stem, containing three domains conserved with DCC, referred to as P1, P2

and P3 (Wilson and Key, 2006) (Figure 3). The P3 domain binds to intracellular proteins that determine the varied NEO1 response. The DCC / NEO1 receptors act as homodimers or form heterodimers with the UNC5 receptor family, sharing their binding to the Netrins ligand (Park *et al.*. 2004).

NEO1 interacts not only with Netrins but also with members of the RGM ligand families (Repulsive Guidance Molecule), and thus, the interaction between NEO1 and RGMa repels axons and generates the collapse of the growth cone (Wilson and Key, 2006). On the other hand, the interaction between Netrin-1 (NTN1), the most studied Netrin ligand, and NEO1 promotes chemoattractive axonal guidance and cell migration as well as cell-cell adhesion (Wilson and Key, 2006). It was shown that NTN1 binds to NEO1, between the Fibronectin domain type III 4 and 5 of NEO1 and that it binds with a stoichiometry of 2: 2 Ligand: Receptor (Xu *et al.*, 2014). Given that the interaction of Neogenin-1 with Netrins promotes neuronal and epithelial cell migration, it can be inferred that both molecules could retain this role in a tumor context.

The family of Netrins belongs to the superfamily of laminin-like proteins, which contain five distinctive members: NTN1, -2, -3, -4 and Netrin-G. Structurally, the organization of these molecules consists of an N-terminal domain, laminin VI, a central domain, laminin V (repeats type EGF V1, V2 and V3), and a single positively charged C-terminal domain. The laminin VI-type portion of the Netrins binds to the DCC / NEO1 and UNC5 receptors and is sufficient to generate a biological response, while the single C-terminal domain has affinity for proteoglycans and can serve to localize the Netrins on the surface cellular or in the ECM (Nikolopoulos and Giancotti, 2005). On the other hand, Netrin-4 (NTN4) is the most distant member of the Netrins family in terms of its primary

sequence and in its globular structural domain, which is more related to laminin than to other Netrins (Yin *et al.*, 2000). NTN4 is broadly expressed in the nervous system during embryonic development and is maintained in adult individuals. NTN4 (Yin *et al.*, 2000) and NTN1 (Guan *et al.*, 2003) are restricted to specific areas, such as the olfactory bulb, the retina, cerebellar granule cells, hippocampal and cortical neurons, as well as in DRG neurons. The latter is relevant, since this fact could provide insights to a potential role of the NTN1 and NTN4 ligands that would be interacting with NEO1 in the cells of the DRG that originate the NB.

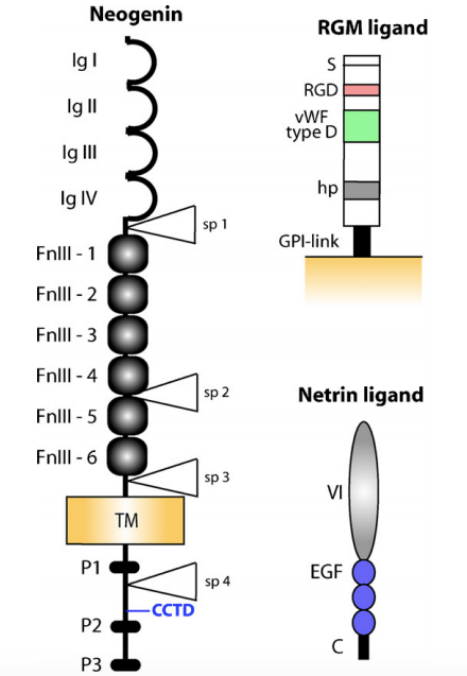

Figure 3: Scheme of the structure of NEO1 and its ligands. NEO1 consists of four Ig-like domains, six repeats of fibronectin type III (FNIII), one transmembrane domain and one cytoplasmic stem with three domains conserved with their DCC homologue, P1, P2 and P3. The P3 domain has 90% homology with the same DCC domain and is the binding site for several intracellular proteins. It also contains a substrate domain for Caspase-3, CCTD. The alternative processing sites described in the mouse sequence are indicated in triangles. In addition, the Netrin and RGM ligands of NEO1 are shown (Adapted from Wilson & Key, 2006)

NTN1, via interaction with DCC in axonal guidance, has effects on the organization of F-actin, *filopodia* formation and the extension of the plasma membrane.

This interaction regulates the growth and morphology of the growth cone and the adherence of these cells (Baker *et al.*, 2006). A relevant precedent for this thesis is that NEO1 activates FAK, by stimulating the phosphorylation on Y397 through its P3 domain, precisely through the interaction with its ligand NTN1. This was observed in three-dimensionally cultured cortical neurons, where phosphorylation of FAK on Y397 is specific for the activation of NTN1 with NEO1, since cells lacking NEO1, but expressing the receptor UNC5 instead (another Netrin receptor) failed to increase FAK phosphorylation (Ren *et al.* 2004). In addition, it was observed that NEO1 modulates the activity of FAK on Y397 in other processes such as myogenesis (Bae *et al.*, 2009). Knowing that FAK is constitutively linked to the P3 domain of NEO1, it is proposed that when NTN1 binds to the receptor, the latter is phosphorylated on tyrosine (NEO1, amino acid 1467) by a mechanism that may be associated with the binding of FAK with Src. The phosphorylation of NEO1 provides binding sites for the Src kinases which causes a positive feedback loop in which these kinases continue to phosphorylate FAK and the receptors. The tyrosine phosphatase, SHP-2, also binds to the phospho-tyrosine site in NEO1 (Ren *et al.*, 2008), suggesting a possible mechanism for the deactivation of this phosphorylation cycle.

A recent study shows that NEO1 modulates the neuronal migration guided by NTN1 as it is the case of the migration of the precursors of the olfactory interneurons that migrate through the rostral migratory current (RMS, Rostral Migratory Stream) from the zone subventricular to the olfactory bulb (O'Leary *et al.*. 2015). However, last year it was published that NTN1, produced by the floor plate of the neural tube, would not be required as a morphogen for the axonal guidance of commissural neurons but rather would act as

a haptotactic guide molecule at short distances, being secreted by the neurons (Dominici *et al.*. 2017). These results advocate a review of the prevalent model and demand further investigation to elucidate the NTN1 as the mean adhesion and / or axonal guidance molecule.

Beyond the context of the development of the nervous system, both Netrins and NEO1 are involved in general processes of organogenesis. Particularly their participation in angiogenesis has been reported, a process that shares the receptors and the axonal guidance ligands. For example, it has been reported that NTN4 participates in angiogenesis in a context-dependent manner, as it was shown that NTN4 is an anti-angiogenic factor that mediates the binding between NEO1 and UNC5B (Lejmi *et al.*, 2008). These authors also indicated that NTN4 binds directly to NEO1 and not to the UNC5B or UNC5C receptors (Lejmi *et al.*. 2008). Similarly, the silencing of UNC5B or NEO1 produced the neutralization of the inhibitory effect of NTN4, suggesting that *in vitro* both receptors are essential for its function (Lejmi *et al.*. 2008). In 2016, Reuten and collaborators (Reuten *et al.*. 2016) showed that NTN4 doesn't bind NEO1 in a direct way, rather via intermediators, contrary as previously reported. Thus, the matter is is still a subject of investigation and therefore this thesis aims to elucidate the correct mechanism of NEO1/NTN4 interaction.

As previously discussed, certain integrins interact with Netrins. For example, both integrin $\alpha_6\beta_4$ and DCC interact with NTN1 and their signaling converge to protect from hypoxia-induced apoptosis in mesenchymal cells (Son *et al.*, 2013). Also, it was observed that in chicken DRG, NTN1 causes the collapse of the growth cone of these cells grown on a high laminin substrate, while these events are absent on other substrates, such as

fibronectin or low laminin concentrations (Lemons *et al.*, 2013). In this last work, this result is related to the direct interaction of NTN1 to integrin α_6 and α_3, and the activation of ITGB1, with an increase in intracellular AMPc (Lemons *et al.*, 2013). It is shown that NTN1 produces the collapse of the growth cone under certain conditions, where the possibility of indirect roles of the interaction of NTN1 with NEO1 or UNC5 is not excluded, since they show that these receptors are also expressed in DRG (Lemons *et al.*, 2013).

Having known the roles of NEO1 in interacting with Netrins, where the ligand / receptor complex is able to promote both axonal guidance and neuronal migration and angiogenesis, it is important to consider what are the consequences of this interaction, in the context of cell migration. Furthermore, it becomes relevant to establish whether this system is de-regulated in cancer. The following paragraphs will comment on the roles of NEO1 and Netrins in cancer.

NEO1, Netrin and Integrins in cancer and neuroblastoma

The NEO1 gene is found on chromosome 15q22, a region infrequently altered in cancer (Vielmetter *et al.*. 1997). In addition, recent studies have shown that the expression of NEO1 and its interaction with NTN1 in Medulloblastoma is associated with the promotion of cell migration (Akino *et al.*, 2014) and that its overexpression in gastric cancer increases cell motility, that is, tumor cells acquire a more migratory phenotype (Kim *et al.*, 2014). NBs express NEO1 during its development and the expression of this receptor is maintained during the cancer process.

With respect to NTN1 and NTN4, its role in cancer is a matter of intense debate, since both proteins appear mainly linked to the process of tumor angiogenesis (Cirulli and Yebra, 2007). Some studies point to a pro-angiogenic function, while others, give arguments for an anti-angiogenic role (Cirulli and Yebra, 2007). The scenario is even more complex since the function of these ligands is highly dependent on the cellular context, the receptor through which they exert their function and their relative concentrations. The lack of expression of NTN4 in glioblastoma cell lines significantly decreases proliferation and motility and increases apoptosis induced by serum starvation (Hu *et al.*. 2012). It was also observed that integrin β_4 interacts with NTN4 and mediates proliferation in these cells (Hu *et al.*. 2012). In addition, high concentrations of NTN4 reduce proliferation, which is probably mediated by UNC5B (Hu *et al.*. 2012). Thus, NTN4 has biphasic functions in the modulation of glioblastoma proliferation. At low concentrations of the ligand (physiological concentrations), proliferation and cell migration are promoted, but at high concentrations, cell growth and angiogenesis in the tumor are inhibited. This is consistent with the result that NTN4 has a low expression in glioblastoma compared with healthy tissue (Hu *et al.*- 2012). On the other hand, in breast carcinoma, the expression of NTN4 is detected mostly in effusions compared to solid tumors (Yuan *et al.*, 2010). These results suggest a biological role of NTN4 in tumor metastasis.

Knowing that NTN4 is expressed in DRG neurons (Yin *et al.*, 2009), it has been suggested that this expression could be altered in a NB context. As already mentioned NTN1, like NEO1, is also expressed during the development of DRGs (Guan *et al.*, 2003). On the other hand, it is known that NB cell lines express ITGB1 (Meyer *et al.*, 2004). In

an assay performed in patients with NB it has been found that the expression of this integrin is common for all the cases analyzed (Young *et al.*, 2013). Another study revealed that in a large fraction of NBs, extracted from patients in stage 4 (final stage of the cancer: tumor metastasis in distant tissues) there was an overexpression of NTN1, which conferred a selective advantage for the survival of the cells of NB. Furthermore, it was observed that disruption of NTN1 expression inhibited metastasis in models of mouse and chicken tumorigenesis (Delloye-Borgeois *et al.*, 2009).

As in NB the expression of NEO1 is maintained and Netrins are important for its progression, it is worth asking what is the role of this interaction in this particular cancer? In turn, it is known that normal tissue expresses these molecules, so it could be expected that its role in migration is maintained in a tumor context and that its intracellular signaling participates in the activation of intracellular signaling of ITGB1, which it is known to be expressed in this cancer. It has been observed that the signaling of intracellular activation of integrins is involved in metastasis processes mainly through the activation of a specific integrin, ITGB1 (Kato *et al.*, 2011), which increases tumor cell metastasis.

In summary, we propose a mechanism by which NEO1 could be involved in cell migration, via interaction with Netrins, allowing FAK activation and thus, generating a cascade of signaling that promotes the activation of ITGB1 (figure 4).

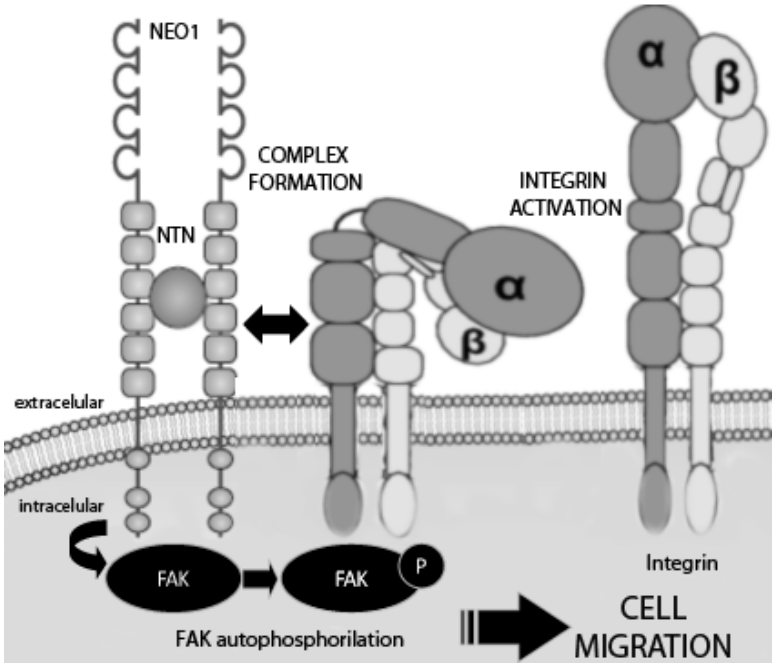

Figure 4: Model of the proposed NTN / NEO1 signaling complex for SK-N-SH cells, a NB cell line. NTN interacts with NEO1, forming a complex with ITGB1, which could promote the auto-phosphorylation of FAK in Y397 and the activation of ITGB1. This signaling promotes cell migration and, consequently, metastasis.

HYPOTHESIS

The NTN / NEO1 complex induces ITGB1 activation mediated by FAK, promoting cell migration, in human NB cells.

GENERAL AIM

To determine if the NTN / NEO1 complex induces the activation of ITGB1 mediated by FAK, promoting cell migration in human NB cells.

SPECIFIC AIMS

1. Determine if NEO1, in its binding to the NTN ligands, promotes cell migration *in vitro* in human NB cells.

2. Elucidate whether the NTN / NEO1 complex promotes the activation of ITGB1 through FAK, promoting the migration of human NB cells.

3. Evaluate the role of NTN / NEO1 in the metastasis of human NB cells *in vivo*.

    3.1. Chick embryo chorioallantoic membrane assay

    3.2. Metastasis assay in immunodeficient mice

RESULTS

These aims are represented in three manuscripts and publications, and then, the articles were attached in a chronological form, entitled and numbered. Then, the figures corresponding to each of the aims are mentioned. The articles are:

1. The Netrin-4/ Neogenin-1 axis promotes neuroblastoma cell survival and migration
2. The Netrin-4/Laminin γ1/ Neogenin-1 complex mediates migration in SK-N-SH neuroblastoma cells
3. Neogenin-1 promotes Integrin β1 activation via FAK leading to Neuroblastoma cell migration

First of all, we evaluated the expression of NEO1 and Ligands (NTN1 and NTN4) in SK-N-SH cells and patient samples:

- NEO1 and Ligands expression in SK-N-SH cells in publication 1, figure 1 (NEO1 and NTN4), page 23 and supplementary figure 1 (NTN1), page 37.
- NEO1 expression in patient samples, publication 3, figure 1, pages 91-92.
- NTN1 expression in patient samples, publication 3, supplemental figure 1, page 103.
- NTN4 expression in patient samples, publication 2, figure 1, page 57 and supplemental figures page 62-63.

1. Determine if NEO1, in its binding to the NTN ligands, promotes cell migration *in vitro* in human NB cells.

- In relation with NTN4: Publication 1, figure 4, page 27, Publication 2, figure3, page 60.
- In relation with NTN1: Publication 3, figure 2, page 93.

2. Elucidate whether the NTN / NEO1 complex promotes the activation of ITGB1 through FAK, promoting the migration of human NB cells.

- NEO1, NTN4, LMγ1 complex formation, publication 1 (NEO1-NTN4), figure 1, page 23; publication 2 (NEO1- LMγ1), figure 3, page 60.
- ITGB1, NTN1 and NEO1 complex formation, publication 3, figure3, page 94.
- FAK is a downstream molecule in NEO1/NTN1 signaling pathway, publication 3, figure 4, page 95.
- NEO1 promotes ITGB1 activation via FAK, publication 3, figure 5, page 96-97.

3. Evaluate the role of NTN / NEO1 in the metastasis of human NB cells *in vivo*.

3.1. Chick embryo chorioallantoic membrane assay: Publication 1, figure 6, page 29.

3.2. Metastasis assay in immunodeficient mice: Publication 3, figure 6, page 98.

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Research Paper

The Netrin-4/ Neogenin-1 axis promotes neuroblastoma cell survival and migration

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ABSTRACT

Neogenin-1 (NEO1) is a transmembrane receptor involved in axonal guidance, angiogenesis, neuronal cell migration and cell death, during both embryonic development and adult homeostasis. It has been described as a dependence receptor, because it promotes cell death in the absence of its ligands (Netrin and Repulsive Guidance Molecule (RGM) families) and cell survival when they are present. Although NEO1 and its ligands are involved in tumor progression, their precise role in tumor cell survival and migration remain unclear. Public databases contain extensive information regarding the expression of NEO1 and its ligands Netrin-1 (NTN1) and Netrin-4 (NTN4) in primary neuroblastoma (NB) tumors. Analysis of this data revealed that patients with high expression levels of both NEO1 and NTN4 have a poor survival rate. Accordingly, our analyses in NB cell lines with different genetic backgrounds revealed that knocking-down NEO1 reduces cell migration, whereas silencing of endogenous NTN4 induced cell death. Conversely, overexpression of NEO1 resulted in higher cell migration in the presence of NTN4, and increased apoptosis in the absence of ligand. Increased apoptosis was prevented when utilizing physiological concentrations of exogenous Netrin-4. Likewise, cell death induced after NTN4 knock-down was rescued when NEO1 was transiently silenced, thus revealing an important role for NEO1 in NB cell survival. *In vivo* analysis, using the chicken embryo chorioallantoic membrane (CAM) model, showed that NEO1 and endogenous NTN4 are involved in tumor extravasation and metastasis. Our data collectively demonstrate that endogenous NTN4/NEO1 maintain NB growth via both pro-survival and pro-migratory molecular signaling.

INTRODUCTION

Cancer is a complex chronic disease, characterized by the uncontrolled growth and dissemination of tumor cells. Within the several varieties of cancer, pediatric solid tumors represent about 30% of pediatric cancers, including brain tumors, rhabdomyosarcoma, Wilms' tumor, osteosarcoma, and neuroblastoma (NB) [1]. In general, these tumors arise as a result of the imbalance between proliferation/apoptosis and cell differentiation during development [2]. Particularly, NB arises from neural crest cells in the symphatoadrenal lineage that develops from the dorsal root ganglion (DRG) and adrenal gland [3]. Little is known about the specific genes and signaling pathways that are involved in the development and spread of this aggressive and highly metastatic disease

[3]. Hence, it is important to understand its etiology and the molecular mechanisms involved in tumor onset and progression control.

In this context, an important molecule is NEO1, which is expressed during the development of the DRG [4]. NEO1 is a member of the immunoglobulin superfamily of transmembrane protein receptors, involved in a variety of features associated with tumor progression [5] including proliferation [6], angiogenesis [7], apoptosis [8], and migration [9] in several tissues, during development and in adult homeostasis [reviewed in [10]). Both NEO1 and its homologue, the Deleted in Colorectal Cancer receptor (DCC) [10], act as dependence receptors that induce apoptosis in the absence of their ligands (dependence factors) [11]. DCC primarily signals upon binding of the Netrin family ligands [12], whereas NEO1 signals through ligands of the Repulsive Guidance Molecule (RGM) family [8]. However, recent research has proposed that NEO1 may also act via Netrin ligands, thus avoiding its pro-apoptotic activity and promoting a survival factor function [13] in certain physiological contexts as described in β-pancreatic islets [14].

The Netrin ligands belong to the superfamily of laminin type proteins, which include five distinct members including Netrin-1, -2, -3, -4, -5 and –G, where NTN1 and NTN4 are the most characterized ligands [13]. Structurally, these molecules consist of an N-terminal domain, laminin VI, a central domain, laminin V (EGF repeated V1, V2, and V3), and one positively charged C-terminal domain. NTN4 is the most distant member of this family because its primary sequence and globular structural domain are more similar to that of Laminin V than to that of other Netrins [15]. Netrins bind to their receptors, DCC/NEO1 via the Laminin type VI domain [13]. Only the C-terminal domain of the ligand has affinity for proteoglycans and thus serves to locate Netrins to the cell surface or in the extracellular matrix (ECM) [16].

NTN1 is a critical axonal guidance protein during embryonic development, cell migration and morphogenesis, and its expression has been reported in the DRG [4]. Regarding NTN4 expression, it widely encompasses the nervous system during embryonic development and it is maintained in adult individuals. Its expression is centered in the olfactory bulb, retina, cerebellar granule cells, hippocampal and cortical neurons, and in DRG neurons [15]. The former is of interest, considering the fact that NEO1 is expressed in the DRG, suggesting a possible interaction between Netrins/NEO1 in cells that give rise to NB.

The Netrin family of ligands is highly involved in a variety of processes associated with tumor progression, however their specific contribution remains controversial. Although NTN1 was initially reported to be downregulated in NB [17], a more recent clinical study revealed that NB tissues from stage-4 patients exhibited an overexpression of NTN1, conferring a selective advantage for survival of NB cells. Disrupting the expression of NTN1 further inhibited metastasis in mouse and chicken models of NB tumorigenesis [18]. Furthermore, studies *in vitro* and patient samples have demonstrated that the interaction between NEO1 and NTN1 is associated with cell migration and invasiveness in medulloblastoma, another pediatric malignancy [19].

Contrary to the description and analyses of NTN1 contribution in pediatric cancer, the expression of NTN4 has not yet been characterized. In glioblastoma, NTN4 has been proposed to depict a biphasic function: at low physiological ligand concentrations, both proliferation and cell migration increase, whereas at high concentrations, tumor cell growth is inhibited. Reduced NTN4 expression in glioblastoma cell lines induced by serum starvation significantly decreases proliferation and motility, increasing apoptosis. This is consistent with the low expression of NTN4 in glioblastoma cells compared to its expression in healthy tissue [20]. Endogenous NTN4 also induces migration and proliferation in gastric cancer cells [21]. In breast carcinoma, NTN4 expression is most commonly detected in solid tumors than in malignant pleural effusions [22]. Combined, these findings suggest a possible biological role for NTN4 in tumor metastasis.

Since both NEO1 and Netrins are expressed in the DRG neuronal progenitors, which give rise to NB, their relationship and interaction might be relevant in the oncogenic context. However, little is known about the function that NEO1 plays in NB progression, or about the autocrine expression of Netrin ligands. Here, we provide evidence about novel roles of the NTN4/NEO1 complex in NB cell migration, survival, and *in vivo* metastasis. Furthermore, our data contribute to the identification and characterization of new therapeutic targets to inhibit NB tumor growth.

RESULTS

Expression of NEO1 and Netrins in NB samples and cell lines

In order to determine the expression of NEO1 and its ligands, NTN1 and NTN4, in primary NB tumors, and to further correlate the patient prognosis with patient survival, we reviewed public available data from R2: Genomics Analysis and Visualization Platform (http://r2.amc.nl). Specifically, we analyzed the Versteeg data set [23] that includes information from 88 patients. Our analysis depicts that high levels of *NEO1* mRNA (n=58) and *NTN4* mRNA (n=32) are associated with overall lower patient survival rates (raw p value: 0,056 and 0,0014 respectively), as seen in Figure 1A and 1B, respectively. This suggests that NEO1 and its ligand NTN4 have potential roles in NB progression. Conversely, higher

NTN1 mRNA expression (n=8) was found to be associated with lower patient survival rate (Supplementary Figure 1A).

Considering this evidence, we first sought to determine the expression of NEO1 and its ligands in two subsets of NB cell lines: SK-N-SH (*MYCN* WT), LAN-1 and NB1691 (*MYCN* amplified). As seen in Figure 1C, NEO1 is expressed in all NB cell lines studied, especially in SK-N-SH. In addition, NEO1 expression was higher in SK-N-SH, when compared to other cancer cell lines such as DAOY (medulloblastoma), U87 (glioblastoma), and HEK293 cells (Supplementary Figure 1B). Whereas NTN1 protein was barely expressed in the SK-N-SH cell line, it was detected in the two *MYCN* amplified NB (Supplementary Figure 1C). In addition, the SK-N-SH cell line did not exhibit RGMa protein expression (Supplementary Figure 1D), whereas LAN-1 did. Western Blot analysis confirmed the expression of NTN4 by the *MYCN* WT SH-SY5Y and SK-N-SH cell lines (Figure 1D). Of note, NTN4 band is predicted at 69 kDa, but we detected a single band at 90 kDa, possibly due to post-translational modifications of the protein [24]. qPCR analysis revealed that SK-N-SH and LAN-1 express *NEO1* (data not shown) and *NTN4* mRNA (Figure 1E). Next, we evaluated the

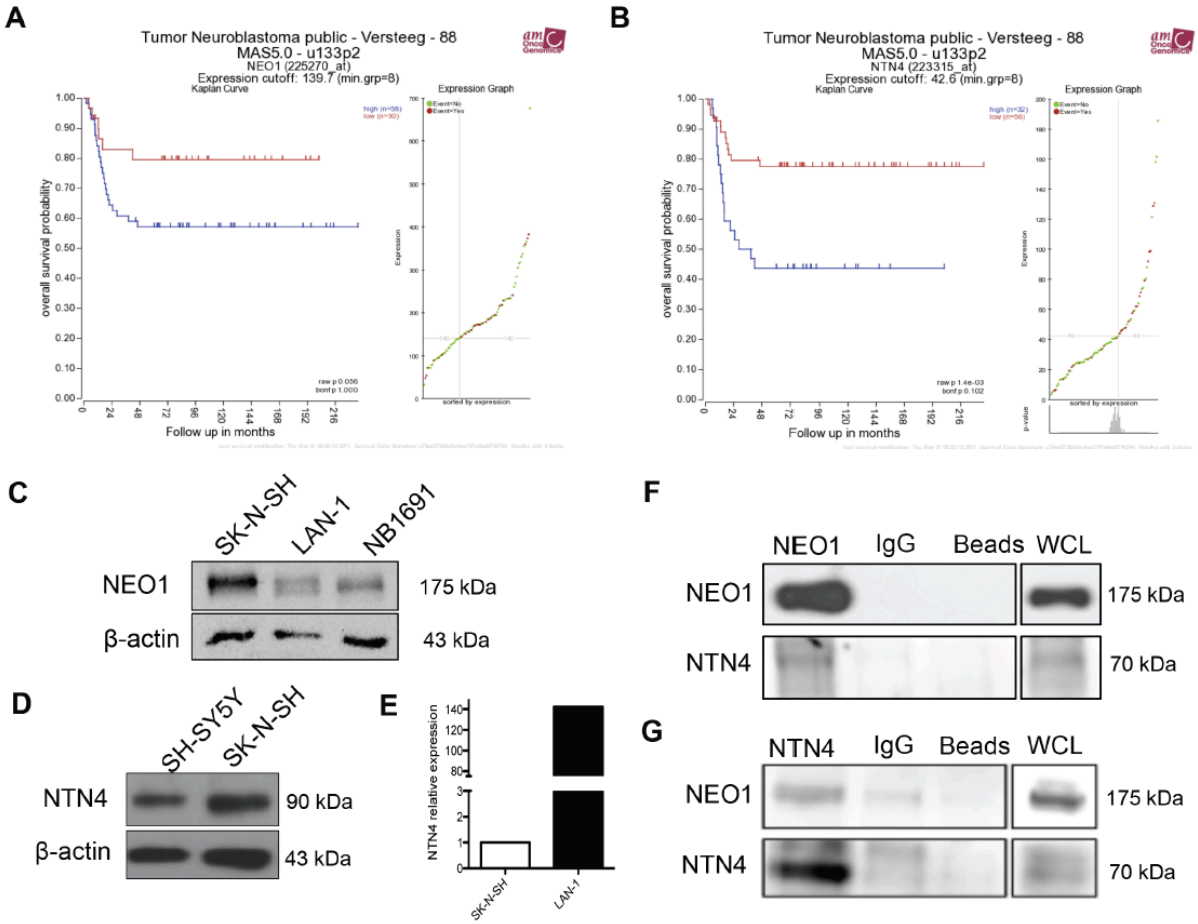

Figure 1: Clinical significance of NEO1 and NTN4 expression and characterization of NB cell lines. A, B. Analysis was performed using R2 (http://r2.amc.nl) and public primary tumor NB database from 88 patients (Versteeg). The data set is separated into two categories, high and low mRNA, depending on where the values lie in relation to the median value: values above the median are high mRNA levels and those below the median, are low mRNA levels. These values are then plotted against patient survival rate in a Kaplan-Meier estimate plot. Observed is the overall survival rate according to mRNA expression of *NEO1* (A) and *NTN4* (B). **C.** Western blot against NEO1 in NB cell lines SK-N-SH, LAN-1 and NB1891. **D.** NTN4 expression in NB SH-SY5Y and SK-N-SH cell lines. Of note, NTN4 band is predicted at 69 kDa, but we detected a single band at 90 kDa, possibly due to post-translational modifications of the protein, as reported in the human Netrin-4 datasheet (R&D systems). **E.** Q-PCR analysis showing NTN4 expression in SK-N-SH and LAN-1 cells. **F, G.** Representative Western blots of protein co-immunoprecipitation assays used to evaluate interaction between NEO1 and NTN4 in SK-N-SH cells. Cells were treated for 1h with exogenous Netrin-4 (200 ng/ml) and then incubated using specific antibodies against either NEO1 (F) or NTN4 (G) followed by Western blot against NEO1 and NTN4.

putative association in a complex between NEO1 and NTN4 in SK-N-SH cells, by co-immunoprecipitation. SK-N-SH cells were incubated for 1h with exogenous Netrin-4 (200 ng/ml) and immunoprecipitation was performed with specific antibodies against NEO1 (Figure 1F) or NTN4 (Figure 1G). Our Western blot analysis shows clearly that NTN4 and NEO1 interact in SK-N-SH, favoring the hypothesis that there could be a functional relationship between these proteins in NB.

Silencing NTN4 increases apoptosis in NB cells

Having assessed the expression and the interaction of NEO1 and NTN4 in both NB tumors and cell lines, we next decided to evaluate the potential contribution of the NEO1/NTN4 complex signaling in a variety of processes associated with tumor progression. It has been demonstrated that NTN4 is a survival factor in other cellular contexts [14] and NEO1 has been shown to behave as a death dependence factor [8]. Therefore, we sought to characterize the role of both proteins in our panel of tumor cell lines by interfering their expression. Knock-down cells for NEO1 (shNEO1) and NTN4 (shNTN4) were generated in SK-N-SH cells and in a *MYCN* amplified LAN-1 cells, using a scrambled sequence as negative control (shSCR). NTN4 expression was reduced by 70% (Supplementary Figure 2A) in SK-N-SH cells and 60% in LAN-1 cells (Supplementary Figure 2B). The expression of NEO1 was reduced by 60% in SK-N-SH (Supplementary Figure 2C) and LAN-1 cells (Supplementary Figure 2D).

In order to evaluate the potential role of NTN4 in cell survival, we first analyzed whether silencing endogenous NTN4 increases apoptosis in LAN-1 cells grown under serum starvation. shNTN4 LAN-1 cells were cultured without serum for 24 h and dead cells were analyzed using propidium iodide staining, while Hoechst was used to stain total nuclei (Figure 2A). We observed an increment of cell death in shNTN4 LAN-1 cells, when compared to shSCR control (Figure 2B). Next, we analyzed whether silencing endogenous NTN4 also increases apoptosis in SK-N-SH cells grown under serum starvation, and if so, whether this could be reverted with exogenous Netrin-4. As NEO1 mediates apoptosis signals through caspase-3 [25], we conducted immunofluorescence on shSCR and shNTN4 SK-N-SH cells labeled with an antibody against the cleaved caspase-3 epitope (Figure 2C). Quantification of the fluorescence demonstrates that shNTN4 cells have a greater cleaved-caspase-3 signal compared to that of the shSCR cells under serum-starvation. This signal was already reduced in the presence of minimal exogenous concentration of Netrin-4 (50ng/mL), and was further diminished with higher Netrin-4 concentrations (Figure 2D). Recombinant RGMa (100ng/ml) was used as a positive control of NEO1-mediated apoptosis avoidance [7, 8]. A TUNEL assay, conducted under serum deprivation, corroborated that shNTN4 SK-N-SH cells showed an increase of TUNEL positive cells compared with the control shSCR cells, depicting higher apoptosis in shNTN4 cells (Figure 2E). shNTN4 cells treated with exogenous Netrin-4 exhibited reduced apoptosis (Figure 2F). Since knocking down the ligand resulted in increased apoptosis, we conclude that endogenous NTN4 behaves as a survival factor in NEO1-expressing NB cells.

Interestingly, we also noticed a diminished proliferation after silencing NTN4 in SK-N-SH cells (Supplementary Figure 3A-3C), although this could be the result of increased cell death in shNTN4 cells.

Apoptosis induced by NTN4 silencing is reversed upon NEO1 knock-down

NEO1 has been suggested to be a dependence receptor in specific cellular contexts driving positive cell signaling (proliferation, migration, survival), but whether or not NTN4 controls its pro-apoptotic role remains unknown. Having demonstrated that NTN4 is a survival factor in NB cells, we next analyzed whether NEO1 acts as its death dependence factor and as such, if its knock-down can revert the apoptosis induced by NTN4 silencing. For this purpose, we transfected siRNAs against NEO1 in shNTN4 SK-N-SH cells. siRNA efficiency was analyzed via Western blot and since siNEO1(1) reduced NEO1 protein levels significantly (Figure 3A), this siRNA sequence was chosen for further analysis. shNTN4 cells were transfected with siNEO1(1) and siControl and 48 h later, cells were serum starved for 48 h and fixed. Apoptosis was evaluated via TUNEL (Figure 3B) and immunofluorescence against Cleaved-Caspase-3 (Figure 3C). Both assays showed a significant decrease in apoptosis in the shNTN4 background when silencing NEO1, indicating that NTN4 is a survival factor that controls NEO1 pro-apoptotic activity.

NEO1 and NTN4 promote *in vitro* chemotactic cell migration

Considering that NEO1 is involved in neuronal cell migration [9], we aimed to address whether this function was relevant in the tumor context and if it was dependent of its ligand NTN4. Using transwell chemotaxis assays, we examined the function of NEO1 in sensing and guiding tumor NB cells, and analyzed whether NTN4, a known chemotactic molecule, influenced this process. Previous studies have revealed a dose-dependent effect of NTN4 in glioblastoma cell proliferation and motility [20]. Therefore, we compared the migratory behavior of shNEO1, shNTN4, and shSCR LAN-1 in the presence of 100ng/ml of Netrin-4 as a chemoattractant in the bottom chamber (representative images shown in Figure 4A). We observed that shSCR and shNTN4 LAN-1 cells migrate positively towards the chamber containing Netrin-4 (Figure 4B). Conversely, shNEO1 cells cannot migrate

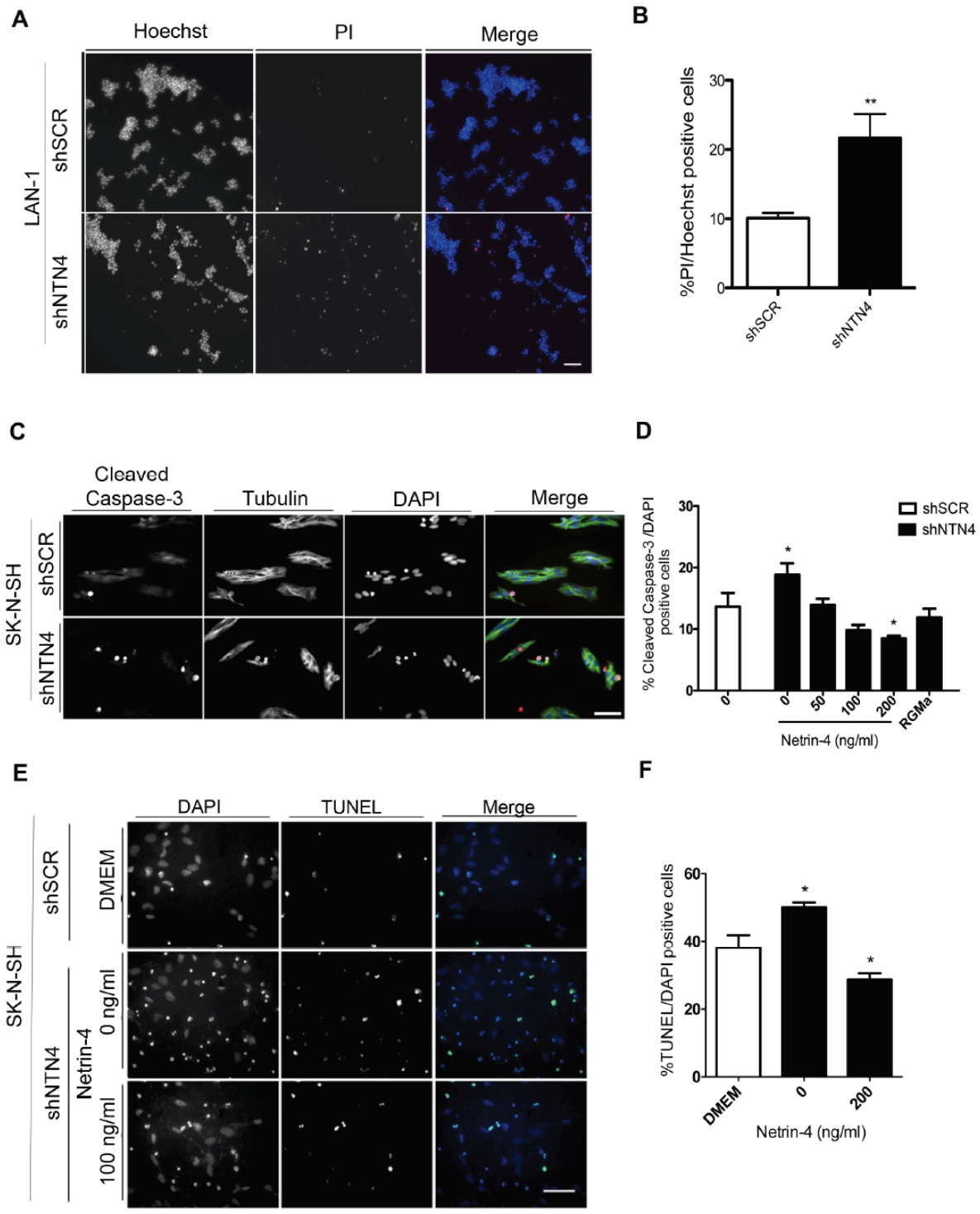

Figure 2: Silencing of NTN4 increases apoptosis in NB cells; exogenous Netrin-4 decreases this effect. A, B. Representative images of propidium iodide (PI) staining in shSCR and shNTN4 LAN-1 cell cultures. Cells were serum deprived for 24 h and stained with PI and Hoechst; cell death quantification is shown in (B) *p<0,05 shSCR v/s shNTN4. Bar: 200µm. **C.** Representative images of immunofluorescence against cleaved caspase-3 in shSCR and shNTN4 SK-N-SH cells. **D.** Quantification of cell death rescue experiments in SK-N-SH cells. Cells were treated for 24 h in serum free media adding human recombinant Netrin-4 at concentrations as described. Human recombinant RGMa (100ng/ml) was used as a control. Bar:100µm **E.** Representative images of TUNEL assays made in shSCR and shNTN4 SK-N-SH cells. Cells were incubated with serum free media with or without NTN4 (200 ng/ml); quantification is shown in **F.** *p<0,05 shSCR v/s shNTN4 (0 and 200 ng/ml). Bar:100 µm.

positively in presence of Netrin-4 (Figure 4B), therefore, these cells cannot sense Netrin-4 as a chemotactic molecule, *ergo* disabling the cell migration execution. Next, we wondered if this mechanism was conserved in *MYCN* WT cells, such as SK-N-SH cells, by using shSCR, shNEO1 and shNTN4 cells, and by increasing concentrations of exogenous Netrin-4 (25, 50, 100 ng/ml) in the bottom chamber (Figure 4C). The migration of shSCR cells augmented with increasing concentrations of NTN4 (Figure 4D). Our results depict a negative chemotaxis response due to the fact that shNEO1 cells did not sense NTN4, which is in line with the results observed for the LAN-1 NB cell line. Strikingly, both shNEO1 and shNTN4 cells migrated less than shSCR cells and their migration did not improve with increasing concentrations of Netrin-4 (Figure 4D). Even when we tested higher concentrations of Netrin-4 (200 and 500 ng/ml) as a chemoattractant stimulus for shNTN4 SK-N-SH cells, we did not observe any increases in cell migration (data not shown). Previous reports have shown that endogenous NTN4 promotes cell migration in gastric cancer cells [21]. Indeed, NTN4 is required for proper endothelial cell migration, adhesion, and focal adhesion contacts, through the binding with $a_6\beta_1$ integrin [26]. This suggests that NTN4 might be necessary to initiate NB cell migration, possibly acting through non-canonical receptors. The difference in shNTN4 cell migration between the two NB cell lines is probably due to the differential NTN1 expression, which is not present in the SK-N-SH cells, but is expressed in LAN-1 cells. This protein partially rescued the shNTN4 phenotype in the second cell line (Supplementary Figure 1C). Together, these results suggest that NEO1 promotes cell migration through NTN4 in NB.

We decided to complement our SK-N-SH cell migration analysis by performing a functional complementary approach, such as the wound healing assay, in presence of low serum (2,5%). shNEO1 cells migrated significantly less, when compared to shSCR and shNTN4 (Figure 4E). Quantification of the wounded area is shown in Figure 4F. The impaired wound closure in shNEO1 cells confirmed the importance of NEO1 in NB cell migration, and thus in tumor migration.

NEO1 regulates apoptosis and cell migration through NTN4

Our previous results demonstrate that silencing NTN4 increases apoptosis, an effect that is reverted after transient NEO1 knock-down (Figure 3). In order to validate whether NTN4/NEO1 acts as a signaling complex in NB we analyzed the effect of NEO1 overexpression in SK-N-SH cells. Relative protein expression analysis revealed a two-fold increase in the levels of NEO1 in NEO1GFP compared to cells that were transfected with an empty vector (EV) (Supplementary Figure 2E, 2F). In agreement with our hypothesis, results indicate that overexpressing NEO1 (NEO1GFP) increased TUNEL positive cells compared to control EV cells, suggesting an increase in cell apoptosis (representative images shown in Figure 5A). Introducing exogenous Netrin-4 reduced the number of TUNEL positive cells, suggesting a reduction of apoptosis (Figure 5B). To determine whether NTN4 acts as a survival factor specifically through NEO1 or as a general survival factor, we overexpressed TrkC, a dependence receptor that acts as a tumor suppressor in NB [27] and repeated TUNEL assays. As expected, TrkC induced apoptosis in SK-N-SH cells (Supplementary Figure 3D). However, exogenous Netrin-4 did not reduce TUNEL positive cells overexpressing TrkC, suggesting that NTN4 acts as a survival factor specifically via NEO1. Taken together, these results indicate that NEO1 triggers apoptosis in the absence of NTN4, whereas the induction of cell death is inhibited if NEO1 binds to its ligand.

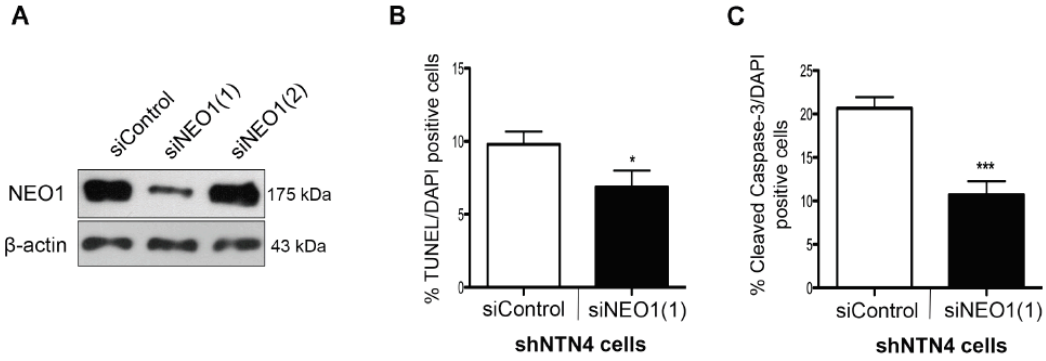

Figure 3: NEO1 knock-down reverses apoptosis induced by NTN4 silencing in SK-N-SH cells. A. Two siRNA sequences were tested to silence NEO1 in NTN4 knock-down cells. Western blot revealed efficient silencing of NEO1 with the siNEO1(1) sequence. **B.** Quantification of TUNEL assay in 48 h serum deprived shNTN4 cells transfected with siRNAs as indicated. *p<0,05 siControl v/s siNEO1(1). **C.** Quantification of immunofluorescence against cleaved Caspase-3 made in 48 h serum deprived shNTN4 cells transfected with either siControl or siNEO1(1) *p<0,05 siControl v/s siNEO1(1).

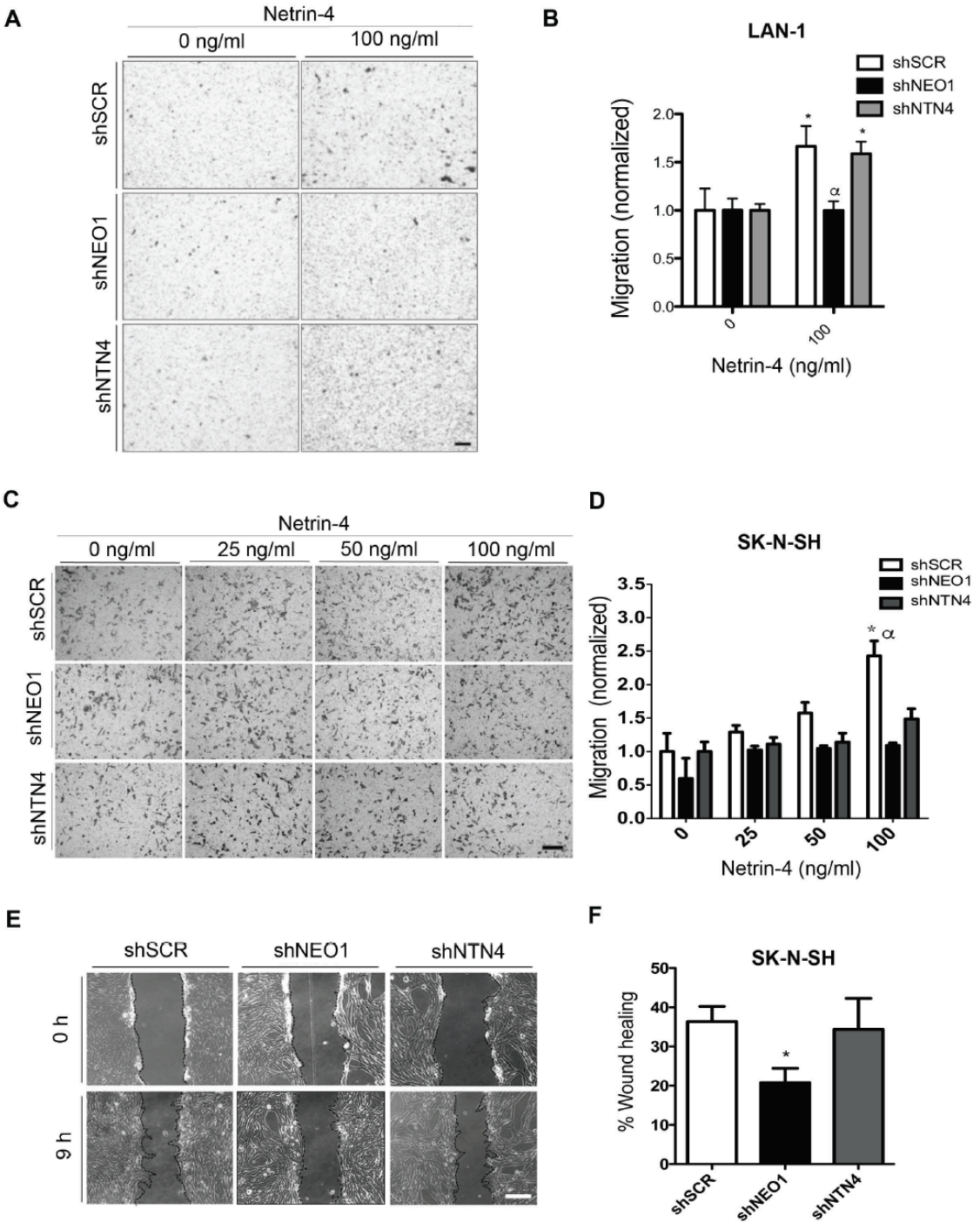

Figure 4: NEO1, acting through NTN4, promotes *in vitro* chemotactic cell migration in NB cells. A. Representative images of transwell assay with LAN-1 knock-down cells. shSCR, shNEO1, and shNTN4 LAN-1 cells migrated for 6 hours under exogenous Netrin-4 (100 ng/ml), added to the serum free media in the botton chamber. Bar:100µm. **B.** Quantification of LAN-1 transwell assay * p<0,05 shSCR or shNTN4 0 v/s 100ng/ml NTN4, α p<0,05 shSCR or shNTN4 v/s shNEO1. **C.** Representative images of transwell assay made with SK-N-SH knock-down cells. shSCR, shNEO1, and shNTN4 SK-N-SH cells migrated for 4 hours under different concentrations of exogenous Netrin-4 present in the serum free media placed in the botton chamber. Bar:100µm. **D.** Quantification of transwell assay * p<0,05 shSCR 0 v/s 100ng/ml NTN4, α p<0,05 shSCR v/s shNEO1 or shNTN4. **E, F.** Wound healing assays of stable knocked-down SK-N-SH cells using shRNAs as indicated. Representative images (E) were quantified after 9 hours (F). Bar:100µm; *p<0,05 shSCR v/s shNEO1.

Finally, we decided to analyze the effect of NEO1 overexpression in the migratory assay. For that, we transfected SK-N-SH cells with either a GFP-tagged NEO1 (NEO1GFP) or the intracellular fragment of NEO1 (NEO1ICDGFP), which lacks the extracellular domain that binds to the ligands. Supplementary Figure 2E shows a scheme of the full-length NEO1 protein indicating the NEO1ICD domain localization. Transfection efficiency obtained was around 50%. We performed a transwell assay with NEO1GFP, NEO1ICDGFP, and EV cells using 100ng/mL of Netrin-4 as a chemotactic stimulus (representative images shown in Figure 5C). As shown in Figure 5D, NEO1GFP overexpressing cells migrated more than the control EV cells (α p<0,05) when the chemotactic stimulus was introduced, while NEO1ICDGFP cells did not exhibit significant migration. The lack of significant migration indicates that NEO1ICDGFP transfected cells cannot sense the NTN4 stimulus efficiently, probably due an interference of NEO1ICDGFP with the downstream signaling activities of the endogenous full length NEO1. Therefore, in NB cells, endogenous NTN4 acts as a survival factor and induces cell migration, both through NEO1 binding. Summarizing these results, we can conclude that, in NB cells, there is a delicate balance between apoptosis and cell migration controlled by the NEO1/NTN4 signaling axis.

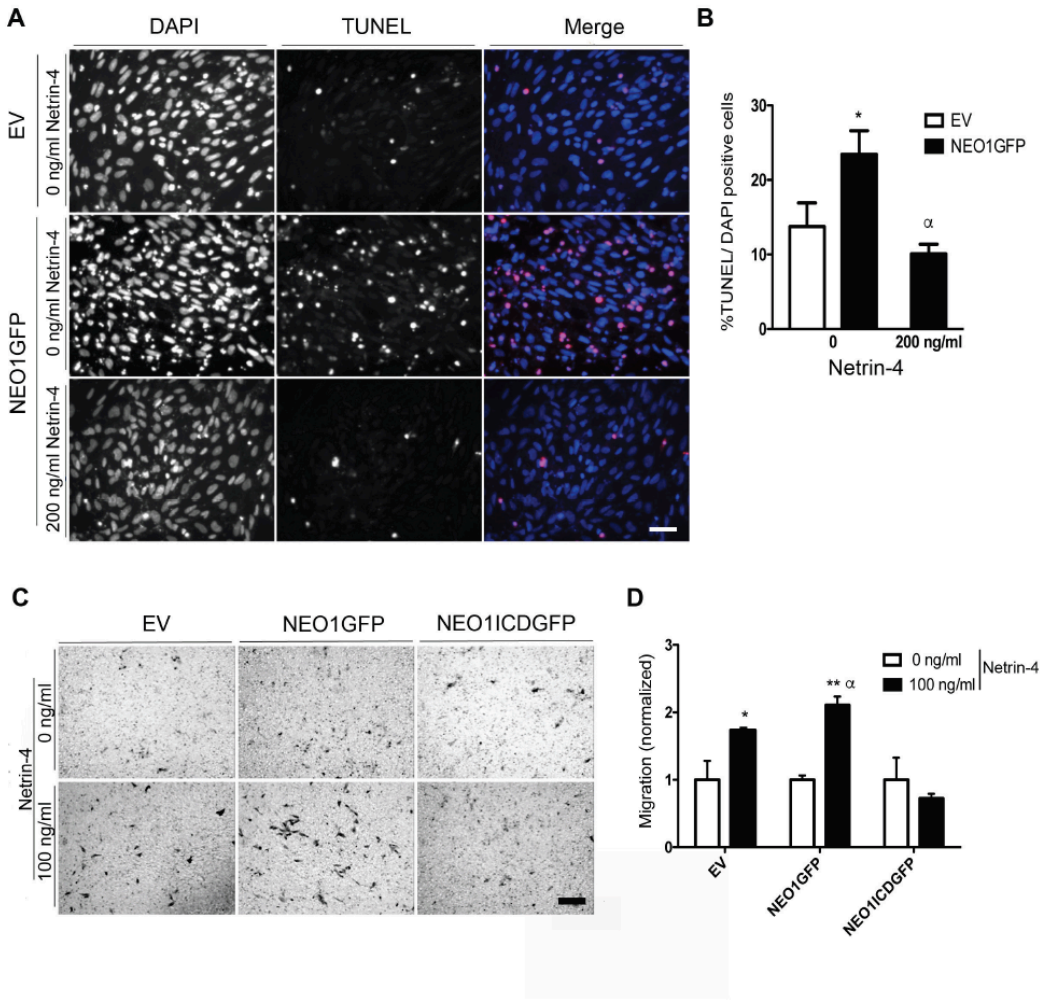

Figure 5: Overexpression of NEO1 induces apoptosis that can be rescued by exogenous Netrin-4 and promotes cell migration through NTN4 chemotaxis. A. Representative images of TUNEL assay made with SK-N-SH cells transfected with NEO1 (NEO1GFP) or empty vector (EV) and treated for 24 h with serum free media with or without Netrin-4 (200 ng/ml). Bar:100 mm. TUNEL positive cells are shown in red and were quantified in **B.** *p<0,05 EV versus NEO1GFP (0 ng/ml), α *p<0,05 NEO1GFP (0n/ml) vs NEO1GFP (200ng/ml Netrin-4). **C.** Representative images of transwell assays using SK-N-SH cells that overexpress NEO1 (NEO1GFP), or its intracellular domain (NEOICDGFP). An empty vector (EV) was utilized as a control. Cells migrated for 4 hours using 100 ng/mL Netrin-4 as a chemotactic stimulus Bar: 100μm. **D.** Quantification of transwell assay shown in (C) * p<0,05, **p<0,001, α p<0,05.

NEO1 and endogenous NTN4 participate in metastasis *in vivo*

After examining the potential role of endogenous NEO1 and NTN4 participating in apoptosis and cell migration, we wanted to ascertain whether they also participate in primary tumor formation and metastasis *in vivo*. We conducted a chick CAM assay and transplanted shNEO1, shSCR, or shNTN4 SK-N-SH cells, to generate primary tumors in chicken embryos (representative images are shown in Figure 6A). The resulting primary tumors were all similar, with no significant differences in tumor size and weights among the different transfected cell batches (Figure 6B). Importantly, we observed that shSCR cells formed secondary tumors in 57,2% of the embryos, characterized as GFP+ small nodules on the CAM, near the primary tumor (Figure 6C), while shNEO1 and shNTN4 transfected cells grew mostly as primary tumors (16,6 % shNEO1 and 22,2 % shNTN4; secondary tumor formation). The latter result suggests that shNEO1 and shNTN4 cells have a reduced capacity to migrate across the CAM.

Tumor metastasis involves the migration, invasion, and proliferation of tumor cells into other tissues and niches. We evaluated metastasis rate in the embryonic chicken lungs by amplifying human *Alu* sequences. The

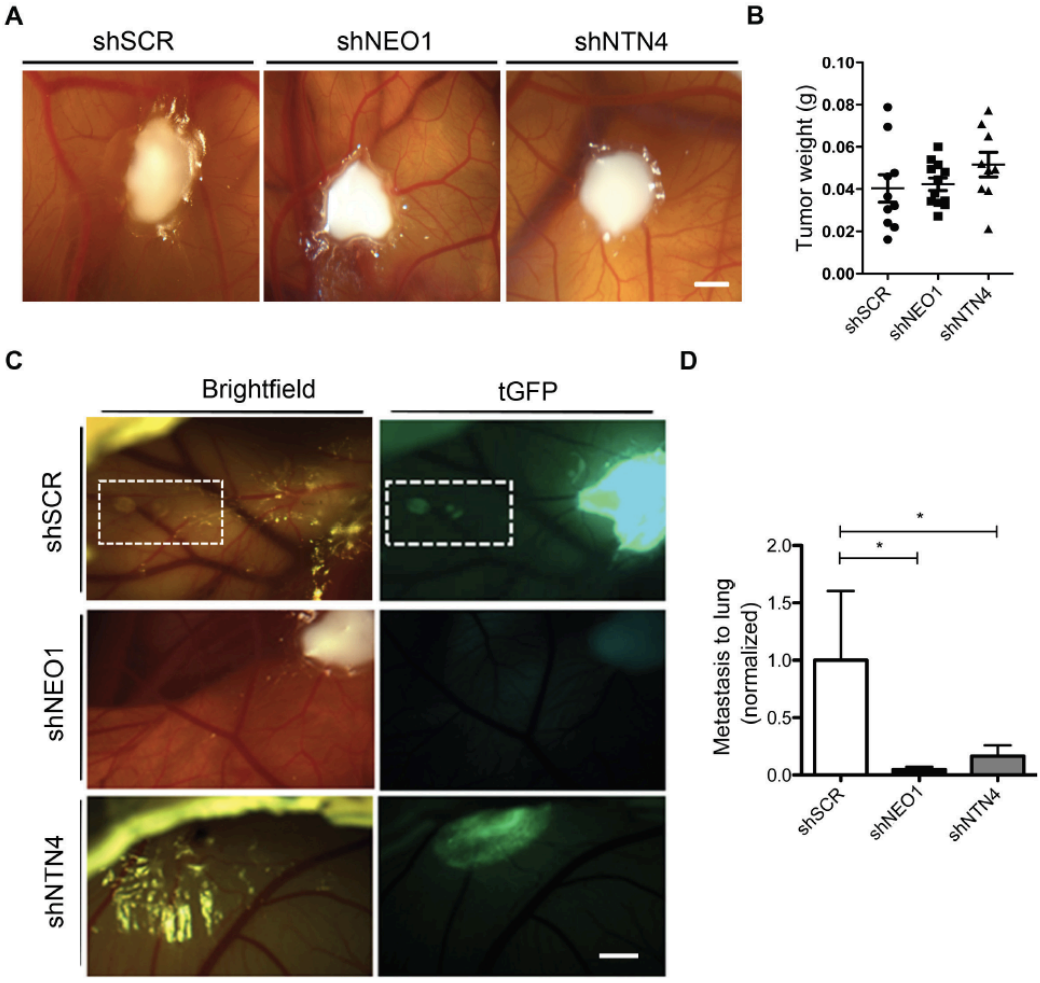

Figure 6: NEO1 and endogenous NTN4 participate in spontaneous metastasis *in vivo*. CAM assays were used to evaluate spontaneous metastasis of SK-N-SH cells dropped onto CAMs of chicken embryos on day 10 of incubation (E10). Stable knock-down cells shSCR, shNEO1, and shNTN4 were used in this assay. After 7 days (E17) embryos were dissected and lung metastasis was analyzed for each cell type. **A.** Representative images of primary tumor in CAM 5 days post-dropping. Bar: 5 mm. **B.** Tumor weight from primary tumor formed by the different cell lines in CAM. **C.** On day 5 post-dropping, secondary tumors in CAM were clearly visible and were formed by shSCR cells that migrated from primary tumors. This process did not occur in the case of shNEO1 and shNTN4 cells. Bar: 5 mm **D.** Quantification of Q-PCR analysis using genomic DNA of human *Alu* sequences in lungs to evaluate metastasis for shRNA cells, compared with chicken *GAPDH* gene. *p<0,05.

PCR results indicate that neither shNEO1 nor shNTN4 cells metastasized into the lungs (Figure 6D). Overall, these results suggest that both NEO1 and NTN4 may potentially participate in metastasis *in vivo*.

DISCUSSION

Tumor metastasis is orchestrated by several cellular processes, such as proliferation, cell survival, apoptosis, and cell migration [28]. In this work, we show that NEO1 is involved in all of these processes, recapitulating its known role in embryonic development [10]. NEO1 [4, 29] and NTN4 [15] are expressed in sites where NB originates, such as in the DRG of the embryonic neural crest, and we hypothesize that their functions persist throughout cancer progression, as a result of an impairment in the normal developmental signaling. Clinically, NEO1 expression endures throughout NB progression [30], and often results in poor patient prognosis. Conversely, loss of DCC expression, a homolog of NEO1, correlates inversely with the degree of NB dissemination [31], acting in this context as a tumor suppressor. In line with this data, *DCC* is not expressed in SK-N-SH cells [31] or in other NB cell lines [30, 31]. This phenomenon is due to the fact that the *DCC* gene undergoes an allelic loss. In fact, reduced expression of DCC [31] has been reported in several types of cancer (prostatic, colorectal and NB), unlike NEO1, which expression is not altered [30]. The expression of NTN4, one of the described ligands of the NEO1/DCC receptor family [32, 33], is also associated with an overall poor survival rate of NB patients. Overall, these results are in agreement with our meta-analysis of repository data, confirming that both NTN4 and NEO1 expression correlates with a worse prognosis, whereas NTN1 expression behaves the opposite. In fact, previous data from other groups have shown that NTN1 loss of expression in both, patient samples and NB cell lines, might contribute to progression of NB [17].

Here, we establish that both, WT and high *MYCN* NB cell lines (represented by SK-N-SH and LAN-1), express NEO1 and NTN4. In particular, we demonstrate that both proteins interact directly in NB cell lines, conforming a signaling complex that contributes differentially in several tumoral progression processes.

Role of NTN4 in apoptosis

Physiologically, NTN4 promotes angiogenesis [34, 24] in endothelial cells [7], driving proliferation, migration, and adhesion. Within the cancer context, NTN4 could have biphasic roles. At low physiological concentrations [24], cells exhibit high proliferation rates in glioblastoma [20] and gastric cancer [21]. At high concentrations, however, proliferation and angiogenesis are inhibited in glioblastoma [20] and colorectal cancer [35]. NTN4 could also act as a survival factor, especially because it blocks the pro-apoptotic activity of its receptors [14]. In fact, NTN4 has been shown to promote survival maintenance in beta pancreatic islets [14]. Here, by using apoptotic markers, we have confirmed that endogenous and exogenous NTN4 act as a survival factors in cells with different levels of endogenous NTN4.

NTN4 as a survival factor through NEO1 signaling

Apoptosis increases when NEO1 is overexpressed, revealing its death dependence function [8]. This effect was reverted using exogenous Netrin-4. In addition, when NEO1 is silenced in a shNTN4 background, the apoptosis induction by the NTN4 knock-down is reverted, indicating that NTN4 is a survival factor modulating dependence receptor NEO1's pro-apoptotic downstream signaling. In addition, Netrin-4 did not reduced the apoptosis of cells overexpressing TrkC indicating that NTN4 acts as a survival factor specifically through NEO1. Thus, the NEO1/NTN4 signaling complex modulates a balance between survival and apoptosis in these cells. NTN1, another NEO1 ligand, is overexpressed in aggressive NB and is also considered a survival factor [18]. Although LAN-1 express NTN1, NTN4 acts probably as the main NEO-1-related survival factor in these cells, since cell death is triggered when NTN4 is silenced. Likewise, it has been shown in NB that NTN1 could act as a survival factor through UNC5H downstream signaling [18].

Usually, dependence receptors act as tumor suppressors [11]. However, NEO1 knock-down does not reduce cell death and its expression is maintained in several cancer cells, such as NB [30] and medulloblastoma [6]. We propose that autocrine NTN4 expression through NEO1 binding allows sustained NEO1 function in these tumors, maintaining cells in a pro-survival state.

NEO1 acts via NTN4 in NB cell migration

In this work, we show that NEO1 is involved in NB cell migration, acting via its ligand NTN4. In other cell types, such as gastric cancer, NEO1 modulates the effect of endogenous NTN4 on motility [21]. The overexpression of NEO1 in gastric cancer increases cell motility, acquiring a migratory phenotype [36], and its knock-down reduces cell migration in the same cell types [21]. Researchers have stated these observations in other contexts, and, so far, no data have demonstrated the dose-dependent chemotactic behavior of NEO1 in NB cells.

Evaluating the effects of cell-matrix and cell-cell interactions via wound healing assays revealed that NEO1 SK-N-SH knock-down cells significantly reduce cell migration compared with shSCR (control) and shNTN4 knock-down cells. Endogenous NTN4, under these conditions, did not influence cell migration. But it is worth mentioning that our experimental conditions

included low serum supplementation, which contains NTN4 as well as other Netrin ligands (data not shown). Thus, we also performed transwell assays in the absence of serum to evaluate the role and contribution of NTN4 to cell migration and the competence of NEO1 to sense NTN4 chemotactic stimulus. Knocking down NTN4 did not result in an increase in cell migration compared to the condition without a chemotactic stimulus, hence suggesting that endogenous NTN4 maintains cells in a pro-migratory state. Contrary to SK-N-SH, LAN-1 cells do express NTN1 and shNTN4 cells migrate sensing NTN4 chemoattraction; most probably NTN1 compensates the NTN4 function in these cells. Furthermore, there is evidence that demonstrates the binding of NTN4 and NTN1 to other non-canonical Netrin receptors, such as Integrins (α6β1, [26]), which might explain a NEO1-independent Netrin compensation. We observed that shNEO1 cells did not migrate more than in a condition without stimulus. These cells cannot sense the NTN4 stimulus, highlighting the importance of NEO1 in guiding cells to migrate. Overexpressing NEO1 resulted in greater cell migration compared to control cells, confirming that NEO1 has an essential role in promoting cell migration.

The dependency of NB cells to migrate through NEO1 was revealed when we overexpressed NEOICD in SK-N-SH. NEOICD cells cannot bind Netrins and, consequently, the cells did not migrate efficiently. This result also confirms the possibility that endogenous NTN4 is binding to the NEO1 receptor on the cell surface, activating its intracellular pro-migratory cascade, possibly through focal adhesion kinase (FAK) signaling. During neuronal migration and axonal guidance, NEO1 binds to FAK [37]. FAK is fundamental for focal adhesion dynamics and cell migration [38]. Therefore, NEO1 promotes cell migration, guiding the cells via the NTN4 chemotactic stimulus. This is relevant in NB cells, because the molecular mechanisms that mediate neural crest delamination are also likely to be involved in NB migration [39]. The data suggest that NEO1, expressed in DRG, is involved in the symphatoadrenal-lineage neural-crest cells migration during embryonic development. DCC is involved in the migration of neural crest cells in the formation of the bowel and pancreas [40], acting through NTN1 as a guidance molecule; as a homolog, NEO1 may also participate in this process. The role of NEO1 is probably conserved, although intracellular pathways are not shared between NEO1 and DCC. In axonal guidance for example, DCC selectively interacts with Src family kinases Fyn and Lck intracellularly [41], while NEO1 interacts additionally with SHIP1 phosphatase. The different molecules involved in each signaling pathway may explain the differences between NEO1 and DCC signaling in tumoral suppression. The evidence provided by others and the results presented here, collectively, indicate that NEO1 acts by primarily promoting tumoral migration and survival through its ligands produced by the tumoral cells [18], stroma [42], or lamina basal [7], while DCC acts mostly as a pro-apoptotic molecule [43], which expression is reduced in several tumors as NB [31] among others.

Convergence of functions of NEO1/NTN4 in tumor progression

NEO1 and NTN4 knock-down cells generate primary tumors in CAMs, with similar weight and size compared with control tumors (shSCR). The CAM itself produces survival factors [44] and its vessels irrigate the tumors, reducing pro-apoptotic signals, which could explain the similar behavior of shSCR, shNEO1 and shNTN4 cells. As CAM cells migrate, the NEO1/NTN4 signaling complex becomes increasingly more important, deduced by the formation of secondary tumors near the primary tumors of SK-N-SH shSCR cells. shNEO1 and shNTN4 cells lacked secondary tumors, reinforcing the idea that the NEO1/NTN4 complex is biologically pivotal for cell migration process *in vivo*. Metastasis is a complex process that involves cell migration and invasion. When we evaluated the role of NEO1 in this latter process, we observed that NEO1 knock-down cells have a reduced capacity to form secondary tumors and to metastasize to lungs, revealing an impaired migration. Thus, NTN4 is also required for metastasis. This result suggests that NTN4 maintains the cells in a pro-migratory state in coordination with NEO1, activating intracellular signals. Physiologically, we hypothesize that as metastasis commences, NEO1 expressing cells sense the NTN4 gradients produced by the Netrin-producing cells, mainly located in the lamina basal of endothelia [7]. Therefore, NEO1 may have an active role in metastasis by sensing ligand gradients, promoting NTN4-guided intravasation, and colonizing new cancerous niches.

This work suggests that NEO1 acts as a tumoral progression-promoting protein, with an active role in metastasis, resembling its function in developmental cell migration. NEO1 itself induces apoptosis in certain contexts but within the context of cancer, in addition to autocrine ligand production, the stroma and/or basal lamina of vessels produce Netrins, thereby increasing the capacity of cells to migrate guided by NEO1. In cancer onset NEO1 might govern cell cycle kinetics and survival [6], while in aggressive tumor cells, NEO1 may function promoting cell migration. Different NTN4 availability might account for a differential behavior of NEO1 *in vivo* [36]. Further work is required to delineate the associated cellular mechanism required for cell survival and migration guided by NEO1 through ligand gradients, such as NTN4.

MYCN amplification and NEO1 and NTN4 expression

As *MYCN* amplification is associated with NB aggressiveness and poor prognosis (MYCN

protein expression as a predictor of neuroblastoma prognosis) [45], we evaluated the relation in between *MYCN* amplification and NEO1/NTN4 expression. According with Versteeg data set [23], there is not difference in *NEO1* expression in tumor samples with or without *MYCN* amplification (Supplementary Figure 4A). By contrast, *MYCN* amplified tumor samples have more *NTN4* expression (Supplementary Figure 4B), which is in line with the expression data observed in NB cell lines analyzed in this work. It is important to emphasize the complexity of the NEO1 signaling pathway, and how NTN4 acts through NEO1 regardless of *MYCN* status. Therefore, NEO1 could be one of the main regulators of survival and migration in NB.

On the other hand, there is controversy about *NTN1* expression in *MYCN* amplified tumors. In the same data set, its shown that *NTN1* is less expressed in *MYCN* amplified tumors (Supplementary Figure 4C), which does not correspond with cell lines analyzed in this work and with other literature, which shows that there is not an association between *NTN1* up-regulation and *MYCN* amplification [18]. NTN1 Kapplan Meier survival plots (Supplementary Figure 1B), show that NTN1 expression is a good survival prognosis factor, which is contrary with the *MYCN* amplification in NB aggressiveness. Importantly, previous report [18], have shown that NTN1 survival function is via UNC5H, and not through NEO1. This controversy shows that NTN1 regulation is complex and needs further analysis.

In conclusion, NTN4 and its receptor NEO1 promote cell migration, survival, and metastasis in NB cells. The NTN4/NEO1 signaling complex balances apoptosis and survival. If NTN4 is not expressed, NEO1 induces cellular apoptosis. However, if NTN4 is expressed, NEO1 signaling promotes cellular survival and migration. All these results underlie that there is a delicate balance between apoptosis and cell migration.

NEO1 signaling is becoming an attractive target for use in cancer therapies [6, 46]. Based on our results, we propose that NEO1 and/or NTN4 are promising targets for use anti-cancer therapies, in particular to inhibit the tumoral metastasis. Considering the ultimate efforts in the clinical and genomic medicine field, directed towards the generation of new therapeutic strategies, we could envision the development of inhibitors of the extracellular recognition of NTN4 by NEO1, allowing thus the modulation of NEO1 activity in an extracellular fashion.

MATERIALS AND METHODS

Cell culture

Neuroblastoma cell lines SH-SY5Y, SK-N-SH, LAN-1 and, NB1691 were cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) with 5% (SK-N-SH) or 10% (LAN-1, NB1691) fetal bovine serum (FBS, Gibco) and supplemented with antibiotics (Penicillin-Streptomycin, 10,000 U/mL). The U87 (glioblastoma), HEK293 (human embryonic kidney) and DAOY (medulloblastoma) cell lines were cultured in DMEM with 10% FBS supplemented with antibiotics.

Western blots

Protein extraction was realized using lysis buffer (SDS 2% w/v, Tris-HCL 80 mM pH 7.5, Glycine 10% w/v) with protease inhibitors (Thermo). After three minutes of sonication at ice-cold temperature, samples were centrifuged (10000 × g) for 5 minutes at 4°C. The antibodies used were anti-Neo1 (#sc-6536, Santa Cruz Biotechnology), anti-DCC (#sc-6535, Santa Cruz Biotechnology), anti-Netrin-1 (AF6419, R&D systems), anti-NTN4 (HYR01, R&D systems), anti-RGMa (AF2459, R&D systems), anti-actin (A5316, Sigma), and anti-tubulin (T9026, Sigma). Primary antibodies were incubated overnight at 4°C in 5% non-fat milk diluted in TBS-Tween 0,01% and secondary antibodies were incubated at room temperature for two hours in the same buffer. Western blots were quantified using integrated density analysis with ImageJ software (National Institutes of Health, USA).

Protein co-immunoprecipitation

SK-N-SH cells were incubated with human recombinant Netrin-4 (200 ng/ml) for 1h. Later, cell extracts were prepared in a buffer containing 20 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, and protease inhibitors by 5 min incubation on ice. Samples were centrifuged at 13,000 × g by 1 min at 4°C, and supernatants (1000 µg total protein) were immunoprecipitated with protein A/G bead-immobilized antibodies for 1h. NEO1 was immunoprecipitated with 5 µg of a rabbit polyclonal antibody (H-175 Santa Cruz) and NTN4 was immunoprecipitated with 5 µg of NTN4 goat polyclonal antibody. Immunoprecipitated samples were solubilized in loading buffer with ß-mercaptoethanol, and analyzed by Western blot as indicated in the Figure 1.

Quantitative PCR

We used real-time quantitative PCR (qPCR) to identify and quantify the expression of *NTN4* in NB cells. The cell cultures were maintained in 10% FBS until 90% confluence, where they were used for RNA extraction using RNAsolv (OMEGA, R6830-02). Purified RNA was used for cDNA synthesis using Revertaid Reverse Transcriptase (Thermo scientific) according to the manufacturer's instructions. The primers used for qPCR analysis were: *NTN4* (Fw: 5' TCAGCACAACACAGAAGGACA3'; Rv: 5' GGATGGCAGGAACACGGTTTG 3'), and 40 PCR cycles were used in the experiments, at an

annealing temperature of 60°C. Gene expression values were graphed as a fold change with respect to GAPDH (Fw: 5' CAAGAAGGTGGTGAAGCAGGC3'; Rv: 5'CCACCACCCTGTTGCTGTAG3'). PCR amplification was verified via gel electrophoresis.

Lentiviral transduction and stable shRNAs cell line generation

To knock-down *NEO1* (shNEO1) and *NTN4* (shNTN4), SK-N-SH and LAN-1 cells were transduced through lentiviral particles that contain shRNAs (pGIPZ backbone) vectors for each gene of interest [6]. A scramble sequence (shSCR) was used as a control. Lentiviral particles were prepared as indicated in [47]. Briefly, HEK 293T cells were triple transfected with pCMV-VSV-G, p8.91, and pGIPZ-shRNA (Openbiosystems). Viral supernatants were harvested 48 hours after transfection, filtered through 0.45-mm cellulose acetate filters, and them were used to transduce SK-N-H and LAN-1 cultured with this medium mixed with DMEM 5% FBS. After 48 hours, the transduction percentage was measured using tGFP encoding in pGIPZ and cells were incubated with the selection marker puromycin (3 µg/ml for SK-N-SH and 1µg/ml LAN-1, Sigma) for an additional 48 hours. Cells were maintained in DMEM with FBS supplemented with puromycin. The knock-down efficiency was measured via Western blot analysis.

NEO1GFP and NEO1ICDGFP overexpression

The plasmids used to overexpress Neo1GFP and NeoICD (intracellular domain of NEO1) fragments were a kind donation from Dr. Patrick Mehlen (Université de Lyon, Centre Léon Bérard, Lyon, France), and were prepared as described in [48]. The backbone used for the plasmids was pEGP-C1, which has an eGFP sequence in frame with NEO1 or NEOICD sequence. The TrkC-overexpression plasmid was also a donation from Dr. Patrick Mehlen and the backbone used for the plasmid was pCDNA3, described in [49]. The transfection was made in SK-N-SH cells using Turbofect (Thermofisher) according to the manufacturer´s instructions.

Wound healing assay

The wound healing assay was carried out using SK-N-SH shNEO1, shNTN4, and shSCR cells as described in [50], with some modifications. Briefly, cells were cultured for 24h to reach confluence. Then, a scratch was made with a micropipette tip in the center of the plate in order to generate a space between cells. The cells were washed with phosphate buffered saline (PBS) and incubated in DMEM 2.5% FBS. The plates were photographed using a microscope (Motic) coupled to digital camera (Leica) at 100x of total amplification, setting this at time 0. Cells were incubated for 9h and then photographed using the same conditions. This experiment was carried out in quadruplicate. The analysis was made using Image J software and the data are shown as a percentage of wound healing (closure) of each cell type.

Transwell migration assays

Tranwell assays were completed using a chamber within an 8µm-pore polycarbonate membrane (Corning). As a haptotactic stimulus, 2µg/µl fibronectin was used (Sigma Aldrich), placed on the bottom of the membrane 12h before performing the assay. As a chemotactic stimulus, different concentrations of human recombinant Netrin-4 (R&D Systems) diluted in DMEM (without FBS) were used; the concentrations are indicated in Figure 4. Briefly, for SK-N-SH, 50,000 shNEO1, shNTN4, and shSCR cells were placed in the upper chamber; the bottom chamber contained NTN4 diluted in DMEM. The cells were incubated for 4h, fixed, and stained using Crystal violet 100% diluted in methanol in a solution 1:5 of NaCl 0,15M. For LAN-1 100,000 shNEO1, shNTN4, and shSCR cells were placed in the upper chamber; the bottom chamber contained 100 ng/ml Netrin-4 diluted in DMEM. The cells were incubated for 6h, fixed, and stained same as SK-N-SH cells.

To determine if NEO1-overexpressed cells migrate more in transwell assay than control cells in the presence of a Netrin-4 stimulus (100ng/mL), SK-N-SH cells overexpressing EV (empty vector), NEO1GFP, or NEO1ICDGFP were used. The results were normalized according to condition without Netrin-4 for each experiment.

Immunofluorescence

Double-immunofluorescence were completed with anti-cleaved Caspase-3 (9661, Cell Signaling) and anti-Tubulin (T9026, Sigma) in SK-N-SH shNTN4 and shSCR cells. The cells were cultured in 24-well plates for 24h. Subsequently, they were deprived of FBS, and exogenous Netrin-4 was added for 24h. Human recombinant RGMa (100ng/mL) was used as a control. Cell nuclei were also stained with a DAPI (Sigma Aldrich).

To evaluate proliferation, SK-N-SH shNTN4, and shSCR cells were incubated with BrdU (Sigma) for 1h in 24-well plates in DMEM 2,5% FBS. Then, the cells were fixed with 4% paraformaldehyde (PFA) and immunostained with anti-BrdU (DAKO) and phosphohistone3 (Millpore) antibodies following the protocol as explained previously. Fluorescence microscopy was performed using an Olympus BX- 51 microscope. A minimum of three independent experiments was realized for each assay.

Propidium iodide staining

LAN-1 cells shSCR and shNTN4 were culture in 96-well plates. After 24 h, cells were serum deprived by 24h. To evaluate dead cells, cells were stained with Propidium iodide (PI) and Hoechst at final concentration of 1 µg/ml and 5 µg/ml, respectively. Cells were analyzed and counted using Cytell Cell Imaging System (GE Healthcare), using Cell Viability BioApp (GEHealthcare). Ten fields were analyzed per well and average per well were grafted. The assay was made by six replicates for each condition and two independent experiments were realized for the assay.

TUNEL assay

TUNEL assays were conducted utilizing the ApopTag® Fluorescein Direct *in Situ* Apoptosis Detection Kit (Merck Millipore), following the manufacturer's instructions, to measure apoptosis in shNTN4, shSCR, and NEO1-overexpressing SK-N-SH cells. Cells were incubated in Netrin-4 (200ng/mL) diluted in DMEM for 24h. We used DMEM without FBS as an incubation control. The assays were analyzed by fluorescence microscopy using an Olympus BX- 51 microscope. TUNEL positive cells and DAPI positive cells were counted and the ratio between both quantifications was graphed as a percentage.

siRNA transfection

To evaluate the contribution of NEO1 in the apoptosis induced by NTN4 knock-down, we transfected siRNAs against NEO1 in shNTN4 SK-N-SH cells using Turbofect (Thermofisher) according to manufacture's instructions. The sequences correspond to siNEO1(1) (SASI_Hs02_00333957) and siNEO1(2) (SASI_Hs01_00151269) and, siControl (SIC002) provided by Sigma Aldrich. siRNA efficiency was evaluated through Western blot against NEO1. Briefly, 1µg of siRNA was transfected into shNTN4 cells and 48 h later, cells were serum deprived for 48h and fixed with PFA 4%. Apoptosis was evaluated via TUNEL and immunofluorescence of Cleaved-Caspase-3. The assays were analyzed by fluorescence microscopy using an Olympus BX- 51 microscope. Positive cells and DAPI positive cells were counted and the ratio between both quantifications was graphed as a percentage. The assays were made by 30 replicates for each condition and two independent experiments were realized for each assay.

Metastasis analysis via CAM assays

Fertilized chicken eggs were incubated at 37.5°C with constant humidity. On the second day of incubation (E2), 2 mL of albumin was removed from the egg. On day four (E4), a rounded window was made in the shell in order to have access to the chick chorioallantoic membrane (CAM), and sealed with adhesive tape. On day ten of incubation (E10), ten million SK-N-SH shNEO1, shNTN4, or shSCR cells were drop-plated on the developing CAM. On day 17 of incubation (E17), the primary tumor of the CAM was weighed and the embryo was dissected. Embryonic lungs were incubated with RNASolv (OmegaBiotek) and kept at -20°C. We extracted genomic DNA following the manufacturer's instructions. Genomic DNA expression levels were analyzed via qPCR analysis using human *Alu* sequences (FW: 5'ACG CCT GTA ATC CCA GCA CTT3'; RV: 5'TCG CCC AGG CTG GAG TGC A3') and genomic chicken GAPDH (FW: 5'GAG GAA AGG TCG CCT GG3'; RV: 5'GGT GAG GAC AAG CAG TGA3') primers. The analysis was made using fold change with respect to chGAPDH and normalized according to lung of control cells (shSCR). Five eggs were used for each condition.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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Author contributions

Verónica Palma, Andrea A. Villanueva and Luis Milla designed the research. Andrea A. Villanueva, Paulina Falcon, Natalie Espinoza, Luis Solano and Esther Hernandez-SanMiguel performed the experiments and analyzed data. Miss Villanueva and Miss Hernandez-SanMiguel, under supervision of Dr. Sánchez, set up and performed the experiments in high *NMYC* neuroblastoma cell lines. Under Dr. Torres assistance, Miss Villanueva undertook the immunoprecipitation protocol. Andrea A. Villanueva and Verónica Palma wrote the paper. Corresponding author, Dr. Palma, drafted the manuscript, conceived and coordinated the study, participated in the design of all of the experiments, discussion of results and in the preparation of the final version of the manuscript and figures.

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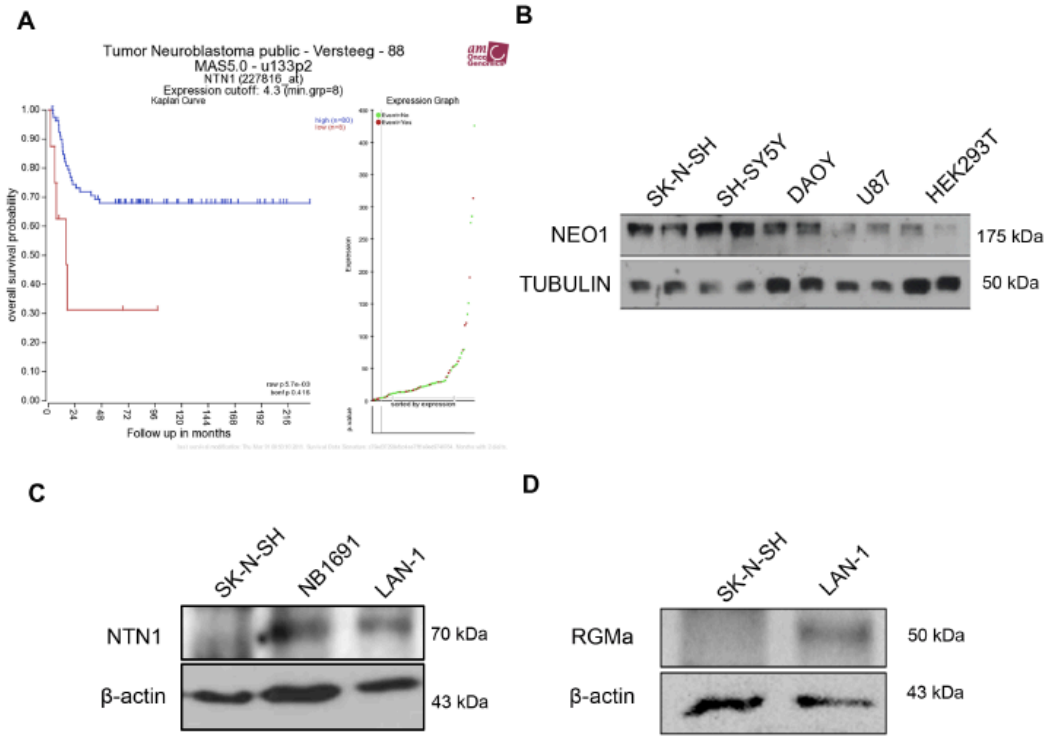
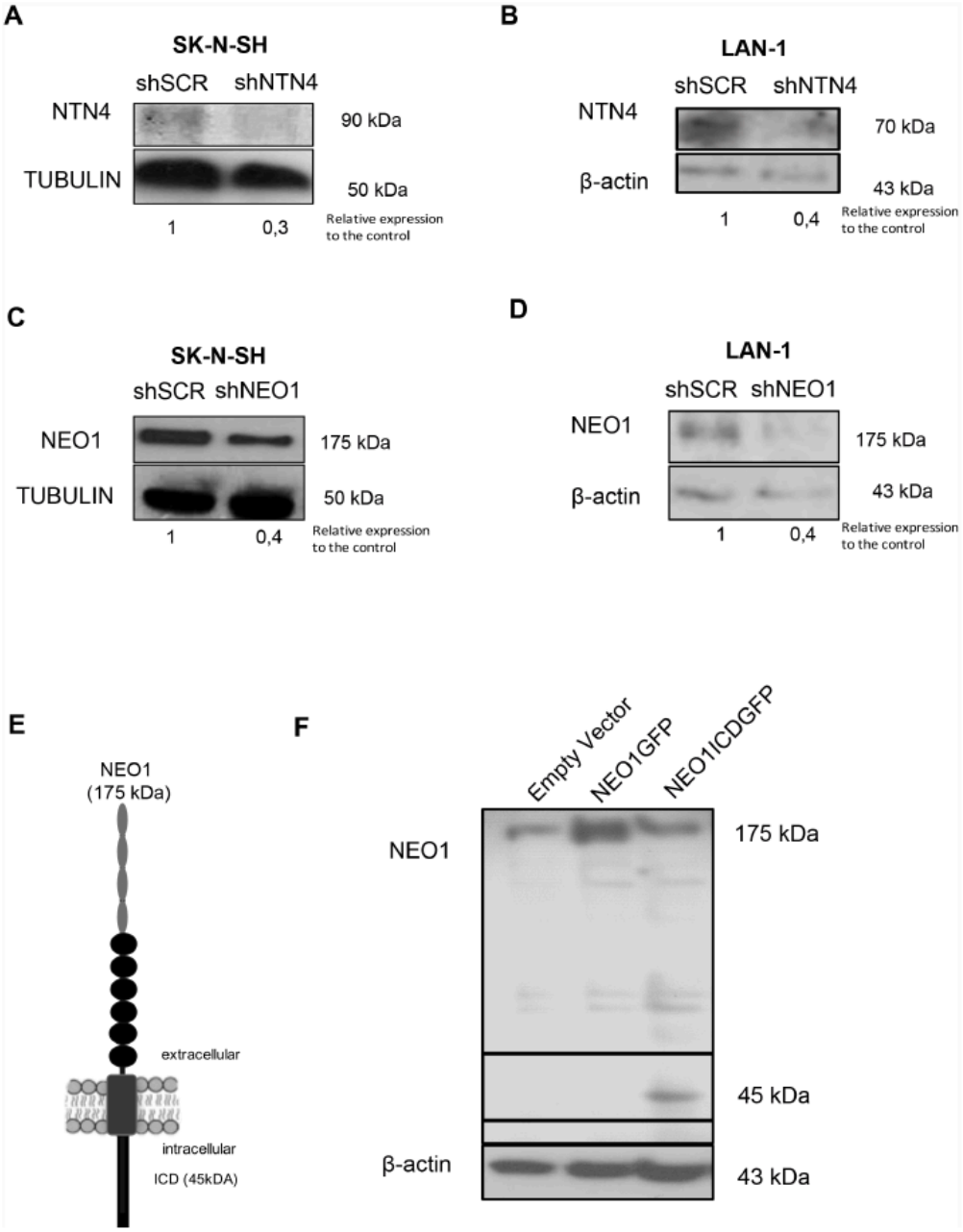
The Netrin-4/ Neogenin-1 axis promotes neuroblastoma cell survival and migration

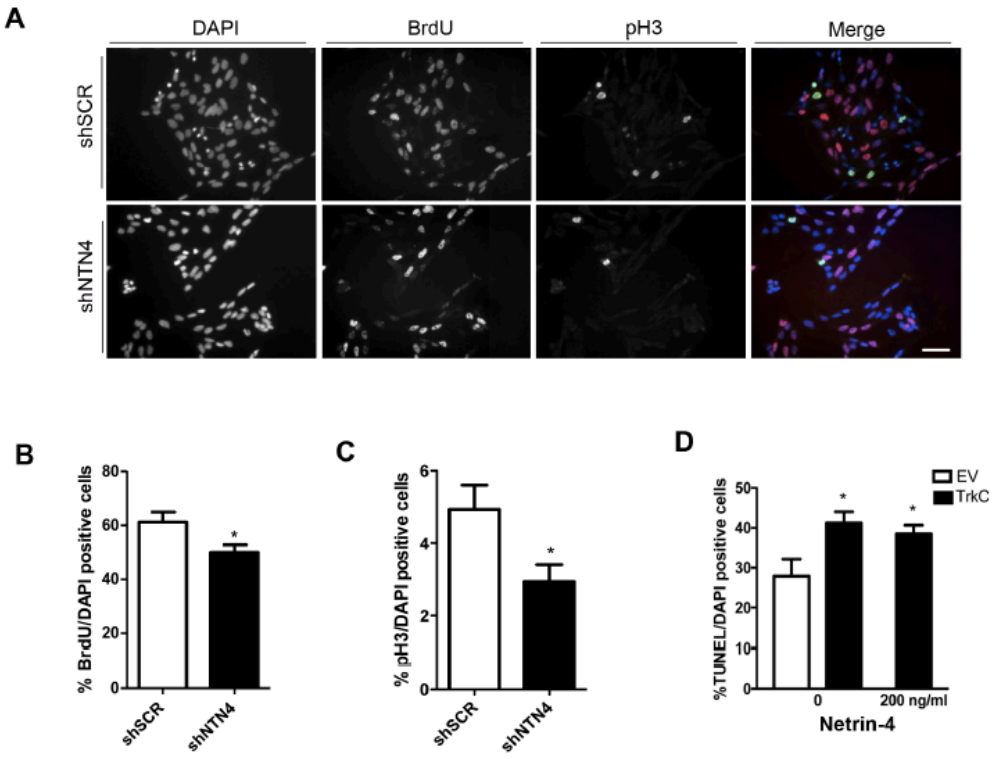
SUPPLEMENTARY FIGURES



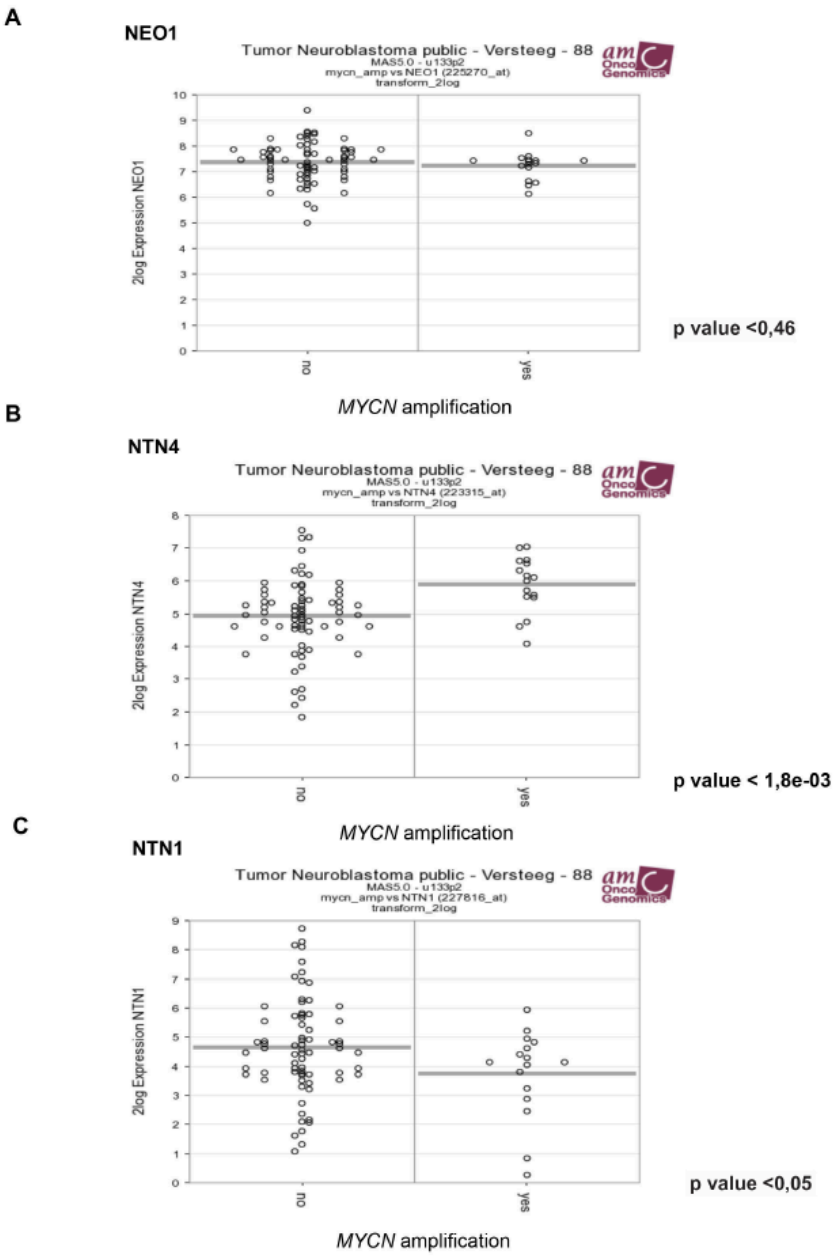
Supplementary Figure 1: Characterization of NEO1 and its ligands NTN1 and RGMa in tumor cell lines. A. Clinical significance of NTN1 expression in NB. *NTN1* mRNA value is plotted against patient survival rate in a Kaplan-Meier estimate plot. **B.** Western blot against NEO1in several tumor cell lines, as indicated. **C.** Western blot of NTN1 in both WT and high *MYCN* NB cell lines. Note that NTN1 is not expressed in SK-N-SH cells. **D.** Western blot against RGMa in representative WT and high *MYCN* NB cell lines. Note that RGMa is not expressed in SK-N-SH cells.



Supplementary Figure 2: NEO1 and NTN4 knock-down and NEO1 overexpression in NB cell lines. A, B. Representative Western blots against NTN4 in stable transduced cells using lentiviral particles containing shRNA for NTN4 or shSCR control in SK-N-SH (A) and LAN-1 (B). Relative expression to the control value was estimated from a triplicate. **C, D.** Representative Western blots against NEO1 in stable transduced cells using lentiviral particles containing shRNA for either NEO1 or shSCR control in SK-N-SH (C) and LAN-1 (D) cells. Relative expression to the control value was estimated from a triplicate. **E.** Schematic representation of NEO1 depicting the structure of the full- length protein and the intracellular NEO1 domain (ICD). **F.** Western blot against NEO1 revealing its expression in cells transfected with an empty vector, NEO1 (NEO1GFP), and NEOICD (NEO1ICDGFP). Note that the 45kDa band is only detected when overexpressing NEOICDGFP (see box).



Supplementary Figure 3: NTN4 knock-down reduces the proliferation in SK-N-SH cells and Netrin-4 treatment cannot reduce apoptosis induced by TrkC in SK-N-SH cells. A. Representative images of immunofluorescence against phospho-Histone-3 (pH3) and BrdU. Bar:100 μm **B, C.** Quantification of BrdU (B) and pH3 (C) positive cells in shSCR or shNTN4. *p<0,05. **D.** TUNEL assay of SK-N-SH cells transfected with TrkC or empty vector (EV) and treated in serum free media for 24 h with or without Netrin-4 (200 ng/ml) EV vs TrkC*p<0,05.



Supplementary Figure 4: *NEO1* and *NTN* expression according to *MYCN* amplification. A-C. Using the public primary tumor NB database from 88 patients (Versteeg data set), *NEO1* (A), *NTN4* (B) and *NTN1* (C) expression was plotted according to *MYCN* amplification in patient samples and expressed as yes (*MYCN* amplification) or no (*MYCN* WT). p value is indicated in the figure for each gene.

Cell Adhesion & Migration

The Netrin-4/Laminin γ1/ Neogenin-1 complex mediates migration in SK-N-SH neuroblastoma cells

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Abstract:	Neuroblastoma (NB) is the most common pediatric extracranial solid tumor. It arises during development of the sympathetic nervous system. Netrin-4 (NTN4), a laminin-related protein, has been proposed as a key factor to target NB metastasis, although there is controversy about its function. Here, we show that NTN4 is broadly expressed in tumor, stroma and blood vessels of NB patient samples. Furthermore, NTN4 was shown to act as a cell adhesion molecule required for the migration induced by Neogenin-1 (NEO1) in SK-N-SH neuroblastoma cells. Therefore, we propose that NTN4, by forming a ternary complex with Laminin γ1 (LMγ1) and NEO1, acts as an essential extracellular matrix component, which induces the migration of SK-N-SH cells.

TITLE: The Netrin-4/Laminin γ1/ Neogenin-1 complex mediates migration in SK-N-SH neuroblastoma cells

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ABSTRACT

Neuroblastoma (NB) is the most common pediatric extracranial solid tumor. It arises during development of the sympathetic nervous system. Netrin-4 (NTN4), a laminin-related protein, has been proposed as a key factor to target NB metastasis, although there is controversy about its function. Here, we show that NTN4 is broadly expressed in tumor, stroma and blood vessels of NB patient samples. Furthermore, NTN4 was shown to act as a cell adhesion molecule required for the migration induced by Neogenin-1 (NEO1) in SK-N-SH neuroblastoma cells. Therefore, we propose that NTN4, by forming a ternary complex with Laminin γ1 (LMγ1) and NEO1, acts as an essential extracellular matrix component, which induces the migration of SK-N-SH cells.

INTRODUCTION

Pediatric solid tumors represent about 30% of pediatric cancers. Neuroblastoma (NB) is an extracranial solid tumor that emerges from neural crest cells during development and it is a highly metastatic cancer [1]. The laminin-related secreted Netrins (Netrin 1-4) act as versatile extracellular cues regulating axon guidance [2], angiogenesis, survival and cell proliferation during embryogenesis as well as in cancer [3]. We have recently shown that Netrin-4 (NTN4) promotes NB progression and metastasis acting as a chemotaxis stimulus for the Neogenin-1 (NEO1) receptor [4]. NEO1, a member of the immunoglobulin superfamily of transmembrane protein receptors, and its homologue, the Deleted in Colorectal Cancer receptor (DCC), have been related to tumor progression, proliferation, angiogenesis, apoptosis, and migration in several tissues [5-9]. However, contrasting with our results, NTN4 was recently proposed to signal independently of classical Netrin NEO1/DCC receptors [10]. In line with the former observation, NTN4 protein structure was determined and proposed as a cell adhesion molecule, acting as an extracellular matrix protein that forms a high-affinity complex with laminin γ1 (LMγ1) [11]. Added to this information, data by Staquicini et al. [12] suggested the formation of a complex between NTN4 and LMγ1 that activates a signaling pathway mediated by a6β1 integrin, participating thereby in the migration of neural stem cells. Hence, in this study, we aimed to examine the short range-effects of NTN4 on NB cell migration. To this end, by using the SK-N-SH cell line, we demonstrate a role for NTN4 acting as a cell adhesion molecule in the extracellular matrix, contained within a NTN4/ NEO1/ LMγ1 ternary complex. Furthermore, our results show that NTN4 is strongly expressed in NB patient samples, in particular in endothelial cells. NTN4 might act both as a cell adhesion and chemotactic stimulus, highlighting the important contribution of the NTN4/NEO1 signaling axis in NB migration and metastasis; a result that might reconcile the apparent controversy in the field and thus provide a new mechanism underlying NB metastasis.

RESULTS

To investigate the role of NTN4 in NB, we first characterized the expression of the ligand in a cohort of 23 NB patient samples. The samples were stratified based on the International Neuroblastoma Risk Group Staging System (INRGSS) which contemplates a pretreatment risk classification system, considering tumor spread and surgical risk factors known as Image Defined Risk Factors (IDRFs) at the moment of diagnosis of the disease. Localized tumors are staged L1 or L2 based on the absence or presence of one or more of 20 IDRFs, respectively. Metastatic tumors are defined as stage M and MS, the latter refers to metastases confined to the skin, liver, and/or bone marrow in children younger than 18 months of age [13]. In order to correlate patient's disease staging with our NTN4 immunohistochemistry analysis, we organized the results in relation to PCNA expression levels, age, tumor stage, patient status, gender and primary tumor sites. Particularly, we evaluated NTN4 presence in tumor cells, stroma and blood vessels (Table 1). Despite the wide spectrum of NB presentation and clinical course [13-15] our data show that NTN4 is strongly expressed in NB. Notably, the number of male patient's biopsies expressing NTN4 is twice the number of female biopsies expressing it. Another intriguing result is that no NTN4 expression was found in tumor cells or stroma of patient's whose primary tumor location was ascribed at the thoracic level. Despite these observations, statistically there is no association between the percentage of NTN4 and these clinical features. Interestingly, NTN4 was intensely expressed in the endothelium (Figure 1) throughout all the samples analyzed, independent of the tumor stage (Figure S1). Corroborating our results, NTN4 indeed has been described as an endothelium lamina basal component in another context such as hemangiogenesis [16]. In a representative NB section, defined by classification criteria as characteristic of a disseminated tumor stage, primitive neuroblasts, identified as small, round and blue cells with almost no cytoplasm, grouped in small nests (Figure 1A, asterisk) and are easily distinguished from the ganglionar apparent cells. The latter are revealed by hematoxylin and eosin staining as cells with abundant eosinophilic cytoplasm, nucleus with vesicular chromatin and a prominent nucleolus (Figure 1A, arrowhead). NTN4 expression in this tumor is localized mostly in blood vessels and stroma (Figure 1B, 1F). PCNA expression in this sample is

very high (PCNA >40%), corroborating the aggressive stage of this NB sample (Figure 1C). Strong NTN4 expression can be found in the endothelium, seeming to delineate the CD31 positive blood vessels (Figure 1D, arrows; inset, Figure S2). Within the tumor cells, NTN4 expression is located in the cytoplasm surrounding the nucleus (Figure 1E, arrowhead). NTN4 expression is also highly upregulated within the extracellular matrix in regions of high cellular density (Figure 1F, asterisk).

Next, we aimed to examine the effects of NTN4 on NEO1 driven cell adhesion and migration. To this end, we evaluated first whether NTN4 behaves as an adhesion molecule in a NB cell line. We performed an adhesion assay with SK-N-SH cells, at different times, using rhNTN4 (2 μg / ml, according to [11]), or mouse Laminin-1 (10 μg/ ml) (mLM-111), as a positive control. As indicated in Figure 2A, NTN4 acts as an adhesion molecule, as corroborated by quantification (Figure 2B), revealing significant differences at 30 min of adhesion, when compared with the PBS control condition. Importantly, NTN4 acted as an adhesion molecule, inducing the adhesion of SK-N-SH cells to a similar extent as compared with mLM-111. In order to determine whether NEO1 is also important to adhesion in SK-N-SH cells, we performed a spreading assay using control cells (shSCR) or NEO1 knockdown cells (shNEO1), demonstrating that shNEO1 cells spread less than control cells on a Fibronectin substrate, indicating that NEO1 indeed contributes to cell adhesion and spreading (Figure 2D). Furthermore, to evaluate the requirement of NEO1 in the context of a cell adhesion induced-migration, provided particularly by NTN4, a transwell assay was tested in shSCR or shNEO1 cells. rhNTN4 and/or mLM-111 were placed at the transwell and the assay was performed according to [17] using low serum as a chemotactic stimulus in the bottom part of the chamber. As shown in Figure 3A shNEO1 cells migrated less than the shSCR cells in both conditions, mLM-111 and rhNTN4, even when using a combination of both molecules. Quantifications revealed no significant differences between shSCR and shNEO1 cells at basal migration (i.e. using PBS as stimulus, Figure 3B), probably due to dispersion of the data. However, when using either mLM-111 or rhNTN4, migration was 2-fold increase in shSCR, with respect to shNEO1 cells, further supporting that NTN4 promotes cell adhesion and migration to a similar extent as for mLM-111. According to the observations

of Reuten et al. [11], cell migration is likely modulated by NEO1, since silencing of the receptor prevented cell migration. Finally, combined use of mLM-111 and NTN4 led to a non–significant increase in cell migration of shSCR cells, when compared to each separate ligand. In our previous work [4], NTN4 was postulated as a chemotactic molecule that promotes migration of NB cells. Now, based on these observations, we reasoned that NTN4 could also act as an adhesion molecule, in conjunction with Laminin LMγ1.

According to Reuten et al. [11], NTN4 does not interact directly with any of the putative Netrin´s family canonical receptors, such as NEO1. However, others and we have shown that NTN4 immunoprecipitates with NEO1 [4,7]. This suggests that the interaction between NEO1 and NTN4 is rather indirect, most probably forming part of a complex with LMγ1. Indeed, studies by Staquinini et al. [12] and Reuten et al. [11] demonstrated an interaction between NTN4 and LMγ1. To assess whether NEO1 also combines with LMγ1 and thus could explain a functional protein complex linking NTN4 with NEO1, we performed co-immunoprecipitation assays. Reciprocal co-immunoprecipitation experiments demonstrated that such association is effective on SK-N-SH cells (Figure 3C, 3D). These results show that NEO1 associates with the LMγ1 chain and presumably, this interaction accounts for the reduction of cell migration observed in NEO1 knockdown cells.

Taken together, our results confirm that NTN4 acts as a cell adhesion molecule, promoting serum-induced cell migration in the NB cell line SK-N-SH through a ternary complex formed by NTN4/NEO1/ LMγ1.

DISCUSSION

The spectrum of NB and prognosis is wide, ranging from an aggressive course with poor survival, differentiation of NB into more differentiated ganglio-neurona, or even spontaneous tumor regression. Due to the high metastatic rate of NB targeted therapies, aimed at modulating those critical processes that are critical for tumor growth and metastasis, are required.

NTN4 is a protein involved in many physiological processes, such as angiogenesis [7], neovascularization [18], axon branching [19] among others. NTN4 has been implicated as a prognosis marker of certain malignancies such as gastric [20] and breast cancer [21]. In both cancers, it has been shown that NTN4 has a role in the migration and/ or metastasis of tumor cells, without specifying the mechanism associated with these functions. Based on our results, we can state that NTN4 is expressed in all NB samples, although with different cellular distribution. By analyzing the patient cohort samples, NTN4 reveals labeling in the endothelium of all sections (Figure S2), probably delineating the basal lamina [22]. In agreement with our observations, NTN4 has been described as integral component of the basal lamina of the endothelium and is highly enriched in the proximal basement membrane of tubules [23]. Indeed, our studies revealed positive LMγ1 staining in blood vessels located within the tumor niche (Figure S2). Moreover, our analysis suggests that NEO1 can also be expressed in blood vessels (Figure S2). Probably, tumor cells are attracted to endothelial capillaries as they disseminate and, NTN4, produced as a chemotactic molecule in this permissive substrate, might facilitate their migration. NTN4 is also observed in the stroma, especially in samples that have the histological appearance of neuropil. In tumor cells, NTN4 labels in a distinctive punctuated pattern, suggestive of strong expression in secretory organelles, observations that need to be further defined. Thus, NTN4 could be acting both in autocrine and paracrine fashion in the tumor niche.

Interestingly, the expression of NTN4 in primary tumors located in the thoracic region was null. Supplementary studies are required aiming to increase the number of samples with this location of NB in order to be able to assure that there is a relationship between the location of the primary tumor and the absence of NTN4.

Historically, NTN4 has been postulated as a chemotactic molecule [4,19]. Nevertheless, a recent study demonstrated that this molecule could be a putative component of the extracellular matrix, through its high affinity with LMγ1, evidencing that secreted NTN4 could function in an autocrine fashion [11]. Our group has shown that NTN4 promotes the migration, survival, and metastasis of NB cells through NEO1 by acting as a chemotactic molecule on NB cells [4]. Here, we show that NTN4 also could act as a cell adhesion molecule inducing cell migration. In addition, we provide evidence that NEO1 contributes to cell spreading, indicating its function in the initiation of cell migration. Transwell migration assays adding rhNTN4 in the underside of the chamber generated a positive cell migration of control NB cells (shSCR), similar to the results obtained with mLM-111. Interestingly, when using a combination of mLM-111 and rhNTN4, cell migration was not significantly different to that observed using separate ligands, although there is a tendency. Knockdown of NEO1 reduces the cell migration of SK-N-SH cells in all conditions, except in the non-stimulated control (PBS), revealing that NEO1 is also indispensable for adhesion-induced migration. This phenomenon could be explained on the basis that NEO1 associates with LMγ1 in this context. It is known that LMγ1 binds to integrin α6β1 [24]. Hence, most probably the interaction of NEO1 with NTN4 shown in [4], could be explained due to the formation of a ternary complex, including NTN4, LMγ1 and NEO1. At this point, we do not rule out that integrins also form part of the complex, a matter that deserves further investigation. Importantly, the combined action of all these molecules may be a key signaling event driving NB migration and dispersion.

MATERIALS AND METHODS

Patient samples

Ethics committees from University of Chile and CONICYT approved this study. General written consent was obtained from all the patients enrolled by HNPG, at diagnosis. All human tumor samples used in this study were diagnosed, and morphologically typified, through histological analysis at the Anatomopathology Center of this institution.

Immunohistochemistry and histological analysis

Paraffin-embedded samples of NB were deparaffinated and rehydrated as in [25]. Immunohistochemical assays were proceeded incubating the tissues with primary antibodies anti-human NTN4 (AF1254, goat, R&D Systems), anti LMγ1 (MAB1920, Millipore), anti CD31 (P8590, mouse, Sigma), anti NEO1 (H-175, Santa Cruz) and anti-human PCNA (13-3900, mouse, Invitrogen), overnight at 4°C and subsequently with the secondary biotinylated anti-goat Igg (R&D Systems) for NTN4 analysis, and biotinylated anti-rabbit/mouse IgG (Vector) for PCNA analysis for one hour at room temperature (25°C). The samples were later revealed with 39-diaminobenzidine (DAB, Roche). Samples were stained with Hematoxilin (Vector Laboratories, Burlingame CA) and Eosin Y (Sigma Aldrich, St Louis, MO). Immunofluorescence samples were incubated an extra hour with a Donkey anti goat Alexa Fluor 555 (Invitrogen). Dapi was used for nuclei staining. Slices were mounted with fluorescence mounting medium (Dako). PCNA percentage was calculated by counting by two independent observers the number of cells marked in quadrants and multiplying by the total number of quadrants present in each sample. 40% was the median obtained for the total of 23 samples. χ square and Fisher's exact test ($n< 5$ samples) were as statistical tests.

Cell culture

The NB cell line SK-N-SH, was cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) with 5% fetal bovine serum (FBS, Gibco) and

supplemented with antibiotics (Penicillin-Streptomycin, 10,000 U/mL). To knock-down NEO1 (shNEO1), SK-N-SH were transduced according to [4] assessing knockdown efficiency via Western Blot. Stable shNEO1 and shSCR (control) SK-N-SH cells were previously established, using puromycin as a selection marker, as indicated in [4].

Cell adhesion assay

48-well plates were incubated overnight at 4ºC with PBS, mouse Laminin-1 (mLM-111) (Invitrogen, extracted from Engelbreth-Holm-Swarm sarcoma) (10μg/ml) or rhNTN4 (2μg/ml). Next, SK-N-SH cells (70,000) were placed into the wells and allowed to adhere at different times (0, 5, 10, 15, 30, 45 and 60 minutes). At indicated time points, cells were fixed and stained using 0.1% crystal violet in 20% methanol saline (0.15 M NaCl). Photographs were taken to evaluate adhesion and results are presented as the number of adherent cells per condition.

Spreading Assay

ShSCR and shNEO1 SK-N-SH cells were seeded on coverslips pre-covered with Fibronectin (2 μg / ml) for 1 h. Then, the cells were fixed with PFA 4% w/ v followed by staining with phalloidin-546 (Thermofisher) and DAPI. Cells were observed and documented by confocal microscopy (Zeiss 710). A total of 23 spreading cells was quantified per condition.

Transwell migration assays

Transwell assays were completed using a chamber within an 8m-pore polycarbonate membrane (Corning). As a cell adhesion stimulus, 10 μg/ml mLM-111 (Invitrogen) and/or 2 μg/ml rhNTN4 (R&D systems) were placed on the underside of the transwell membrane 12h before performing the assay, dissolved in Phosphate buffered saline (PBS), which was used as a control. As a chemotactic stimulus DMEM 5% Fetal Bovine Serum (FBS) was used at the bottom of the chamber. Briefly, 50,000 shNEO1 or shSCR SK-N-SH cells were placed in the upper chamber. The cells were incubated for

2h, fixed and stained with crystal violet solution. All results were normalized with respect to the PBS condition of the shSCR cells.

Protein Co-Immunoprecipitation

SK-N-SH cells were used to prepare cell extracts with a buffer containing 20 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, and protease inhibitors by 5 min incubation on ice. Samples were centrifuged at 13,000 × *g* by 1 min at 4°C, and supernatants (1000 µg total protein) were immunoprecipitated with Dynabeads protein A (Thermofisher) bead-immobilized antibodies for 1h. NEO1 was immunoprecipitated with 2 µg of a rabbit polyclonal antibody (H-175, Santa Cruz) and LMγ1 was immunoprecipitated with 2 µg of mouse monoclonal antibody (MAB1920, Millipore). Immunoprecipitated samples were solubilized in loading buffer with ß-mercaptoethanol, and analyzed by Western blot as indicated in (Figure 3).

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TABLE I: Characterization of NTN4 expression in patients with NB. Percentage of NTN4 positive samples distinguishing for tumor cells, stroma and / or blood vessel expression, according to specific clinical characteristics of the patients. We do not found association between percentage of NTN4 and clinical features. Asterisk for p value from Fisher's exact test.

Clinical Feature		% of NTN4 positive tumor cells Samples	% of NTN4 positive Stroma Samples	% of NTN4 positive Blood vessel Samples	$\chi 2$	df	p
Gender	Male	78	78	100	1.23	2	0.54
	Female	36	50	100			
Age	>18M	63	45	100	1.34	2	0.51
	<18M	42	75	100			
Tumor Stage	Disseminated (M, MS)	63	50	100	0.49	2	0.78 / 0.86*
	Localized (L1, L2)	47	67	100			
PCNA	>40%	45	45	100	0.53	2	0.77
	<40%	58	75	100			
Primary Tumor Sites	Cervical	100	100	100	0.54	8	0.71/ 0.84*
	Thoracic	0	0	100			
	Abdominal	33	83	100			
	Retroperitoneal	60	60	100			
	Adrenal	60	40	100			
Patient Status	Dead	60	40	100	0.43	2	0.81/ 0.81*
	Recovered	53	67	100			

FIGURES

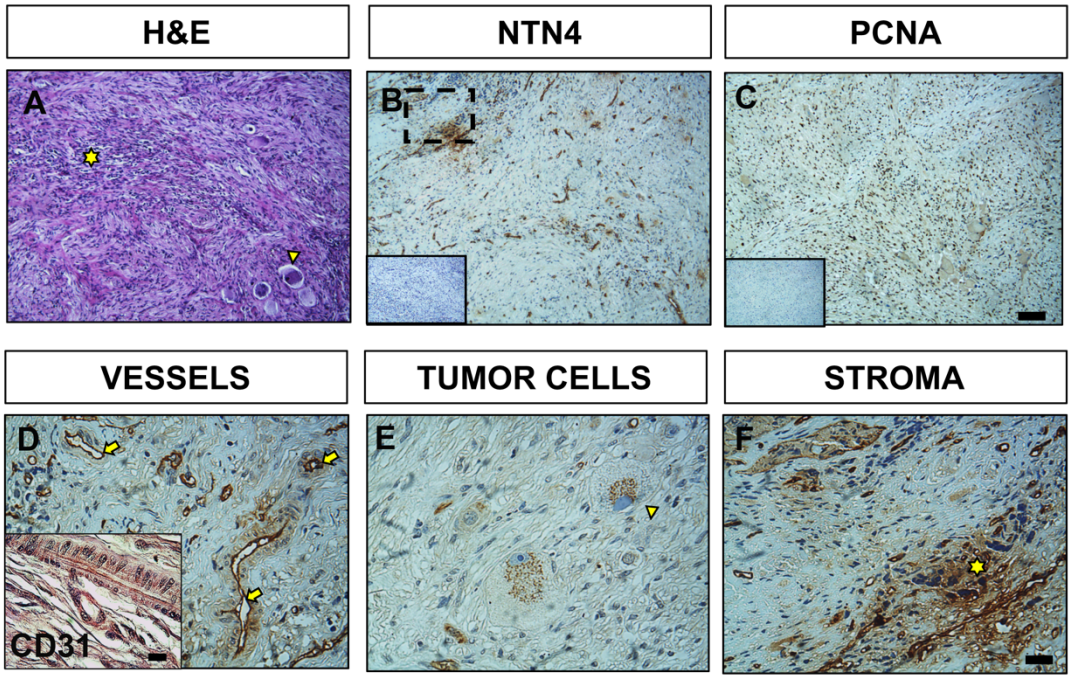


Figure 1: **Immunohistochemistry of NTN4 expression within NB.** Representative light microscopy images of a neuroblastoma sample from a female patient (age > 18M); tumor with retroperotineal location and in disseminated stage. (A) Hematoxylin and eosin staining reveals presence of ganglionar-differentiated cells (yellow arrowhead) and primitive neuroblasts (yellow asterisk). (B) Immunohistochemistry of NTN4 with its corresponding negative control (inset). (C) PCNA staining. (D) NTN4 is expressed preferentially in the endothelium (yellow arrows), as confirmed by CD31 staining (inset). (E) Detail of ganglionar-differentiated cells with strong NTN4 cytoplasmic expression in a characteristic punctuated pattern (yellow arrowhead). (F) Close up image of (B) as indicated, highlighting NTN4 expression within the stroma (yellow asterisk). A, B, C; Scale bar = 40 μm. D, E, F; Scale bar = 10 μm; inset = 10 μm.

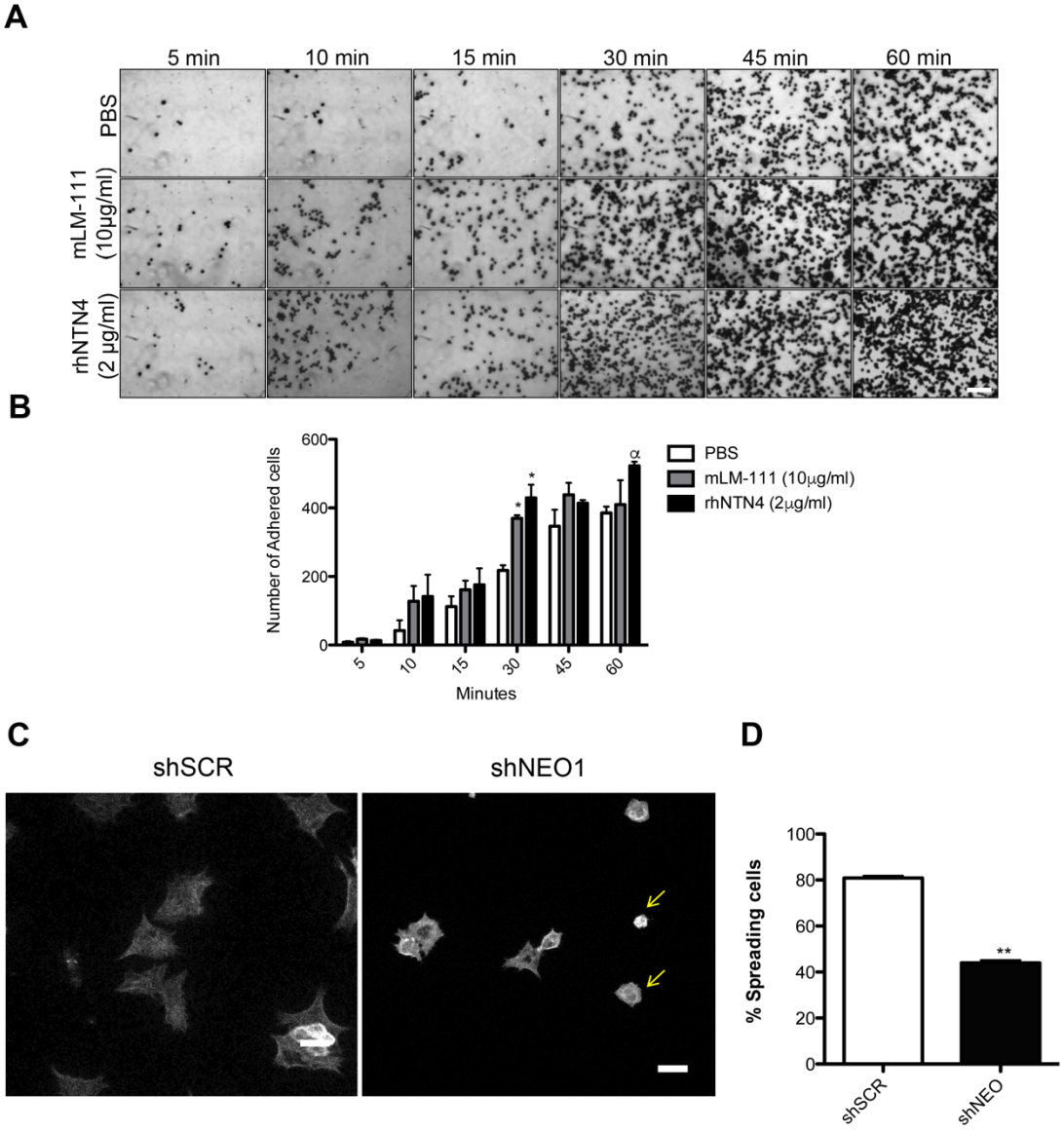


Figure 2: **NTN4 as a cell adhesion molecule and NEO1 contributes to cell spreading of SK-N-SH cells.** (A) Representative images are shown for cell adhesion assays, using 2 µg/ml rhNTN4 or 10 µg/ml mLM-111 (positive control) at the indicated time points (N=3). Scale bar =100 µm. (B) Quantification of cell adhesion assays shown in A. N=3, n=15 fields per condition at time points indicated in A. Bonferroni posttest, *p<0,05 PBS versus mLM-111 or rhNTN4 in 30 min of adhesion, α p<0,05 PBS versus rhNTN4 in 60 min of adhesion. (C) Representative images of spreading assay performed with shSCR

and shNEO1 SK-N-SH cells which spreaded into Fibronectin (2µg/ml) for 1h. Falloidin staining was used to evaluate cell spread. Yellow arrows indicate the different phenotype in spreading of the shNEO1 cells. Bar = 100 µm. (D) Quantification of the spreading assay, where results are expressed as percentage of spreaded cells. n=23, ** $p<0.01$ shSCR versus shNEO1 spreaded cells (black asterisk) or shNEO1 spreaded cells versus no spreaded cells (white asterisk).

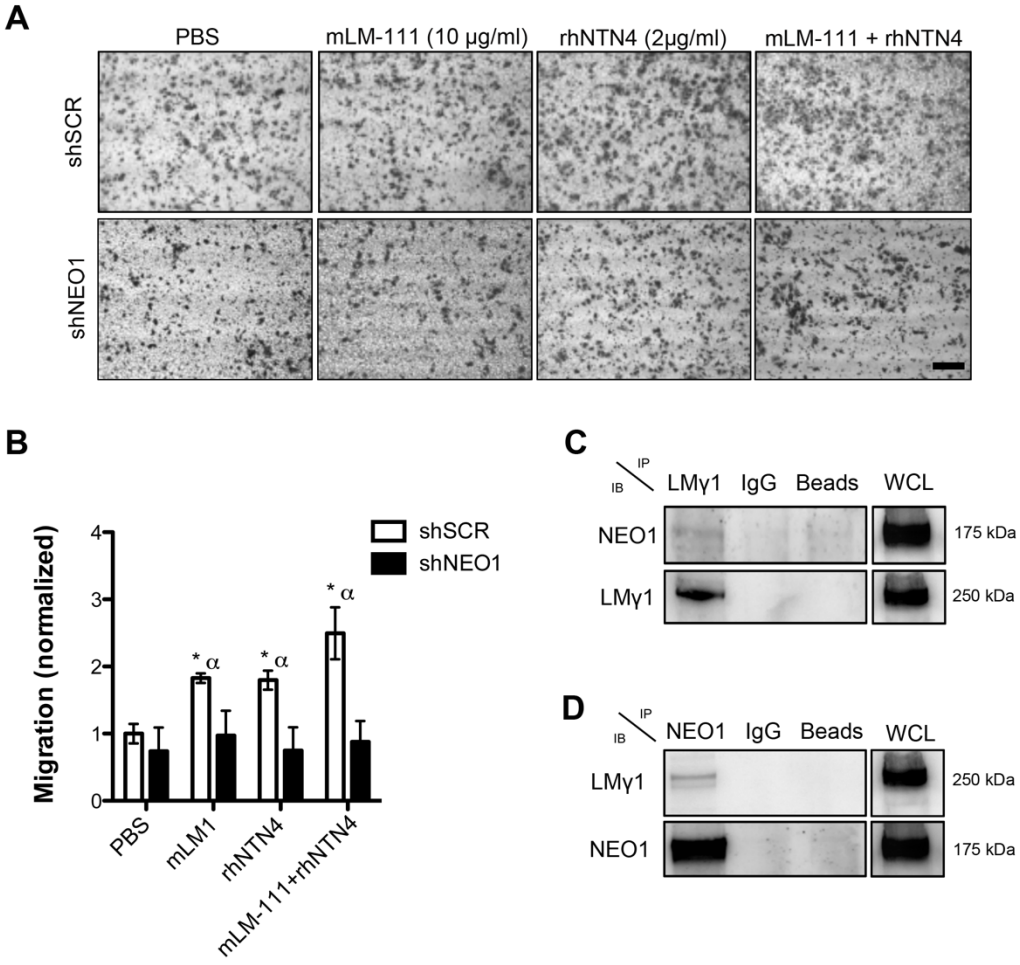
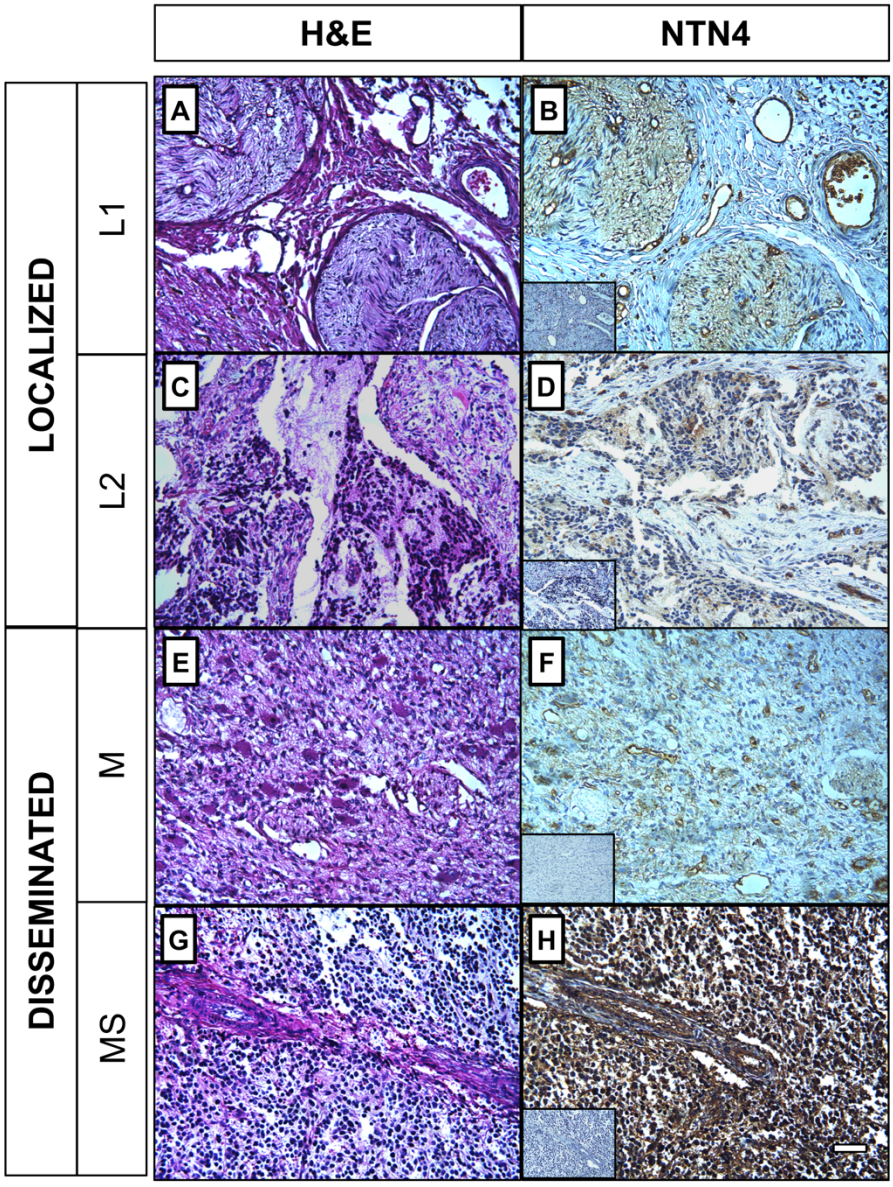


Figure 3: **NTN 4 induces cell migration in neuroblastoma cell line, SK-N-SH, through NEO1/Lamimin γ1 interaction** (A) Representative images of transwell assays using rhNTN4 and mLM-111 as adhesion molecules in SK-N-SH cells shSCR and shNEO1. Briefly, transwell assays were performed in chambers with an 8µm-pore membrane. Chambers were pre-treated with PBS, 10 µg/ml mLM-111 and/or 2 µg/ml rhNTN4 and placed on the underside of the membrane for 12h before performing the assay. As a chemotactic stimulus DMEM medium supplemented with 5% FBS was used. Cell migration was allowed for 2 h and analysis was performed as described in the material and methods. Data were normalized with respect to shSCR cells (PBS condition). Scale bar 100 µm. (B) Quantification of transwell assays obtained in C. Migrated cells were counted using inverted microscopy at 100x magnification. Five fields per condition were

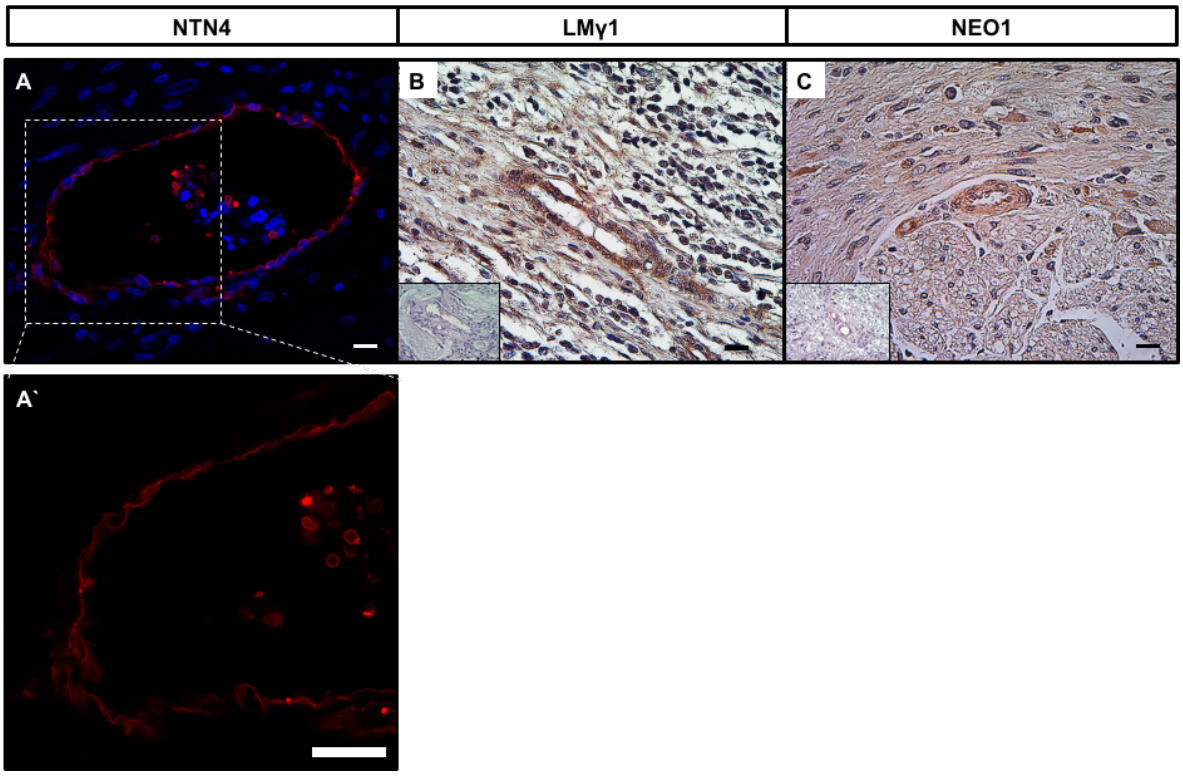
counted and data represent the average from three independent experiments. Data was normalized to shSCR cells (PBS condition). Mann Whitney t-test *p<0,05 shSCR PBS versus shSCR mLM-111 , rhNTN4 or both, α p<0,05 shSCR versus shNEO1 in the same cell adhesion stimulus. (C), (D) Representative Western blots of protein co-immunoprecipitation assays used to evaluate interaction between NEO1 and LMγ1 in SK-N-SH cells. Cells were lysed and incubated using specific antibodies against either LMγ1 (C) or NEO1 (D) followed by Western blot against NEO1 and LMγ1.

SUPPLEMENTAL DATA



Supplemental figure 1:
NTN4 expression within NB tumors stratified based on the International Neuroblastoma Risk Group Staging System (INRGSS) .
(A), (C), (E), (G) Hematoxylin and eosin staining of NB sections at stages as indicated. (B), (D), (F), (H) Analysis of NTN4 expression within the corresponding NB sample.

Each immunohistochemistry is shown with its corresponding negative control (inset). Scale bar = 10 μm.



Supplemental figure 2:
NTN4, LMγ1 and NEO1 expression in blood vessels within NB tumors.
(A, A') Representative images of NTN4 staining (red) in blood vessels of the same patient shown in figure1. DAPI (blue) was used for nuclei staining. (B) LMγ1 immunostaining identifies basement membrane. (C) NEO1 immunohistochemistry shows localization on blood vessels. Negative controls are shown as insets. Scale bar = 10 μm.

Neogenin-1 promotes Integrin β1 activation via FAK leading to Neuroblastoma cell migration

Running title: Neogenin-1 induces Integrin β1 activation through FAK

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Keywords:

Netrin-1, Neogenin-1, Integrin β1 activation, FAK, Cell migration, Metastasis

ABSTRACT

Neuroblastoma (NB) is an extracranial solid tumor that emerges from neural crest cell progenitors during development, and is characterized by being a highly metastatic cancer. Focal Adhesion Kinase (FAK), which is upregulated in NB, promotes cell migration by activating Integrin β1 (ITGB1) and promoting focal adhesion turnover, leading to tumor progression and metastasis. FAK is known to bind the axonal guidance receptor Neogenin-1 (NEO1), leading to FAK autophosphorylation and neuronal cell migration, by mechanisms that remain elusive. Most importantly, the relevance of such signaling axis in malignancy (i.e. neuroblastoma) has not been assessed. Here, we evaluated the role of FAK as a critical downstream hub of NEO1 that leads to ITGB1 activation and NB cell migration. Patient samples analysis revealed that NEO1 is highly expressed in tumor cells in all NB stages. In fact, shRNA-mediated knockdown of NEO1 decreased migration of human NB SK-N-SH cells, as shown in Transwell assays, using the Netrin-1 (NTN1) ligand as a chemoattractant. In addition, NEO1 was found to associate in a complex with ITGB1, FAK and NTN1, whereas NEO1 was necessary for FAK activation in SK-N-SH cells. Accordingly, FAK was required for NEO1-mediated cell migration and ITGB1 activation, as shown by immunofluorescence using a specific conformational antibody. Analysis of metastasis in an immunodeficient mice model confirmed that NEO1 is important to drive NB metastases. Our work suggests for the first time that NEO1 is a tumor progression-promoting protein, with an active role in metastasis. We propose a mechanism whereby NEO1, via interaction with NTN1, is involved in NB cell migration and metastasis. NEO1 likely plays this role by associating with FAK, allowing its autophosphorylation and complex formation with ITGB1, inducing thereby ITGB1 activation. Our results provide further information on the potential use of NEO1 as a therapeutic target to reduce metastasis in NB.

INTRODUCTION

Neuroblastoma (NB) is the most common extracranial solid tumor of infancy[1]. It is derived from neuroblasts of the sympathetic nervous system, and usually arises in the adrenal gland or sympathetic ganglia[1]. Since > 50% of diagnosed cases are metastatic, it is important to know the mechanisms underlying its malignant dissemination. Cell migration is a key event in tumor cell metastasis[2] and the dynamics of cellular adhesions play a fundamental role in this regard. The so-called focal adhesions (FAs) are supramolecular complexes formed upon engagement and activation of integrins, the main receptors of the extracellular matrix (ECM), whose specificity depends on the extracellular domains of the α and β subunits that form the integrin heterodimer[3]. The Focal Adhesion Kinase (FAK) is a critical factor that promotes both the assembly and disassembly of focal adhesions and hence is a master regulator of cell migration[4]. FAK is a common downstream signaling protein of growth factor, axonal guidance receptors, and integrins, integrating signals that converge in cell migration, growth, and survival[5]. FAK is upregulated in human NB cell lines and tumors, suggesting that FAK is important for NB cell viability and migration[6]. This is in accordance with compelling studies showing that FAK is upregulated in a variety of tumors, including ovarian serous cystadenocarcinoma, breast invasive carcinoma, and colorectal adenocarcinoma which correlates with poor prognosis and increased metastasis[7,8].

Recently, we identified Neogenin-1 (NEO1) being relevant to NB cell migration and metastasis[9]. NEO1 is a multifunctional receptor belonging to the immunoglobulin superfamily of transmembrane receptors, involved in axonal guidance, neuronal differentiation, morphogenesis, and cell death[10]. NEO1 is ubiquitously expressed during embryonic development, particularly in regions where there is robust cell proliferation, differentiation and migration[11]. NEO1 was described as an homologue of DCC (Deleted in colorectal cancer), as these proteins share about 50% amino acid identity and possess the same secondary structure, consisting of an extracellular domain that contains four loops type Immunoglobulin and six repeated regions type Fibronectin-III (FNIII), followed by a single transmembrane region and a cytoplasmic stem, containing three domains conserved with DCC, referred to as P1, P2 and P3[10]. The P3 domain binds to

intracellular proteins that determine the varied NEO1 responses[12,13]. The DCC / NEO1 receptors act as homodimers or form heterodimers with the UNC5 receptor family, sharing their binding to the Netrin ligands[14]. Netrin-1 (NTN1), the most characterized Netrin ligand, interacts with NEO1, leading to axonal guidance and cell migration, as well as cell-to-cell adhesion[10].The binding between NTN1 and NEO1 involves the FNIII domains 4 and 5 of NEO1[15].

Since both NEO1 and NTN1 are expressed during the development of the sympathetic nervous system[16], their signaling may be relevant within the context of NB oncogenesis and progression. Interestingly, NEO1 promotes the autophosphorylation of FAK on tyrosine 397 (Y397) in cortical neurons and both proteins have been shown to interact in the rat brain [12]. Thus, it has been proposed that FAK is a downstream signaling molecule of NEO1. Since FAK was suggested to activate Integrin β1 (ITGB1) during cell adhesion[17], and ITGB1 is implicated in NB progression[18], it can be inferred that all these molecules have a critical role in NB metastasis. In this study, we sought to elucidate the downstream signaling mechanism associated with NEO1-mediated cell migration and metastasis in NB. Specifically, we show that intracellular signaling triggered by the interaction between NTN1 and NEO1 promotes the activation of ITGB1 via FAK, leading to cell migration and consequently, metastasis.

MATERIALS AND METHODS

Patient samples and analysis of public databases

Ethics committees from University of Chile and CONICYT approved this study. General written consent was obtained from all patients enrolled by HNPG (Hospital de Pediatría Dr. Prof. Juan P. Garrahan), at diagnosis. All human tumor samples used in this study were diagnosed, and morphologically typified, through histological analysis at the Anatomopathological Center of this institution. Public databases of NB gene expression were visualized from R2: Genomics Analysis and Visualization Platform (http://r2.amc.nl) using MegaSampler analysis to evaluate NEO1 and NTN1 expression across databases. The databases used were Hiyama, Lastowska and Veegsted. Hiyama database comprises 51 NB samples that were resected either from the patients who died of tumor progression or those whose tumor regressed or matured spontaneously. Lastowska database comprises 30 NB samples and these were obtained from patients of all stages. Versteeg database comprises 88 human NB samples. Importantly, NEO1 expression comparing *MYCN* amplification was analyzed across those databases, using MegaSampler from R2.

Immunohistochemistry and histological analysis.

Paraffin-embedded patient samples of NB were deparaffinated and rehydrated as described in[19]. Immunohistochemical assays were proceeded by incubating the tissues with primary antibodies anti-NEO1 (1:50, sc-15337, Santa Cruz biotechnologies), anti-NTN1 (1:40, AF6419, sheep, R&D systems) and anti-PCNA (1: 100, 13-3900, mouse, Invitrogen) antibodies in 2.5% horse serum (from the Vestatin kit). Biotinylated secondary antibody was incubated for 2 h, and the ABC kit (Vestatin) was used, revealing with the 39-diaminobenzidine (DAB, Roche) substrate. Hematoxylin was used as a counterstain (Vector Laboratories, Burlingame CA). Images were taken at 100X and 400X. PCNA percentage was calculated from two independent observers, by counting the number of

cells marked in quadrants and multiplying by the total number of cells present in each sample, quantified counting hematoxylin stained cells. 40% was the median obtained for the total of 21 samples. χ square and Fisher's exact test ($n< 5$ samples) were used as statistical tests.

Cell culture

The NB cell line, SK-N-SH (ATCC® HTB-11), was cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM, Gibco) with 5% Fetal Bovine Serum (FBS, Gibco) and supplemented with antibiotics (Penicillin-Streptomycin, 10,000 U/mL, Gibco). The HEK293FT (human embryonic kidney) cells were cultured in DMEM with 10% FBS supplemented with antibiotics.

Lentiviral transduction and stable shRNAs cell line generation

To knock-down NEO1 (shNEO1), SK-N-SH cells were transduced with lentiviral particles that contained shRNA vectors (pGIPZ backbone); and two different shRNA sequences were used to knock-down this protein. A scramble sequence (shSCR) was used as a control. These lentiviral particles were generated using HEK293FT cells, with the $CaCl_2$ transfection method[20]. HEK 293FT cells were triple transfected with pCMV-VSV-G, p8.91, and pGIPZ-shRNA (Openbiosystems). After 48 h, the conditioned medium (viral supernatants) of these cells was collected and added in a 1: 1 ratio to the media of SK-N-SH cells. After 48 hours, the transduction percentage was measured using tGFP encoding in pGIPZ and cells were incubated with the selection marker puromycin (3 μg/ml, Sigma) for additional 48 h. Cells were maintained in DMEM with FBS supplemented with puromycin. The knock-down efficiency was measured via Western blot analysis.

Transwell migration assays

Tranwell assays were completed using a chamber with an 8 μm pore polycarbonate membrane (Corning). As an underside cover, 2 μg/ml of fibronectin (Sigma Aldrich) was used, placed on the membrane 12 h before performing the assay. As a chemotactic stimulus, different concentrations of recombinant human Netrin-1 (rhNTN1, R & D Systems) were used, all dissolved in DMEM without serum. Briefly, 50000 shNEO1 and shSCR SK-N-SH cells were used, which were placed in the upper chamber; the lower chamber contained various concentrations of rhNTN1 diluted in DMEM. The cells were incubated for 4 h, fixed and stained using 100% crystal violet diluted in methanol in a 1: 5 solution of 0,15 M NaCl. Photos of each condition where taken and five fields per condition were counted.

Protein co-immunoprecipitation

Protein co-immunoprecipitation was performed as indicated in[9] with variations. To evaluate the interaction between NEO1, ITGB1 and its ligand NTN1, SK-N-SH cells were incubated with rhNTN1 (200 ng/ml) in DMEM without serum for 1 h. In order to evaluate the interaction between NEO1 and FAK, the cells were treated with NTN1 (25ng/ml) in DMEM without serum for 1 h. Subsequently, cell extracts were prepared in a buffer containing 20 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, and protease inhibitors and incubated by 5 min on ice. The samples were centrifuged at 13000 g for 1 min at 4° C, and the supernatants (1000 μg of total protein) were incubated with 2 μg of anti-NEO1 antibodies (# sc-6536, Santa Cruz Biotechnology), anti-Netrin-1 (AF6419, RYD Systems), anti pFAK Y397 (# 44-624G, Thermofisher), total anti-FAK (# 05-537, Millipore) or anti-ITGB1 (# sc-8978, Santa Cruz Biotechnology), immunoprecipitated with Dynabeads protein A (Thermofisher) bead-immobilized antibodies for 1 h. Immunoprecipitated samples were solubilized in loading buffer with ß-mercaptoethanol, and analyzed by Western blot.

Western blot

Protein extraction was performed using lysis buffer (SDS 2% w / v, Tris-HCl 80 mM pH 7.5, Glycine 10% w / v) with protease inhibitors (Thermofisher). After three minutes of sonication on ice, samples were centrifuged (10000 xg) for 5 minutes at 4° C. Samples were resolved in 8% polyacrylamide gels, and proteins were transferred to 0.45 μm nitrocellulose membranes by wet transfer overnight. Primary antibodies were incubated overnight at 4° C in 5% non-fat milk (except for pFAK and NTN1, which is 5% BSA in TBS- 0.01% Tween or Buffer 8 (RYD systems)) diluted in TBS-Tween 0,01%, and the secondary antibodies were incubated at room temperature for 2 h in the same buffer. The antibodies used were anti-NEO1 (# sc-6536, Santa Cruz Biotechnology, 1: 200), anti-Netrin-1 (AF6419, RYD Systems, 1: 400), anti-actin (A5316, Sigma, 1: 1000), anti-tubulin (T9026, Sigma 1: 1000), anti-pFAK (# 44-624G, Thermofisher, 1: 1000), total anti-FAK (# 05- 537, Millipore 1: 1000), anti-ITGB1 (# sc-8978, Santa Cruz Biotechnology, 1: 300). Western blots were quantified using integrated density analysis with ImageJ software (National Institutes of Health, USA).

Spreading assays and active ITGB1 analysis by immunofluorescence

shSCR and shNEO1 SK-N-SH cells were seeded on coverslips pre-covered with Fibronectin (2 μg / ml, Sigma-Aldrich) for 1 h. Then, the cells were fixed with PFA 4% w/v, stained with phalloidin-546 (Thermofisher) and DAPI. Cell spreading was analyzed by confocal microscopy (Zeiss 710). To evaluate the activation of ITGB1 and the requirement of FAK, pEGFP-NEO1 or the empty vector (pEGFP) were overexpressed and a spreading assay was performed. Briefly, NEO1 was overexpressed in SK-N-SH cells, by using Turbofect (Thermofisher) as a transfection agent. To this end, a pEGFP plasmid cloned with the complete NEO1 DNA sequence (full length) coupled to eGFP was used. As an empty vector control (EV), the pEGFP plasmid was used. The transfections were performed according to the manufacturer's instructions and the expression of GFP was evaluated at 36 h after transfection by epifluorescence microscopy.

Next, the cells were seeded on coverslips pre-coated with Fibronectin (2 μg / ml) for 1 h, in DMEM containing rhNTN1 (25 ng / ml). Previously, cells were treated with the FAK autophosphorylation inhibitor (PF271, TOCRIS), or vehicle (DMSO), for 1 h before spreading and the same stimuli were maintained during this test. Then, the cells were fixed with PFA 4% w/v in PBS and the immunofluorescence of activated ITGB1 (clone Huts-4, MAB2079Z, EMD Millipore, 1: 300) and total ITGB1 ((# sc-8978, Santa Cruz Biotechnology, 1: 100) was performed. The assay was evaluated by confocal microscopy (Zeiss 710) and 400X images were taken. The GFP fluorescence channel was used to select for transfected cells and the fluorescence intensity of the cell periphery was quantified according to the parameters given by the ImageJ software (https://imagej.nih.gov/ij/). For quantification, a 2-3 μm cell perimeter was considered, using Phalloidin staining as a reference for cell body.

Spheroid formation and migration assay based on spheroids

SK-N-SH shSCR and shNEO1 were used to perform this assay. Spheroids were generated from 1000 cells seeded in a nonadherent T25 bottle (Corning), with DMEM-F12 and B27 (Thermofisher, 1:50), for 5 days. Once the spheroids were formed, they were harvested and seeded on plates covered with Fibronectin (2 μg/ml) in the presence of DMEM 5% SFB. After 12 h, they were stained with phalloidin-546 (Thermofisher) and DAPI and observed by confocal microscopy (Zeiss 710) counting the cells that migrated away from the spheroids. In addition, some experiments included the use of the FAK autophosphorylation inhibitor (PF562,271, TOCRIS) at 1μM, applied at the moment when the spheroids were placed on Fibronectin to perform the migration assays.

Spontaneous metastasis assays

Ethics committees from Universidad de Chile, Instituto de Salud Carlos III and CONICYT approved the animal use and care in this study. shSCR or shNEO1 SK-N-SH cells were used, stably transduced and subsequently transduced with a plasmid coding for

the enzyme luciferase, in order to evaluate metastasis to different organs by luminescence. Briefly, 1.5 million shSCR or shNEO1 cells, mixed with matrigel (1: 5), were injected into both flanks of male immunodeficient mice (NSG strain). One week after the injection, the tumor growth was quantified revealing the luminescent activity of the primary tumors, with the use of luciferin (12.5 mg by intraperitoneal injection) in mice, anesthetized with isoflurane. The luminescence was recorded with the IVIS *in vivo* imaging system, every 2-3 days for several weeks. After 5 weeks post injection, the mice were treated with intraperitoneal luciferin, sacrificed, and the primary tumor was extracted in addition to the following organs: liver, lung, spleen and kidney. All tissues were analyzed with IVIS equipment and presence or absence of metastasis was determined for each organ in the different conditions (shSCR or shNEO1). Five mice were used for each cell line. With the extracted primary tumor, an RNA extraction was performed and *NEO1* transcript levels were determined to determine if the knock-down remained stable during the 5 weeks procedure.

RESULTS

NEO1 is expressed in NB patient samples

In order to determine the contribution of NEO1 in NB progression, we first evaluated the expression of this protein in NB patients (n=21) by immunohistochemistry on paraffin-embedded samples, categorized according to INRGSS (International Neuroblastoma Risk Group staging system)[21]. INRGSS classified the tumors in Localized Stages (L1 and L2), when the tumor is confined in a region, or Disseminated Stages (M, MS), where tumors are metastatic and aggressive. MS is different than M, because metastases are confined to the skin, liver, and/or bone marrow in children younger than 18 months of age[21]. Figure 1 A-D shows the expression of NEO1 in a patient sample corresponding to a localized stage (L1) (B, low magnification and D, high magnification). The staining is mostly restricted to tumoral cells, but can also be found in blood vessels (Figure 1B, F arrowhead). At a Disseminated stage (M), as shown in figure 1E-H (F, low magnification and H, high magnification), the NEO1 expression persists in tumoral cells. When the percentage of NEO1 positive samples is analyzed according to clinical features (Gender, Age, tumor stage, PCNA expression (data no shown), primary tumor sites, patient status) we do not find any significant correlation (Table 1).

Analysis of NEO1 expression across public NB databases using MegaSampler from R2: Genomics Analysis and Visualization Platform (http://r2.amc.nl), revealed that NEO1 expression is similar in different databases (Supplementary Figure 1A) (details of each database provided in the Materials and Methods section). Interestingly, when the NEO1 expression data is sorted by *MYCN* amplification in each database (Supplementary Figure 1B), samples without this amplification have more NEO1 expression than MYCN-amplified samples. According to this information, we decided to use the MYCN/WT cell line SK-N-SH, in order to evaluate the possible signaling mechanism associated to NEO1 expression in NB (upcoming paragraphs). In this respect, we have previously shown that NEO1 expression is highest in this cell line, when compared with another NB cell lines[9].

NTN1 is the main Netrin ligand of NEO1[10]. By analyzing the expression of this protein in the NB Samples (Supplementary Figure 2), we found important differences between localized and disseminated stages. NTN1 expression is prominent in localized stages and can be found in stroma, large vessels and tumor cells. Nevertheless, NTN1 expression was almost absent at disseminate stages. Furthermore, when we evaluated the relation between the percentage of NTN1 positive samples and specific clinical features (Supplementary table 1), the NTN1 positive samples associated with localized stages and recover patient status, inferring that NTN1 could be a positive prognosis factor (Supplementary table 1). The individualized data of NEO1 and NTN1 expression for each patient sample are shown in Supplementary table 2. Collectively, these results show that NEO1 is expressed in NB patient samples, mostly in tumor cells, and persisting throughout different NB stages, while NTN1expression is restricted mostly to localized stages.

NEO1 promotes cell migration induced by NTN1

Having revealed the persistent expression of NEO1 in NB samples, we next sought to address the function of NEO1 in NB SK-N-SH cells. In order to determine the role of NEO1, we generated knockdown cells for NEO1 through the use of shRNA and lentiviral transduction. Two shRNA sequences were tested for NEO1 targeting (Seq.1 and Seq. 7), although only Seq. 7 decreased the expression of NEO1 significantly (60%.), as measured by western blot (Supplementary figure 3). Thus, these cells were used for all the remaining experiments.

Due to the antecedent that NEO1 promotes cell migration in NB[9], evaluated the chemotactic migration of SK-N-SH cells, exposed to different concentrations of rhNTN1, since Netrins are known to act as chemotactic molecules[22]. We performed transwell assays with both shSCR (control) and shNEO1 cells, using different concentrations of rhNTN1 (5, 15, 25 ng/ml) in the bottom chamber, allowing cell migration for 4 h. Figure 2A shows representative images of transwell assay and the quantification of this experiment is shown in figure 2B, indicating that at 15 ng/ml as well as at 25 ng/ml, there is a significant

increase in the migration of shSCR cells compared with the control condition (without NTN1). Moreover, we observed that at the same concentrations of rhNTN1, there is a decrease in migration for shNEO1 cells compared to shSCR. In conclusion, NEO1 promoted NTN1-induced cell migration in SK-N-SH cells.

NEO1/NTN1 form a complex with Integrin β1 (ITGB1) in SK-N-SH cells

To determine the mechanisms underlying NEO1-dependent cell migration, we evaluated if NEO1 can form a complex with NTN1 and ITGB1. NTN1 is known to associate with ITGB1 in interneurons, promoting cell migration[23] although it cannot be excluded the possibility that NEO1 is involved in this process. In order to evaluate the association between NEO1, NTN1 and ITGB1, we performed protein co-immunoprecipitation assays in SK-N-SH cells, upon treatment with rhNTN1 (100ng / ml) for 1h. Immunoprecipitations were made for NEO1 (Figure 3 A, B), NTN1 (Figure 3 C, D) and ITGB1 (Figure 3 E, F), and data showed that NEO1 associates with NTN1 (Figure 3 A) and ITGB1 (Figure 3 B). Furthermore, NTN1 was found associated with NEO1 (Figure 3 C) and ITGB1 (Figure 3 D), whereas ITGB1 was also found associated with NTN1 (Figure 3 E) and NEO1 (Figure 3 F). Collectively, these results allow us to suggest the existence of a ternary complex between NEO1, NTN1 and ITGB1, which may have a relevance in NB cell migration.

FAK is a downstream target of NEO1 during cell migration

FAK is a crucial signaling component that is activated by numerous stimuli, which converge in cell migration[24] within which is integrin signaling[25]. FAK was previously shown to interact with NEO1 in whole brain lysates[12]. Accordingly,, we aimed to evaluate the interaction of NEO1 with FAK via co-immunoprecipitation, using the NB cell line SK-N-SH. In fact, immunoprecipitation of FAK and subsequent blotting against NEO1, revealed that both molecules associate in a complex (Figure 4A). Next, we evaluated the activation of FAK through an analysis of the autophosphorylation site of FAK at Y397

(pFAK Y397), known as the initial activating phosphorylation site of FAK[4], in NEO1-immunoprecipitated FAK (Figure 4 B). To this end, cells were treated with rhNTN1 (25ng/ml) for 1h, lysed and used for immunoprecipitation of NEO1. Phospho-Y397-pFAK was measured via western blot. Of note, NTN1 treatment induced this phosphorylation, indicating that FAK is a NEO1-associated molecule and NTN1 induces the activation of FAK precisely when associated to NEO1.

Having established that FAK is a downstream molecule of NEO1, we evaluated if FAK is important for NEO1 induced cell migration. To this, we conducted an spheroid-based cell migration assay using the FAK inhibitor PF562,271 (PF271)[26]. Briefly, spheroids of shSCR and shNEO1 cells were placed into fibronectin-coated plates and allowed to migrate for 12 h, in presence of 1μM of PF271 (inhibitor efficiency shown in Supplementary figure 4 B). Next, the spheroids were fixed and stained with phalloidin, as shown in Figure 4 C. Migrated cells from the spheroids where quantified as detailed in the materials and methods (Figure 4 D). The treatment with the inhibitor significantly decreased cell migration in both shSCR and shNEO1 conditions. However, the extent of inhibition by PF271 was similar in shNEO1 cells, when compared to shSCR cells, so it could be assumed that the autophosphorylation of FAK in Y397 is important for the migration of these cells and that the kinase is an intracellular NEO1 effector.

NEO1/NTN1 induces Integrin β1 activation via pFAK

Since NEO1/NTN1 associates with ITGB1 and FAK is a downstream molecule of NEO1 signaling, we further evaluated how these components might interact in our experimental model. Therefore, we conducted a co-immunoprecipitation , showing that FAK and ITGB1 associate in SK-N-SH cells (Supplementary figure 4 A). It has been reported that FAK induces the activation of ITGB1 in human fibroblasts[17]. To evaluate this possibility, we performed a spreading assay on SK-N-SH cells undergoing spreading onto fibronectin-coated plates for 1 h, and then fixed and labeled against active ITGB1 using a conformational HUTS-4 antibody[27]. Total ITGB1 immunofluorescence was suited as control (Supplementary figure 4 B). Furthermore, we compared spreading capacity in

PF271 inhibited and DMSO control treated cells. To quantify, confocal microscopy images were taken in the lower plane of spreading cells and then, using ImageJ software, the fluorescence intensity was quantified, for active and total ITGB1 in the immediate 3μm layer inside the cell periphery (without considering the nuclear mark). Results are shown as the ratio active / total ITGB1 for each condition. As the result of this quantification (Supplementary figure 4 C), we revealed that the inhibition of pFAK Y397 reduces ITGB1 activation. These results show that FAK induces ITGB1 activation.

Considering that NEO1 / NTN1 is associated with ITGB, FAK is required for the induction of migration mediated by NEO1 and that FAK activates ITGB1, we next evaluated whether NEO1 promotes the activation of ITGB1 through FAK autophosphorylation. NEO1 is a dependence receptor[9] and hence its overexpression in a long term induces cell death. Thus, we overexpressed NEO1 in SK-N-SH cells at low concentration (WB in supplementary figure 3 C) and realized a spreading assay using the same conditions as previously reported, in presence of PF271 and stimulating with rhNTN1 (25 ng/ml). Figure 5A shows representative confocal images of the assay. Figure 5B reveals the effect of exogenous ligand (rhNTN1) stimulation in terms of the activation of ITGB1. Only the cells that overexpress NEO1, treated with rhNTN1, significantly increased the activity of ITGB1 compared with the control of the same treatment. This result indicates that NTN1 is required for NEO1 to induce the activation of ITGB1. Moreover, when overexpressing NEO1 cells, in the presence of NTN1, are treated with PF271 (Figure 5 C), activation of ITGB1 is significantly reduced. The latter indicates that FAK autophosphorylation is required for the induction of ITGB1 activation downstream of NEO1.

NEO1 promotes metastasis *in vivo*

After determining that NEO1 promotes *in vitro* cell migration of NB cells and having established a possible mechanism associated with this process, we decided to evaluate the role of NEO1 in the *in vivo* metastasis. Accordingly, immunodeficient mice (NSG strain) were injected in both flanks with NEO1 knock-down cells (shNEO1) or

control cells (shSCR), and spontaneous metastasis in each organ were analyzed 5 weeks later. Figure 6A, compares the growth curve for shSCR versus shNEO1 SK-N-SH primary tumors, indicating no significant differences in tumor growth. Five weeks after implanting the primary tumors, metastasis was evaluated in different organs (lung, liver, kidneys, spleen). Figure 5B shows the similar size of representative primary tumors at 5 weeks. In order to determine that silencing of NEO1 was not lost during the assay, receptor mRNA levels were measured in the primary tumors for both conditions verifying that silencing is stable and maintained in an *in vivo* context (Supplementary figure 5). In Figure 5C, secondary tumors are shown in the different organs. It is noteworthy mentioning that there is practically no metastasis in the shNEO1 cells, except in the lung, while the shSCR cells generate metastasis in all the organs analyzed (Figure 5D). The individual data of each mouse analyzed, and the qualitative luminescence intensity can be observed in Supplementary table 3. These results show that NEO1 promotes metastasis in *vivo*.

DISCUSSION

NB is a pediatric tumor arising from embryonic sympathoadrenal lineage of the neural crest[28] and the first cause of death from pedriatric cancer for children under five years. NB is a very aggressive tumor, where <50% of cases diagnoses is metastasize[29], intriguing about the mechanisms associated with this process.

NEO1 is a multifunctional receptor involves in differentiation, cell death, angiogenesis, axonal guidance and, in the last few years, cell migration in the context of development[30]. NEO1 is also relevant for the cell migration of several cancers, such as gastric cancer[31] and NB[9], but the associated signaling mechanisms have not been elucidated. The aim of this work was to evaluate how NEO1 induces chemotactic cell migration through its ligand, NTN1, and consequently metastasis of NB. This work shows that NEO1 associates with ITBG1 and NTN1, inducing FAK phosphorylation and ITGB1 activation through FAK. This mechanism would explain how NEO1 induces NB cell migration. This latter information is important to stablish a general mechanism of NEO1 function.

Clinical significance of NEO1 expression in NB patient samples

A previous report from our group[9], showed that NEO1 expression in NB public database is correlated with a low survival rate, indicating a possible role of NEO1 in the pathogenesis of this cancer. Analyzing NEO1 expression across diverse datasets reveals that expression levels of NEO1 are stable at different NB stages, a similar result to the one we observed with our cohort of patients. Furthermore, the NEO1 expression is mostly restricted to tumor cells and is persistently in all tumoral stages analyzed. Thus, this elevated NEO1 level suggests a selective advantage acquired by cancers cell to migrate and metastasize.

To date, amplification of *MYCN* remains the best-characterized genetic marker of risk in NB. An aberrant expression of *MYCN* has been associated with tumor aggressiveness, resistance to chemotherapy and the inability to differentiate[32].

Interestingly, when NEO1 expression is evaluated in different NB datasets considering the reported *MYCN* amplification we observed that NEO1 is preferentially expressed in patient samples without this amplification. ITGB1 also correlates negatively with the *MYCN* amplification in NB[33]. Hence, a differential mechanism of cell migration, active depending upon *MYCN* amplification could be proposed, a matter that requires further research. Since both NEO1 and ITGB1 are more expressed in patient samples without *MYCN* amplification and both are important for NB cell migration, we considered interesting evaluating whether there is a functional relationship between these proteins in the process of cell migration and metastasis in the SK-N-SH (non-*MYCN*-amplified) cells.

NEO1 has several ligands, such members of NTN family as NTN1. NTN1 is the most characterized member of the laminin-related Netrin family composed by NTNs 1-4. Nowadays, there is a growing collection of information regarding the different biological roles that NTN1 displays in a variety of cancer types, but which signaling pathway is activated downstream is an issue that remains to be resolved. In our analysis, NTN1 is mostly expressed in localized stages, indicating that NTN1 is being secreted by tumor cells of the NB primary tumors, while metastasized tumors showed decreased NTN1 expression. This result is contrary to results reported by[34]. Most likely, the variability between cohorts and differences on how the expression was analyzed explain this discrepancy. NTN1, being a secreted factor, is found in multiple sources. It has been found outside the central nervous system in the blood plasma[35] and urine[36] as well as in endothelial cells[22], medulloblastoma[36] and colorectal tumor cells[37], among others. In this work we evaluated the expression via immunohistochemistry, showing that NTN1 is secreted by primary tumor cells, while disseminated tumors showed decreased ligand expression. We speculate that at disseminated stages probably NTN1 acts as a chemoattractant molecule secreted by another tissues, inducing tumor cells to migrate to another tissues, and hence, mediating metastasis. Keeping in mind that NEO1 is a dependence receptor, and as such requires a ligand to execute a non-apoptotic/positive signaling we propose that at disseminated stages, probably others ligands become more expressed. In fact, we have recently shown that NTN4 is strongly expressed by blood vessels (publication under revision). Therefore, it is relevant to consider the tumor

microenvironment, including the tumor stroma, and other elements, such as the endothelial niche, as being essential to sustain tumor growth and metastasis.

Mechanisms associated to NEO1/NTN1 complex association with ITGB1 and cell migration

In the present study we show that NEO1 induces cell migration through NTN1 mediated chemotaxis in SK-N-SH cells. This result is concordant with data exposed in 2015 by the O' Leary group[30]. They showed that in a physiological context NTN1 induces neuroblast cell migration via its receptor NEO1. More recently, similar results were reported in gastric cancer[38].

Hence, we aimed to determine the interaction between the above-mentioned molecules. Previously, it has been reported that NTN1 binds ITGB1[23], regulating the migration of interneurons during development. Nevertheless, the authors did not evaluate a possible NEO1 association with integrins in this process. Here we show that NEO1 associates with ITGB1 and its ligand NTN1, through co-immunoprecipitation, forming a ternary complex. Our result could reconcile the disparities of NTN1 reported functions according to ECM or concentrations. For example, when studying axon guidance in cultured dorsal root ganglions, NTN1 causes collapse of growth cones extending on high levels of laminin-111, but not on low levels of laminin-111 or fibronectin[39]. This differential phenomenon could be explained by the NTN1 concentration used, because NTN1 has different functions according to cell type analyzed (e.g. reduces chemotaxis of neutrophils[40] or concentration high or low concentrations[22], binding different receptors such as UNC5[41], which binding has a chemorepulsive function. Hence, according to ligands concentration, NTN1 could command different processes, mediating chemoattraction via NEO1 or repulsion through UNC5 family.

In axonal guidance[12] and muscle development[42], NEO1 induces FAK autophosphorylation in Y397, exposing other p-FAK domains and promoting further FAK activation. In this study we found not only interaction between NEO1 and FAK but also

an induction of FAK Y397 phosphorylation when cells are treated with exogenous NTN1. In addition, p-FAK Y397 inhibitor reduces cell migration of control cells to similar extent when compared to NEO1 knock-down cells in a cell migration assay, indicating that FAK is downstream of NEO1 signaling pathway. NEO1 interacts with FAK through its intracellular P3 domain, as reported also for its homologue DCC [12]. Recent findings show that once the NTN1/DCC signaling pathway is activated, a binding of the P3 domain of the receptor to the focal adhesion targeting (FAT) domain of FAK is produced, as evaluated through crystallography[43] Despite the fact that the FAK FAT domain is the binding domain to talin and/or paxilin[44], there are different recognition sites in the FAT domain when bound to the DCC P3 domain binding. Also, this binding recruits to FAK close to the cell membrane, which could exerts a concerted effect for FAK signaling, within which could be the activation of integrins. Indeed, FAK is syndicated as an important molecule in integrin activation[17] (supplementary figure 4), associated in nascent focal contacts[45], where integrin activation is initiated, and FAK is an integrator between receptors and integrin signaling[46]. Having shown that FAK is a downstream molecule of NEO1/NTN1 signaling and having established an association between NEO1/NTN1 and ITGB1, we aimed to determine if NEO1 is involves in ITGB1 activation in SK-N-SH NB cells.

As predicted, the overexpression of NEO1 in presence of NTN1 treatment induces ITGB1 activation. Of note, the binding of NEO1 with its ligand NTN1 is required to induce ITGB1 activation, since in absence of exogenous NTN1, no significant augment in active ITGB1could be found. Also, pharmacological inhibition of phosphorylation of FAK Y397, significantly reduces levels of ITGB1 activation in NEO1 overexpressed cells, showing that phosphorylation of the kinase is important to promote this activation. Once FAK results autophosphorylated in Y397, a recruitment of Src and Src-family kinases and the increased phosphorylation of other proteins has been reported[4]. Thus, the association between NEO1 and ITGB1 could not only promote the ITGB1 activation but also lead to other focal adhesion protein activation. Clearly, the possible intricate network of interplay between these proteins warrants further investigation.

In summary, here we propose a mechanism whereby NEO1 in interaction with NTN1 associates with ITGB1 and induces its activation via FAK phosphorylation in SK-N-SH cells. Further studies are warranted to explore whether this mechanism could be generalized to NEO1 signaling in malignant NB cells.

NB metastasis promoted by NEO1

The fact that that NEO1 knock-down cells promote less lung metastasis in a chorioallantoic membrane assay (CAM)[9], let us to evaluate in this work the potential role of NEO1 in metastasis using a immunodeficient mouse model and a spontaneous metastasis approach. Control cells metastasized into spleen, liver, kidney in 60% of mice analyzed, and lungs in a 80%. Meanwhile, knock-down cells, exclusively metastasized to lungs in 100% of mice analyzed. The latter could be explained since the knockdown is a 60% of reduction of NEO1 levels and still some NEO1 positive cells could metastasize. In support to our findings, lungs are a preferential niche in several cancer metastases[47], because they are very blood irrigated and present an intricate vasculature, promoting extravasation of tumoral cells. Our result indicates that NEO1 promotes NB SK-N-SH cells metastasis in a immunodeficient mice model. NTN1, although expressed by tumor cells, is mostly located either in adjacent endothelial cells or stroma, suggesting a relevant contribution to this pathology acting as chemotactic molecule. Therefore, it is relevant to consider the tumoral/stromal/endothelial niche as being essential to sustain tumor growth and metastasis.

In conclusion, NEO1 binds to its NTN1 ligand, signaling downstream with ITGB1/FAK and promoting metastasis in NB. These findings may be beneficial to the understanding of the cellular mechanisms of NEO1 function. Future studies in preclinical models need to address if this molecular crosstalk is preserved and could represent possible target. Our final goal is to translate our results into better therapeutic strategies, through precision medicine, contributing to the diagnosis and treatment of NTN1/NEO1-driven tumors.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Table I

Characterization of % of NEO1 positive Samples (within tumor cells, blood vessels or stroma). Percentage of NEO1 positive samples according to specific clinical characteristics of the patients. We do not found association between percentage of NEO expression and clinical features. Asterisk for p value from Fisher's exact test.

Clinical Feature		% of NEO1 positive Samples	$\chi 2$	df	p-value	Fisher's p-value
Gender	Male	78.0	0.112	1	0.737	1
	Female	77.8				
Age	>18M	83.3	0.015	1	0.9	1
	<18M	81.8				
Tumor Stage	Disseminated (M, MS)	80.0	0.03	1	0.86	1
	Localized (L1, L2)	83.3				
PCNA	>40%	90.0	1.014	1	0.314	0.586
	<40%	72.7				
Primary Tumor Sites	Cervical	66.7	0.938	4	0.919	1
	Thoracic	100				
	Abdominal	83.3				
	Retroperitoneal	80.0				
	Adrenal	75.0				
Patient Status	Dead	75.0	0.257	1	0.612	1
	Recovered	85.7				

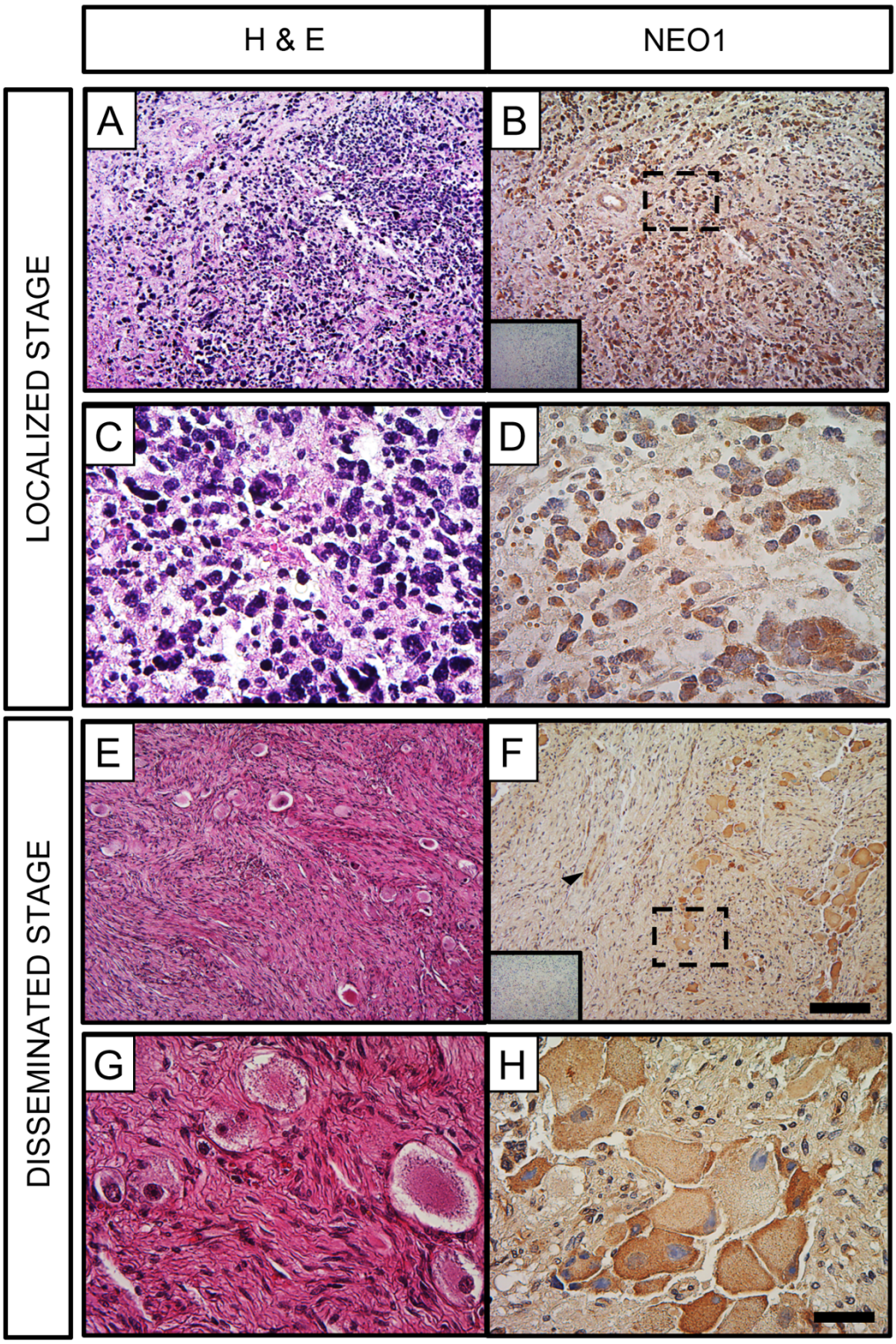

H & E
NEO1
LOCALIZED STAGE
DISSEMINATED STAGE
A
B
C
D
E
F
G
H

Figure 1: NEO1 is expressed in NB samples independently of tumoral stage. Immunohistochemically (IHC) analysis of NEO1 expression in NB samples. In all IHC Hematoxylin was used as a counterstaining A- D: Representative images of a NB patient sample classified at Localized Stage according to INRGSS. A, C: Hematoxylin-Eosin (H&E) staining, B: NEO1 expression (brown). Dotted square shows the area represented at higher magnification in D. E- H: Representative images of a NB patient sample classified at Disseminated Stage according to INRGSS. E, G: H&E staining, F, H: NEO1 expression. Dotted square shows the area represented in high magnification in H. Negative control of the antibody are shown as inset in B and F. Arrowhead indicates NEO1 staining in blood vessels. A, B, E, F: Low magnification Bar: 100 μm, C, D, G, H: High magnification Bar: 20 μm.

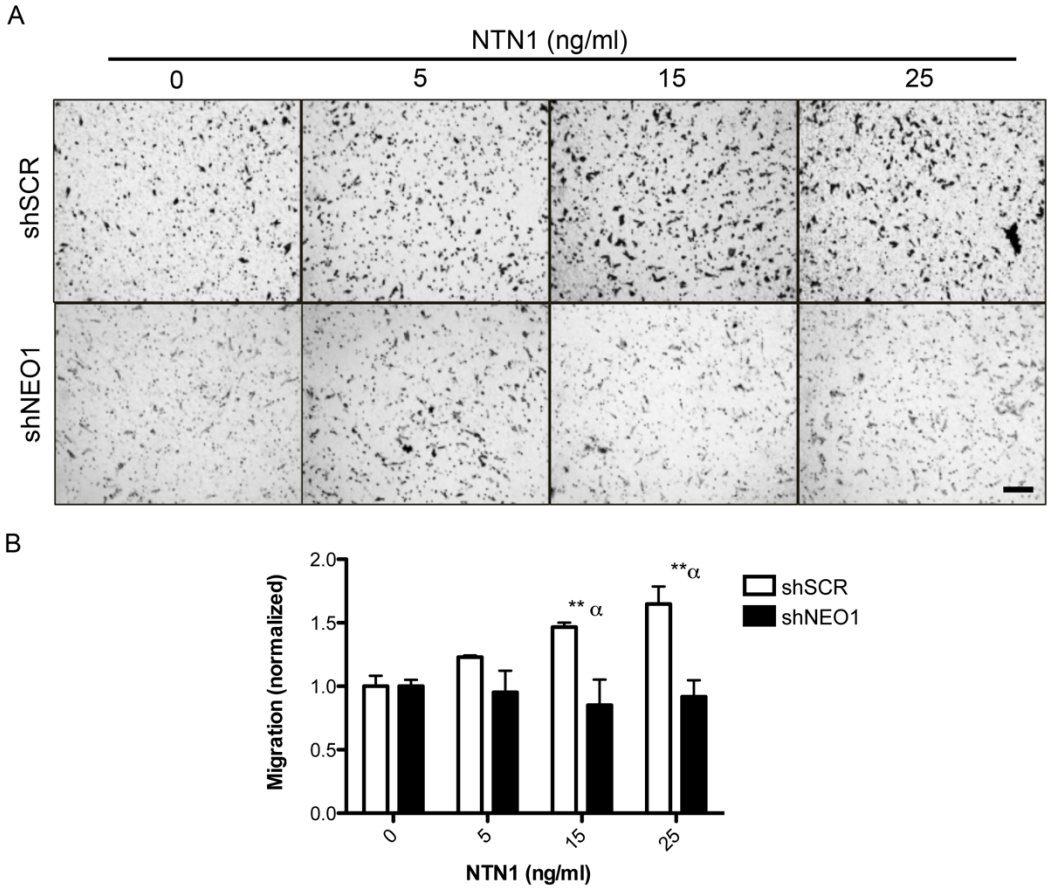


Figure 2: NEO1 promotes chemotactic NTN1-mediated cell migration. A: Representative transwell assay images performed with shSCR and shNEO1 SK-N-SH cells which migrated for 4 hours in increasing concentrations of NTN1 indicated in Figure. Bar = 100µm. B: Quantification of the photographs taken for each condition. Values are expressed as induction times of migration relative to the condition without chemotactic stimulus (0 ng / ml NTN1) for shSCR and shNeo1 cells. N = 3, * $p < 0.05$ 0 v / s 25 ng / ml NTN1.

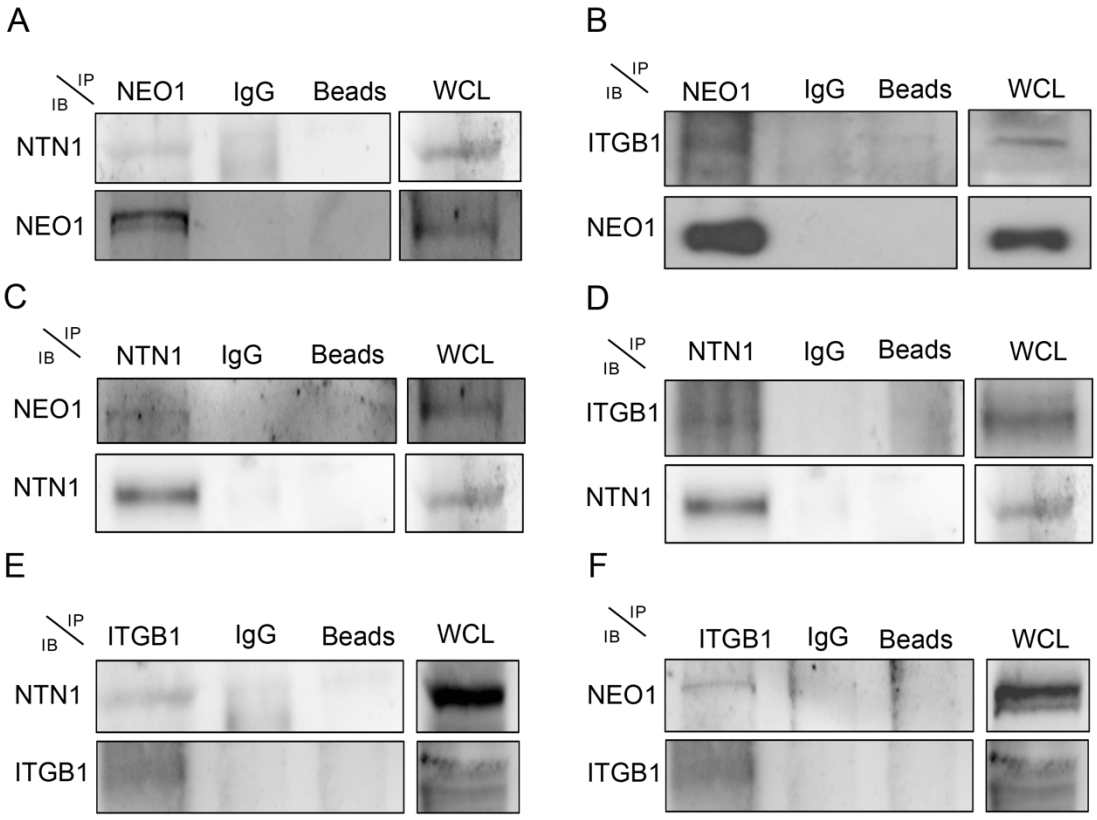


Figure 3: NEO1/NTN1 form a complex with Integrin β1 in SK-N-SH cells. A, B: Representative western blots (WB) of protein co-immunoprecipitation assays used to evaluate interaction between NEO1 with NTN1 (A) and, ITGB1 (B). C, D: Representative WB of protein co-immunoprecipitation assays used to evaluate interaction between NTN1 with NEO1 (C) and, ITGB1 (D). E, F: Representative WB of protein co-immunoprecipitation assays used to evaluate interaction between ITGB1 with NTN1 (E) and, ITGB1 (F). N=2.

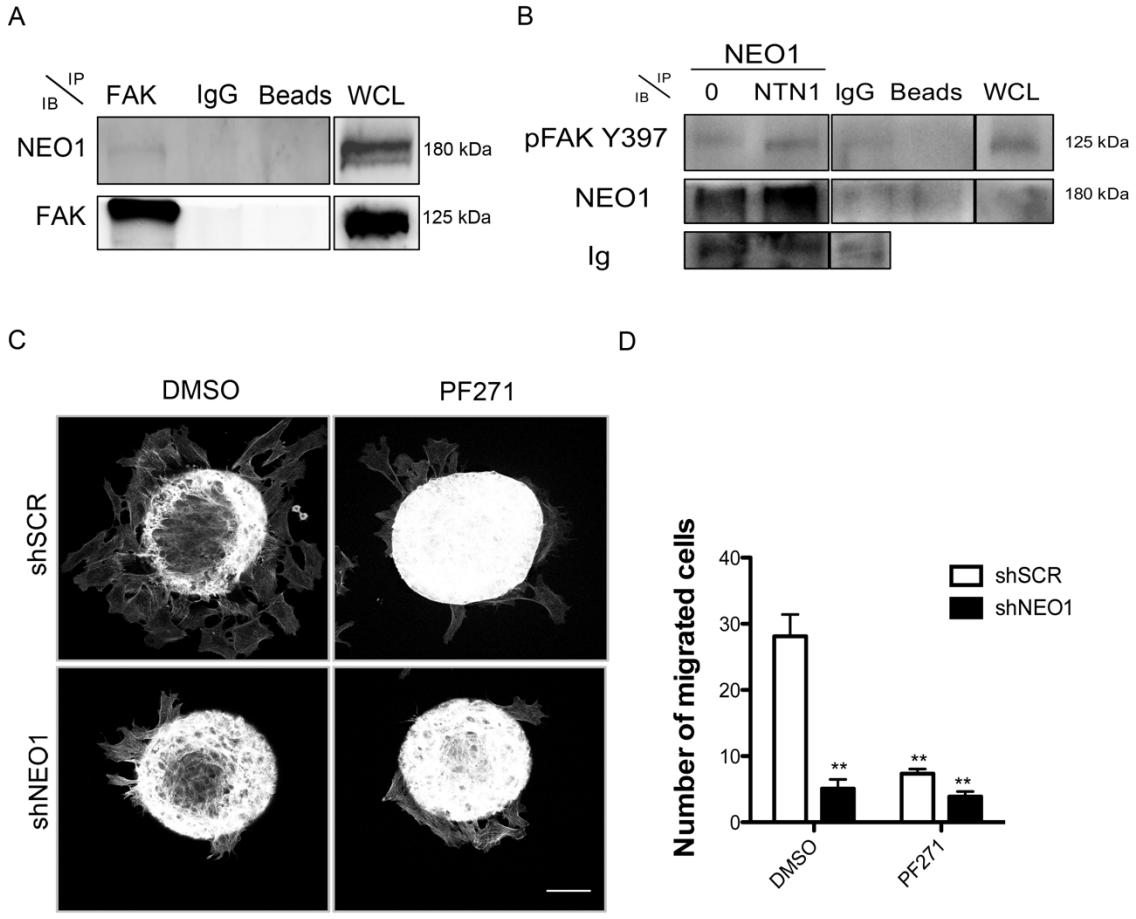


Figure 4: FAK is required for NEO1-mediated cell migration. A: WB of protein co-immunoprecipitation; FAK was immunoprecipitated and NEO1 was evaluated. B: WB of protein co-immunoprecipitation showing NEO1 immunoprecipitation in cells treated for 1 h with NTN1 (25 ng / ml) followed by evaluation of FAK autophosphorylation at Y397. C: Representative images of confocal microscopy of spheroid based migration assay on fibronectin for 1 h, comparing shSCR versus NEO1 knock-down cells treated with or without the PF271. The images reveal F-actin labeling. D: Quantification of cells that migrated away from the spheroid for each condition tested. ** $p < 0.01$ shSCR DMSO versus shSCR PF271, shNEO1 with and without inhibitor.

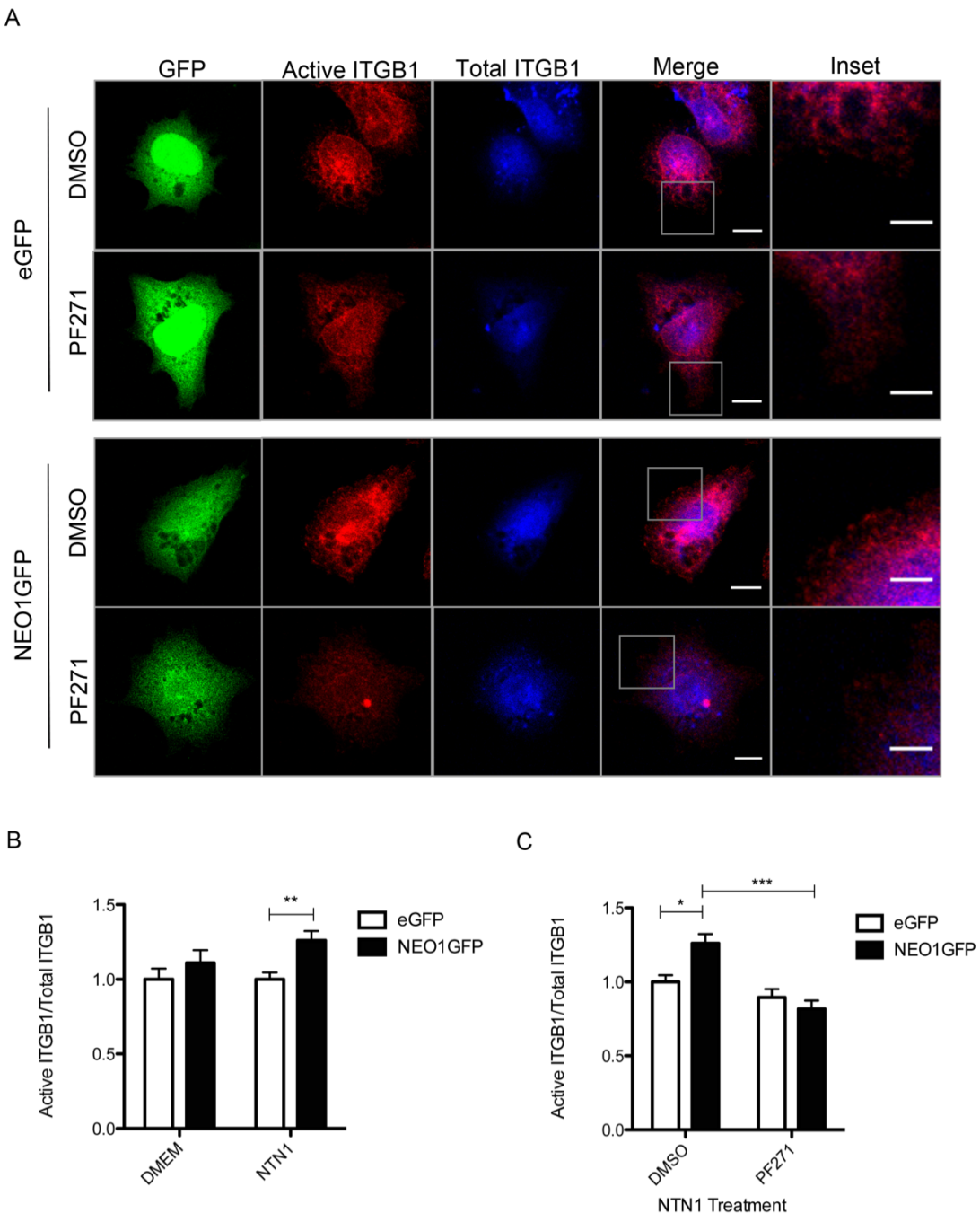

A
GFP
Active ITGB1
Total ITGB1
Merge
Inset
eGFP
DMSO
PF271
NEO1GFP
DMSO
PF271
B
Active ITGB1/Total ITGB1
1.5
1.0
0.5
0.0
**
eGFP
NEO1GFP
DMEM
NTN1
C
Active ITGB1/Total ITGB1
1.5
1.0
0.5
0.0
*
***
eGFP
NEO1GFP
DMSO
PF271
NTN1 Treatment

Figure 5: The NEO1/NTN1 complex induces Integrin β1 activation via pFAK. A: Representative confocal microscopy images of a spreading assay in NEO1 overexpressing SK-N-SH cells (NEO1GFP) versus control eGFP cells in presence of PF271 or vehicle control (DMSO). Immunofluorescence was made using activated ITGB1 (red) and total ITGB1 (blue) antibodies along with transgenic expression of eGFP (green) evaluation. The photos were taken at 400x and the inserts correspond to areas used for quantification Bar: 10 μm. B, C: Quantification of fluorescence intensity between the different conditions for active ITGB1 in relation to total ITGB1 in GFP + cells. Quantification considered the cell edge (2-3 μm) labeled by by the F-actin marker. It was quantified the activation of ITGB1 according to NTN1 treatment (C) and PF271 treatment in NTN1 treated cells. * $p < 0.05$. $n \geq 30$ cells per condition.

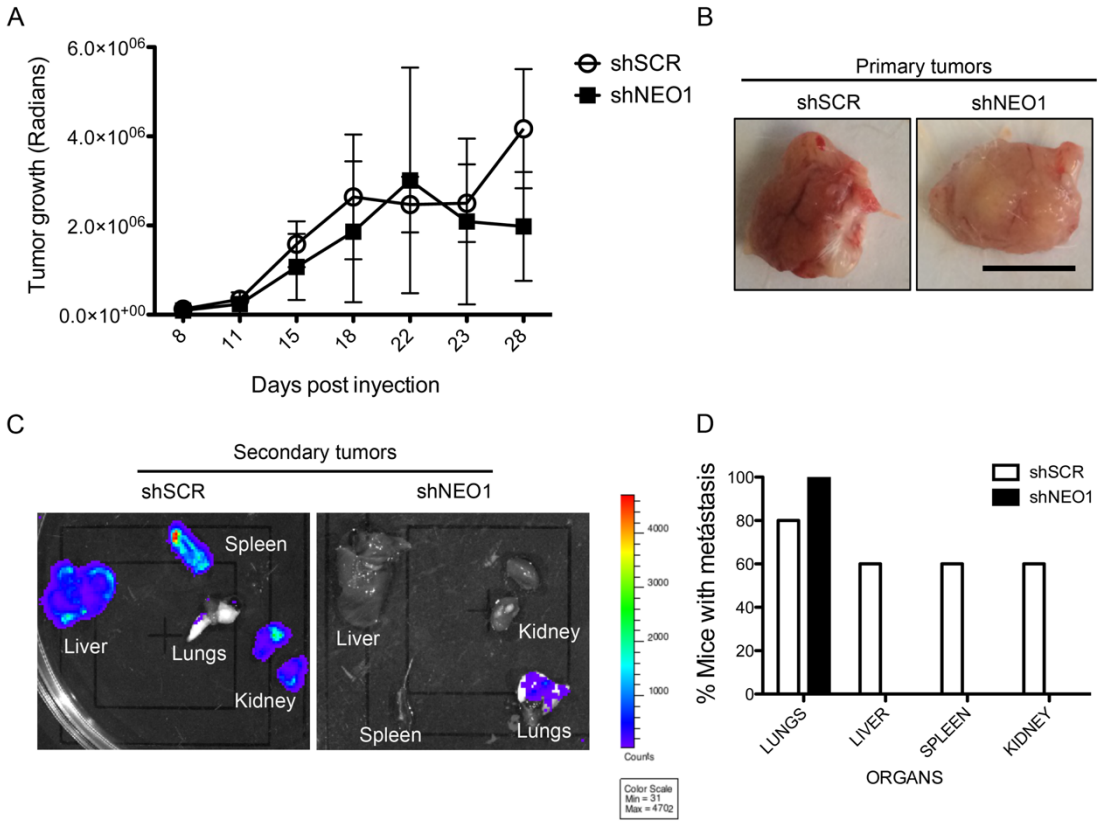


Figure 6: NEO1 promotes metastasis *in vivo*. Stable luminescent shSCR and shNEO1 cells were injected in flank of NSG mice. After 5 weeks, primary tumor and several organs were extracted and analyzed using IVIS Ilumina III *in vivo* imaging system. A: Tumor growth of shSCR and shNEO1 primary tumors. B: Representative images of primary tumor for each condition. Bar: 1 cm. C: Representative images of organs visualized in IVIS. D: Graphic representation of metastasis results. Five specimens per injected cell type were analyzed. Presence or absence of metastasis in each organ was scored. Percentages of metastasis were indicated for each cell type in each organ.

Supplemental information

Supplementary table 1: Characterization of % of NTN1 positive Samples (within tumor cells, blood vessels or stroma). Percentage of NTN1 positive samples according to specific clinical characteristics of the patients. We do not found association between percentage of NTN1 and clinical features. Asterisk for p value from Fisher's exact test.

Clinical Feature		% of NTN1 positive Samples	$\chi 2$	df	p-value	Fisher's p-value
Gender	Male	77.8	1	2.74	0.098	0.18
	Female	41.7				
Age	>18M	40	1	1.17	0.278	0.40
	<18M	63.6				
Tumor Stage	Localized (L1, L2)	66.7	1	4.30	0.038	0.06
	Disseminated (M, MS)	16.7				
PCNA	>40%	40	1	1.82	0.177	0.37
	<40%	70				
Primary Tumor Sites	Cervical	66.7	4	4.58	0.333	0.392
	Thoracic	0				
	Abdominal	83.3				
	Retroperitoneal	60				
	Adrenal	50				
Patient Status	Dead	25	1	4.02	0.045	0.08
	Recovered	78.6				

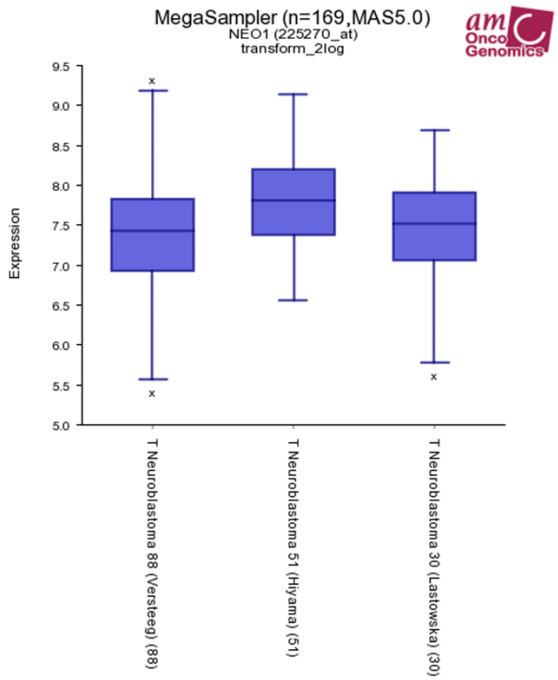
Supplementary table 2: Qualitative NEO1 and NTN1 expression in patients with NB.
NEO1 and NTN1 expression within tumor cells, stroma and / or blood vessel.

		NEO1			NTN1		
Patient	Stage	Tumor cells	Blood vessels	Stroma	Tumor cells	Blood vessels	Stroma
1	L1	+	-	-	++	++	+++
2	L1	++	-	-	-	-	-
3	L1	++	+	-	+++	++	+++
4	L1	-	+	-	-	+	-
5	L1	++	+	-	+	+	-
6	L1	-	+	-	-	+	++
7	L1	+	++	+	-	-	-
8	L2	++	-	-	-	-	-
9	L2	+	-	-	+	-	+
10	L2	-	-	-	-	-	-
11	L2	-	-	-	+	++	+++
12	L2	+++	-	-	+++	-	++
13	L2	+++	-	+	+++	+	++
14	L2	-	-	-	-	-	-
15	M	+	+	-	-	-	-
16	M	+	-	-	-	-	-
17	M	++	+	-	++	-	++
18	M	++	-	-	-	-	-
19	M	-	-	-	-	-	-
20	M	+++	-	+	-	-	-
21	MS	+++	++	+	-	-	+

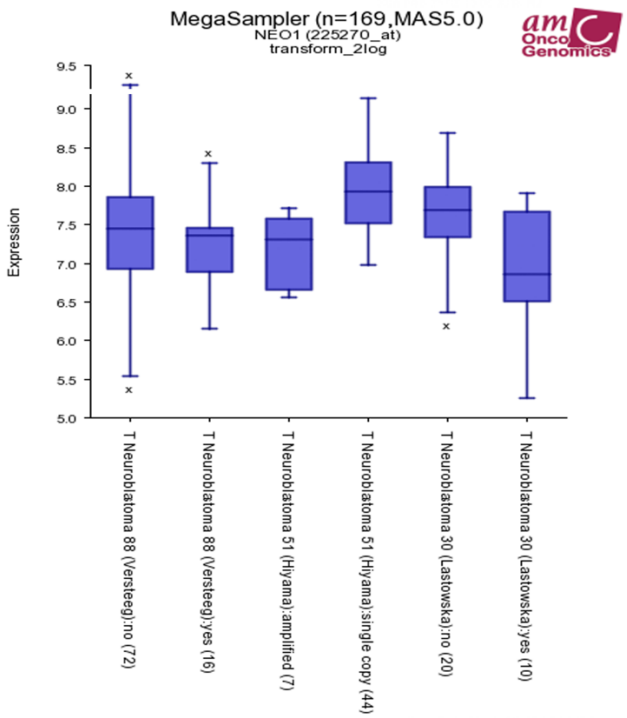
Supplementary table 3: Individualized data of secondary tumors in each organ per condition according to luminescence

CONDITION	LUNGS	LIVER	SPLEEN	KIDNEY
shSCR (1)	+	-	-	-
shSCR (2)	+	++	+++	+++
shSCR (3)	-	+	+	+
shSCR (4)	++	++	+++	+
shSCR (5)	+	-	-	-
shNEO1 (1)	+	-	-	-
shNEO1 (2)	+	-	-	-
shNEO1 (3)	+	-	-	-
shNEO1 (4)	++	-	-	-
shNEO1 (5)	++	-	-	-

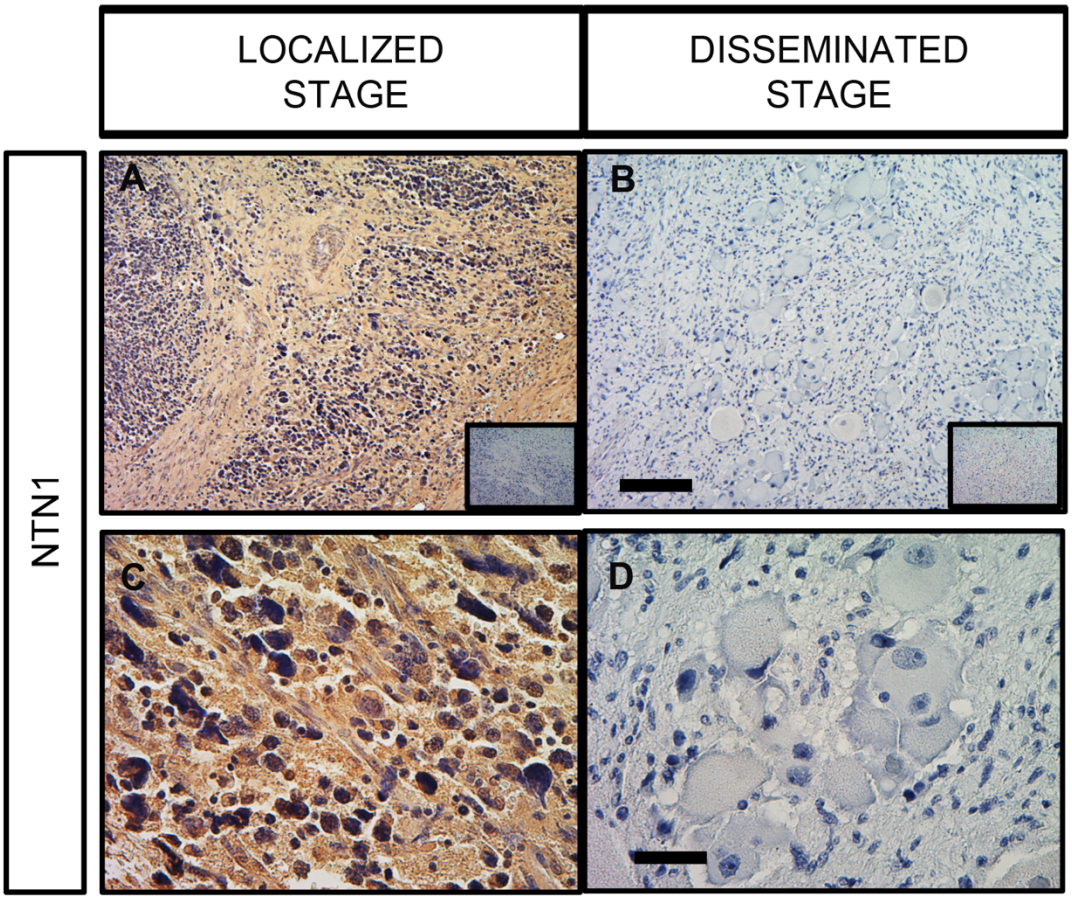
A



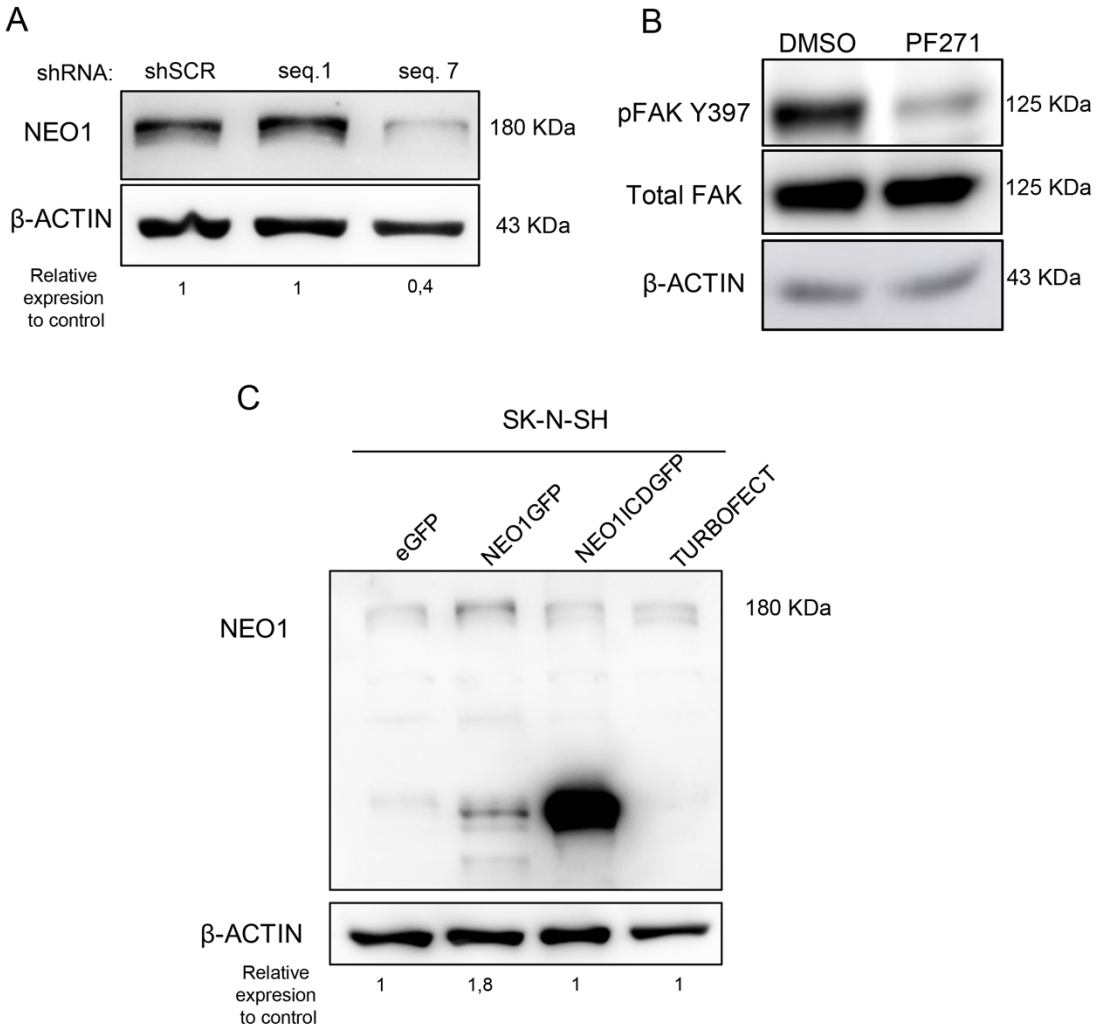
B



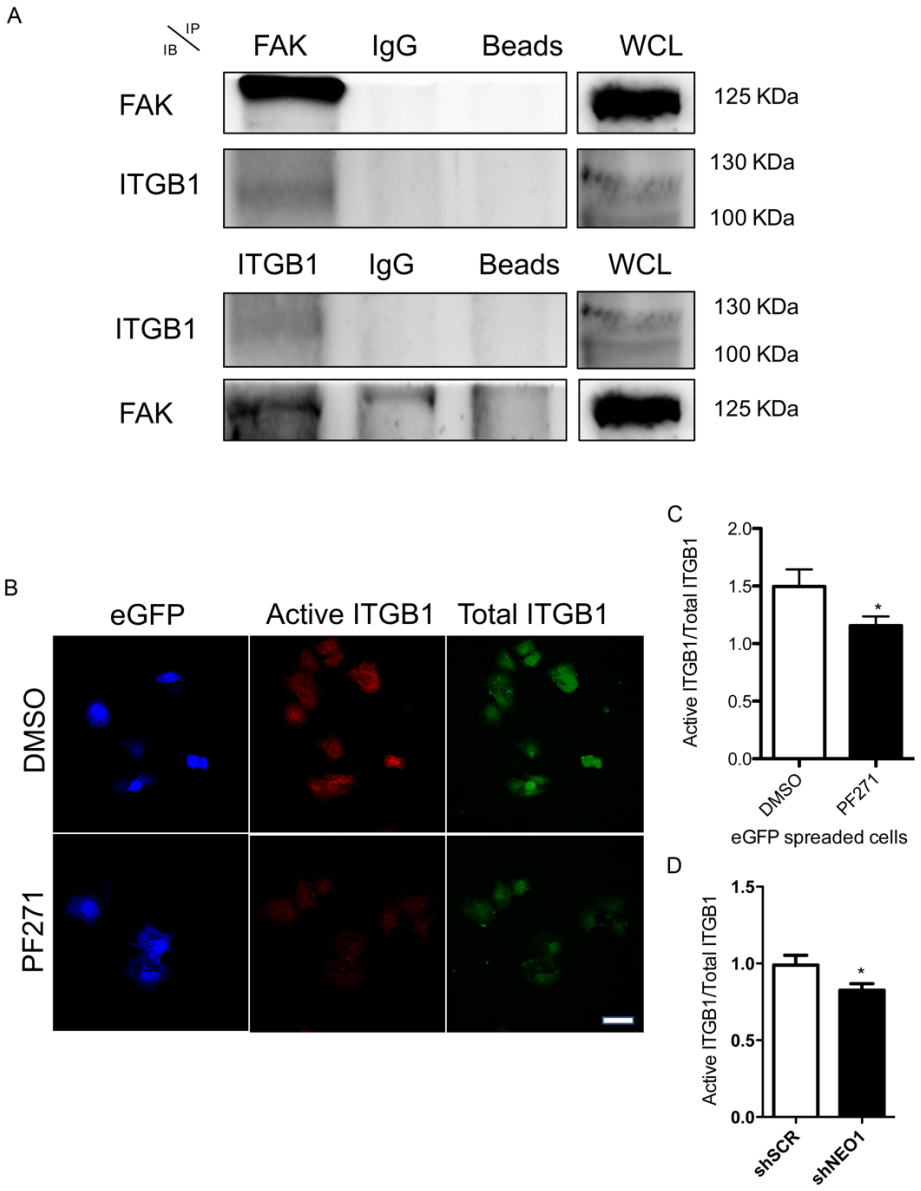
Supplementary Figure 1: Mega-sampler analysis of NEO1 expression across data sets using R2 software. A: analysis of NEO1 expression across data sets, B:NEO1 expression across datasets according to *MYCN* amplification.



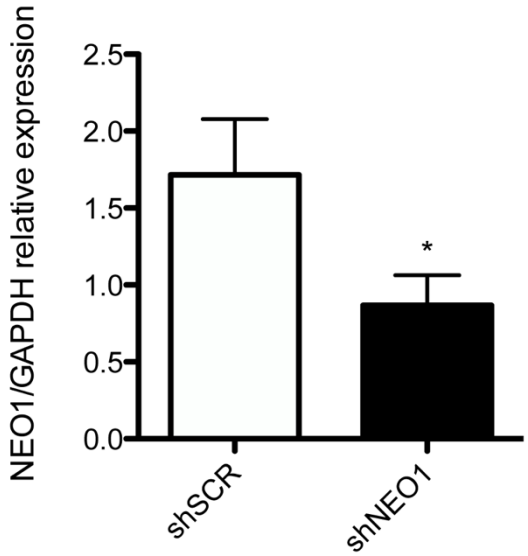
Supplementary Figure 2: Immunohistochemistry of NTN1 expression within NB. Representative light microscopy images of neuroblastoma samples from tumors in either localized (A) or disseminated stages (B). Hematoxylin was used for counterstaining A, C: Representative images of a NB patient sample classified at Localized Stage according to INRGSS at low magnification. A: Low magnification, C: High magnification, Negative control of each antibody is shown as an inset. B, D: Representative images of a NB patient sample classified at Disseminated Stage according to INRGSS at low magnification, B: Low magnification, D: High magnification. Low magnification Bar: 100 μm, High magnification Bar: 20 μm.



Supplementary Figure 3: A. NEO1 knockdown efficiency by WB. Two shRNA sequences against NEO1 were used to knockdown it, as referred in the image at seq. 1 and seq. 7. The inhibition quantity was indicated in the figure. B: WB showing the efficiency of the FAK autophosphorylation inhibitor at Y397 (PF271) at 1μM concentration in SK-N-SH cells. The inhibitor vehicle corresponds to a DMSO. C: NEO1 overexpression analysis. The overexpression of NEO1 (NEO1GFP) is shown in the figure.



Supplementary Figure 4: A. Representative WBs of coimunoprecipitation between FAK and ITGB1, as indicated in the figure. B: Representative confocal images of ITGB1 activation in SK-N-SH cells treated with PF271 in a spreading assay by 1h in fibronectin. C: Quantification of active ITGB1 in SK-N-SH cells treated with PF271, as indicated in C. D: Quantification of active ITGB1 in shSCR and shNEO1 cells in a spreading assay by 1h in fibronectin and confocal analysis was made as indicated previously.



Supplementary Figure 5: Relative expression of NEO1 in primary tumors at the moment of dissection. GAPDH expression was used as housekeeping control. * p<0,05

GENERAL CONCLUSIONS

NB is a pediatric cancer that emerges during embryonic development, from uncontrolled growth of neural crest cells of the sympatho-adrenal lineage (Cheung and Dyer, 2013). NB derives from undifferentiated cells and, as such, is a very aggressive cancer, with higher rates of cell migration and metastasis (Cheung and Dyer, 2013). In this thesis, we reveal the role of NEO1, a multifunctional transmembrane receptor, in the progression of NB, by describing its high expression in patient samples and public databases, and by establishing its function in tumor cell migration. Our work suggests for the first time that NEO1, in association with its ligands NTN1 and/or NTN4, is a tumor progression-promoting protein, with an active role in NB metastasis.

This research is an extension of previous work in which we demonstrated that NEO1 is a critical downstream mediator in Sonic Hedgehog/GLI (SHH/GLI) dependent tumorigenesis. Here, we further extended the original observation that NEO1 is a direct SHH target, highly expressed in SHH/GLI driven tumors, such as Medulloblastoma (Milla *et al.,* 2014) and Basal Cell carcinoma (Casas *et al.,* 2017), as well as NB, another cancer in which aberrant SHH pathway activation has been described (Xu *et al.,* 2012b). Considering these results, we propose that an increase in the activity of NEO1 is critical in the development of neoplasms associated with a deregulation of SHH / GLI.

NEO1 binds the NTN and RGM family ligands (Wilson and Key, 2006) and its signaling mechanisms are complex, given the number of ligands that NEO1 binds to, as well as the multifaceted physiological roles attributed to the receptor (Wilson and Key, 2006). In this thesis, we established a role for NEO1 in NB cell migration and metastasis.

NEO1 binds to its NETRIN ligands, either directly, as in the case of NTN1, or indirectly, as demonstrated for NTN4 by forming a signaling complex with Laminin γ 1 (LMγ1), signaling downstream with ITGB1 /FAK.

NEO1 and ligands NTN4 and NTN1 expression were analyzed in a cohort of NB patient samples. These samples were stratified, based on the International Neuroblastoma Risk Group Staging System (INRGSS) which considers a pretreatment risk classification system, considering tumor spread and surgical risk factors known as Image Defined Risk Factor, at the moment of diagnosis of the disease (Monclair *et al.,* 2009). NEO1 expression was mostly located in tumor cells and found homogeneously independently of the stage analyzed. Also, according to public databases NEO1 expression is less in MYCN genetic amplificated samples, a classical marker of NB (Bordow *et al.,* 1998). This could be important at the moment to evaluate the function of NEO1 in NB cell migration.

Thus, the NEO1 expression pattern suggests a selective advantage acquired by cancer cells to migrate and metastasize. In addition, NEO1 expression in NB is correlated with a low survival rate, which indicates that NEO1, despite its pro-apoptotic function in the absence of ligands, is associated tumor progression. Due to the apparent paradox that NEO1, being a death dependence receptor, is over-expressed in NB we hypothesized that it is the presence of its NTNs ligands, which switches off the receptor's ability to function as a tumor suppressor. Tumor cells constitutively overexpressing the ligand could therefore escape a pro-apoptotic regulation. Indeed, NTNs, although expressed by tumor cells, are mostly located either in adjacent endothelial cells or stroma, suggesting a relevant contribution to this pathology acting as chemotactic molecules. NTN4 is mostly

expressed in vessels and stromal cells in both localized and disseminated tumors. NTN1 is directly secreted by tumor cells within NB, while disseminated tumors showed decreased NTN1 expression. These observations could be explained on the basis of the different signaling complex formed by NEO1 and its ligands (NTN and RGM families). I*n vivo*, NEO1 ligands can be secreted by multiple sources, including blood (Oksala *et al*., 2013) and endothelial cells (Prieto *et al.,* 2017). Therefore, it is relevant to consider the tumor microenvironment, including the tumor stroma, and other elements, such as the endothelial niche, as being essential to sustain tumor growth and metastasis.

Regarding NTN4, recent findings show that NTN4 does not bind directly to NEO1 (Reuten *et al.,* 2016), rather NTN4 would act as an adhesion molecule that binds directly to LMγ1. Indeed, in our study we determined that NTN4 acts as a cell adhesion molecule during NEO1-mediated NB cell migration. We established that NTN4, by forming a ternary complex with LMγ and NEO1, acts as an essential extracellular matrix component, which induces SK-N-SH cell migration (Villanueva et al, accepted with minor revision, resubmitted to *Cell Adhesion and Migration*, July 2018). In summary, we conclude that NTN4, through its interaction with Laminin γ1 and NEO1, plays an important dual role in NB progression by acting both as an adhesion and chemotactic molecule.

NEO1 promotes cell migration through the sensing of chemotactic patterns of NTN1, as has been shown in this thesis and previous reports (O´Leary et al., 2015). However, it was recently published that NTN1, produced by the floor plate of the neural tube, would not be required as a morphogen for the axonal guidance of commissural neurons but rather, it would act as an haptotactic guide molecule at short distances, by

being secreted by neurons and neuronal progenitors located in the ventricular zone (Dominici *et al.,* 2017). Further research is required to elucidate all the functions of this ligand. At least in our model, NTN1 acts as a chemotactic molecule.

Despite the importance of their intimate relationship, how NTN1/NEO1 stimulate intracellular pathways in cancer, remains unclear. The NEO1-dependent anti-apoptotic signaling pathway is still largely unmapped. Therefore, we studied the NTN1/NEO1 interactions at the cell surface. Interestingly, our results suggest a potential mechanism where NTNs act as dependence factors for NEO1 in NB, reducing its proapoptotic activity (Matsunaga *et al.,* 2004), thus modulating the adhesion, motility and subsequent metastasis of SK-N-SH cells via ITGB1 activation through FAK.

NEO1 promotes NB cell migration through the sensing of chemotactic ligands, as NTN1 or associating to cell adhesion molecules such as NTN4 and LMγ1. The NTN1/ NEO1 complex induces cell migration, forming a complex with ITGB1 and inducing the autophosphorylation of FAK in Y397. Those processes induce ITGB1 activation at the edge of the cell, where focal adhesions are dynamic. This mechanism explains how NEO1 promotes chemotactic cell migration, and, consequently NB metastasis in *in vivo* models.

Our final goal is to translate our results into better therapeutic strategies, through precision medicine, contributing to the diagnosis and treatment of NTN1/NEO1-driven tumors. Despite progress in understanding the molecular basis of cancer, challenges must be overcome to improve the overall efficacy of strategies to combat cancer more efficiently.

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