Functional analysis of the cytoplasmic tail of the netrin-1 receptor Deleted in Colorectal Cancer (DCC) in neurite outgrowth

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Abstract

Netrin-1 is an axon guidance cue that has been shown to have a profound influence on neuronal development. Netrin-1 acts in a bifunctional manner, either as an attractant or a repellent depending on the composition of the receptor population on its target. The interaction between netrin-1 and deleted in colorectal cancer receptor (DCC) has been found to mediate the attraction of migrating axons while the interaction between netrin-1 and UNC5 has been associated with growth cone collapse. Thus the analysis of the interaction between netrin-1 and its receptors as well as the downstream biochemical effects produced is key to understanding netrin-1's role in neuronal development. We identified several phosphorylated residues on the intracellular side of DCC by mass spectrometry analysis, and a ProQ phospho-protein stain was used to confirm that at least one of these sites (S1178) is phosphorylated in response to netrin-1. In this thesis we first analyzed the function of different regions of the cytoplasmic domain of DCC on netrin-1-mediated neurite outgrowth and downstream signaling. We next investigated the roles the identified phosphorylation sites play in netrin-1-mediated neurite outgrowth, as well as analyzing the effect of these phosphorylation sites on signaling events downstream of netrin-1/DCC. Lastly we analyzed the effect of these phosphorylation sites on the transport of DCC to the cell surface. This thesis presents data indicating important functional roles for the phosphorylation of serine and threonine residues on the cytoplasmic domain of DCC in netrin-1-mediated neurite outgrowth.

Résumé

Le facteur de croissance nerveux, nétrine-1 dont le rôle est de guider la croissance de l'axone des neurones, exerce une influence cruciale durant le développement neuronal.
La nétrine-1 pourrait être à la fois un facteur d'attraction et de répulsion dépendamment de la nature et la composition des éléments présents sur l'axone cible. En effet, l'interaction nétrine-1/DCC (deleted in colorectal cancer) induit une attraction et une migration des axones tandis que l'interaction nétrine-1/UNC5 est associée avec une réponse répulsive et un effondrement du cône de croissance. Ainsi, l'étude de l'interaction et des effets en aval de la nétrine-1 et ses récepteurs est primordiale afin de déterminer le rôle joué par la nétrine-1 dans le développement neuronal. En utilisant la spectroscopie de masse et la technique de détection de protéines phosphorylées, ProQ, nous avons identifié plusieurs sites de phosphorylation sur le domaine intracellulaire (cytoplasmique) de DCC et confirmé que le site S1178 est phosphorylé en réponse à la stimulation par la nétrine-1. Dans cette thèse, nous analysons d'abord l'effet des différentes régions du domaine cytoplasmique de DCC sur la croissance des neurites ainsi que la signalisation en aval en réponse à la nétrine-1. Ensuite nous examinons les rôles que les sites de phosphorylation identifiés jouent sur la croissance de neurites ainsi que la signalisation en aval de nétrin-1/DCC. Finalement, nous analysons l'effet de ces phosphorylations sur le transport de DCC à la surface cellulaire. Cette thèse présente des résultats originaux élucidant des rôles fonctionnels de la phosphorylation des résidus sérine et thréonine du domaine cytoplasmique de DCC dans le cadre de la croissance de neurites en réponse à la nétrine-1.

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Contribution of Co-Authors

Tristan Dellazizzo Toth: I conducted the neurite outgrowth experiments (Fig. 3, 8), Western blots (Fig. 4, 9), cell surface biotinylation experiments (Fig. 10), Phos-Tag blots (Fig. 11) and conducted the statistical analysis for all experiments. I also contributed to the planning and organization of this project and wrote this thesis with the support of Dr. Nathalie Lamarche-Vane.

Dr. Chantal Piché: Chantal originally identified the different serine and threonine phosphorylation sites on the cytoplasmic tail of DCC through the use of mass spectrometry (Fig. 5).

Dr. Joseph Tcherkezian: Joseph generated the DCC C-terminal deletion mutants (Fig. 2) and performed the *in vitro* kinase assay finding that the C-terminal domain of DCC is phosphorylated *in vitro* by activated ERK1/2 (Fig. 6).

Vilayphone Luangrath: Vilayphone generated the DCC C-terminal phospho-null mutants (Fig. 2) and performed the Pro-Q phospho-protein stain experiment (Fig. 7).

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Dr. Nathalie Lamarche-Vane: Dr. Lamarche-Vane supervised this project and contributed to all aspects of the project as well as providing all the materials and laboratory facilites necessary to complete this project. Dr. Lamarche-Vane edited the thesis.

List of Abbreviations

A	Alanine
AC	ADF/cofilin
ADF	Actin depolymerizing factor
ADP	Adenosine diphosphate
Akt	Protein kinase B
Arf	ADP ribosylation factor
ATP	Adenosine triphosphate
C3	Complement component 3
C4	Complement component 4
C5	Complement component 5
CA	Constitutively active
Cdc42	Cell division control protein 42
CDM	CED-5, DOCK180 and MBC family
C. elegans	Caenorhabditis elegans
Comm	Commissureless
CNS	Central nervous system
DCC	Deleted in Colorectal Cancer
DMEM	Dulbecco's Modification Eagle Medium
DNA	Deoxyribonucleic acid
DOCK180	Dedicator of cytokinesis protein 1
DRG	Dorsal root ganglion
DSCAM	Down syndrome cell adhesion molecule
E#	Embryonic day
EGFR	Epidermal growth factor receptor
Elk-1	ETS domain-containing protein Elk-1
Ephexin	Eph-Interacting exchange protein 1
ERK1/2	Extracellular-signal-regulated kinase 1/2
ERM	Ezrin–radixin–moesin
EphA	Ephrin receptor A
EphB	Ephrin receptor B
F-actin	Filamentous actin
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FERM	N-terminal four-point-one, ezrin, radixin, moesin
FN	Fibronectin
FPLC	Fast protein liquid chromatography
GAP	GTPase activating proteins
GDI	Guanine dissociation inhibitors
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factors
GEFD	GEF domain
GFP	Green fluorescent protein
Grb2	Growth factor receptor-bound protein 2
GST	Glutathione S-transferase

GTP	Guanosine triphosphate
HEK-293	Human embryonic kidney 293 cells
HRP	Horseradish peroxidase
IG	Immunoglobulin
IP	Immunoprecipitation
IP3	Inositol 1,4,5-trisphosphate
LB	Lysogeny broth
LIM1	LIM domain kinase 1
LIM2	LIM domain kinase 2
MAPK	Mitogen activated kinase-like protein
MEK1	Dual specificity mitogen-activated protein kinase kinase 1
MIG10	Abnormal cell migration protein 10
NFAT	Nuclear factor of activated T-cells
NFATC4	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 4
PAK1	P21 activated kinase 1
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
Rac1	Ras-related C3 botulinum toxin substrate 1
RhoA	Transforming protein RhoA
RhoD	Rho-related GTP-binding protein RhoD
RhoG	Rho-related GTP-binding protein RhoG
RIPA	Radioimmunoprecipitation assay buffer
Rnd	Ribonuclease D
ROBO	Roundabout
S	Serine
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	Small interfering ribonucleic acid
srGAP	SLIT-ROBO Rho GTPase activating protein
SWISS 3T3	Swiss albino mouse 3-day transfer, inoculum 3 x 10^5 cells
T	Threonine
Trio	Triple functional domain protein
UNC5	Uncoordinated protein 5
V	Valine
WASP	Wiskott-Aldrich syndrome protein
WT	Wild-type
Y	Tyrosine

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Chapter 1

Introduction and Literature Review

1.0 Axon Guidance in Development

Human cognition is dependent on a remarkably intricate network of connections between neurons in the human brain. Each one of the billions of neurons in the human brain synapses with, often hundreds of other neurons creating an information processing system of staggering complexity (Tessier-Lavigne & Goodman, 1996). How then, during embryonic development, does this precise wiring of this immensely complex circuit occur? During development, these connections are formed when the axons of newly differentiating neurons migrate to their targets. At the tip of the migrating axon there is a growth cone, which is composed of a central region containing microtubules and a dynamic periphery composed of constantly changing actin based structures, chiefly filopodia and lamellipodia (Dent and Gertler, 2003). The growth cone is able to sense and react to chemotropic cues in its environment. It is these axon guidance cues that, through attracting the migrating axons towards their correct targets or repelling them away from incorrect ones, play a large role in creating the correct patterning of neuronal connections in the human body (Tessier-Lavigne & Goodman, 1996). Thus the study of these guidance cues is essential for understanding the development of the human nervous system.

1.1 The Rho GTPases

One important mechanism that most axon guidance cues use to induce a response in a migrating growth cone is to modulate the activity of Rho GTPases within the growth cone. The Rho family of small GTP-binding proteins consists of 20 small (all less than 25kDa) mammalian proteins that all have an intrinsic GTPase activity, meaning that they hydrolyze guanosine triphosphate (GTP) into guanosine diphosphate (GDP) (Tcherkezian and Lamarche-Vane, 2007). These GTPases cycle between an active, GTP-bound state and an inactive GDP-bound state.

This GDP-GTP cycle is regulated by three families of proteins: The GEFs (Guanine nucleotide exchange factors) stimulate the release of GDP, allowing for GTP to bind, while the GAPs (GTPase activating proteins) enhance intrinsic GTPase activity leading to the inactive state of the Rho GTPase, and the GDIs, (Guanine dissociation inhibitors) which sequester Rho GTPases in their GDP-bound, inactive state and regulate their intracellular localization (Tcherkezian and Lamarche-Vane, 2007). The three most studied members of Rho GTPase family are transforming protein RhoA (RhoA), Ras-related C3 botulinum toxin substrate 1 (Rac1) and cell division control protein 42 (Cdc42). The activity of these three GTPases each play a distinct role in cytoskeletal dynamics at the growth cone: Rac1 stimulates F-actin polymerization and the formation of lamellipodia, which are important for axon guidance due to providing the tension necessary for neurite extension (Antoine-Bertrand, et al., 2011b). Cdc42 also stimulates F-actin polymerization and induces the formation of filopodia that through being able to sense the extracellular environment, act to guide the growth cone (Antoine-Bertrand et al., 2011b). RhoA activity is involved in reorganizing the growth cone cytoskeleton through inducing actomyosin contraction and growth cone collapse (Antoine-Bertrand et al., 2011b). Through affecting the activity of Rho GTPases, particularly RhoA, Rac1 and Cdc42, guidance cues are able to induce the cytoskeletal changes necessary for an axon to grow, retract or turn and thus they are capable of guiding the axon to its target.

1.2 The Classic Guidance Cues

There are four classic families of axon guidance cues: the ephrins, the semaphorins, the slits and the netrins. These guidance cues affect growth cone cytoskeletal dynamics via one of several different mechanisms: long range chemo-attraction, long range chemo-repulsion, short

range contact dependent attraction and short range contact dependent repulsion (Tessier-Lavigne & Goodman, 1996). All four of these guidance cues also modulate Rho GTPase activity in order to produce the cytoskeletal changes necessary for the correct axon guidance. This will be discussed in the next section.

1.2.1 Ephrin

The ephrins are a group of membrane bound ligands, which operate through interacting with Eph receptors. Ephrins can be generally split into two classes, with each ligand generally binding to a receptor in its own class: ephrin-A ligands are glycosylphosphoinositol (GPI) anchored and bind to EphA receptors and ephrin-B ligands have a transmembrane and cytoplasmic domain and bind to EphB receptors (Wilkinson, 2000). A key function of epherin in nervous system development is to mediate cell-contact dependent repulsion, such as in guiding retinal ganglion neurons to their proper targets on the tectum (Wilkinson, 2000). Eph receptors often form homo and heterodimers upon ephrin binding, mediated by a cysteine-rich extracellular region, with the heterodimers next forming tetramers in a 2:2 ratio (Aoto and Chen, 2007). Eph receptors and ephrin ligands are capable of both forward and reverse signaling. Forward signaling refers to pathways associated with Eph receptor activation, while reverse signaling are those that with ephrin activations (Murai and Pasquale, 2003). Upon ephrin-Eph interaction, the phosphorylation of the juxtamembrane domain of the Eph receptor occurs and downstream, there is the activation of different RhoGEFs. EphA receptors modulate the activity of the GEF Eph-Interacting exchange protein 1 (ephexin), leading to the activation of RhoA and the inactivation of Rac1, the net effect of which is growth cone collapse (Shamah et al., 2001). However, EphB activity leads to the activation of Cdc42 through the GEF intersectin and this

interaction has been demonstrated to be essential for dendritic spine formation in cultured neurons (Irie and Yamaguchi, 2002).

1.2.2 Semaphorin

The semaphorins are a group of axon guidance cues that includes both secreted and membrane bound proteins (Kolodkin, et al., 1993). The 20 different semaphorin genes in mice and humans and 5 in *Drosophila* which are divided into 8 classes, with vertebrates expressing classes 3-7, and are defined by a highly conserved cysteine-rich sema domain (Yazdani and Terman, 2006). The semaphorins are known to play a wide variety of roles including the formation and function of the endocrine, hepatic, musculoskeletal, renal, reproductive, cardiovascular, immune, gastrointestinal and respiratory systems (Yazdani and Terman, 2006). However they were initially characterized by their role in neuronal development and axon guidance (Kolodkin, et al., 1992). They generally mediate axon guidance through repulsion, preventing migrating axons from entering improper areas. Through their interaction with their plexin receptors, which also contains a conserved sema domain, there is activation of Rho A leading to actin rearrangement and growth cone collapse as demonstrated in *in vitro* assays where semaphorin III (also called collapsin) is able to induce collapse of dorsal root ganglion (DRG) growth cones (Luo, et al., 1993; Koppel et al., 1997). In addition, Rac1 along with two other Rho GTPases, Rnd and RhoD have been shown to directly interact with plexin and Sema3A induced growth cone collapse requires the Rac-GAP activity of α 2-Chimaerin. (Antoine-Bertrand et al., 2011b). Interestingly, semaphorins appear to have a role in how the nervous system responds to injury, acting to limit the ability of axons to regenerate (Moreau-Fauvarque, et al., 2003) and prevent the growth and sprouting of axons involved in pain

sensation (Tang, et al., 2004). Semaphorin function has also been shown to have a role in proper neurological functioning in adults, with aberrant functioning being linked to conditions such as Alzheimer's disease, epilepsy, motor neuron degeneration, retinal degeneration, Parkinson's disease and schizophrenia (Yazdani and Terman, 2006).

1.2.3 Slit

Slits are a class of conserved secreted proteins that with their receptors, the robos are important in mediating axon guidance during development through repulsion (Andrews and Parnavelas, 2007). The classic role of slit and robo is in the guidance of commissural neurons that are crossing the midline. In particular slit-mediated repulsion is necessary for keeping ipsilateral neurons from crossing over and for keeping contralateral axons from recrossing the midline after the initial crossover (Rajagopalan et al., 2000). Disruption of the *slit* gene in *Drosophila* leads to midline defects including axons aberrantly crossing or stalling at the midline, while in *robo* loss of function mutants, there is an excess of axons crossing the midline including contralateral axons that re-cross (Dickson and Gilestro, 2006). Mammals have three *slit* genes expressed in the Central Nervous System (CNS) at midline cells and disruption of the three mammalian slit genes in mice leads to similar to, although less severe defects as those seen in *Drosophila slit* mutants (Dickson and Gilestro, 2006). The regulation of Rho GTPases has been shown to be essential for slit/robo mediated axon guidance. When slit is absent, there is a basal level of interaction between robo receptors and slit-robo GAPs (srGAP-1, -2, and -3) that maintains a low level of Cdc42 and RhoA activity, however upon slit interacting with robo, there is an increase in the interaction of srGAP with Cdc42 and decreases its interaction with RhoA, thereby reducing Cdc42 activity and increasing RhoA activity and leading to axon repulsion

(Antoine-Bertrand et al., 2011b). Interestingly post-translational regulation of robo is essential for its functioning at the midline. All neurons in the spinal cord express robo mRNA, however only the growth cones of ipsilateral neurons but not contralateral commissures, prior to crossing the midline, express robo protein (Kidd et al., 1998a). However after crossing the midline these commissures then express high levels of robo protein, and it is this modification of robo protein expression that is thought to ensure that these axons cross the midline only once (Kidd et al., 1998b). In *Drosophila*, it is thought that the gene *commissureless (comm)* controls this change in robo protein expression, initially suppressing robo expression so that the axon is able to cross the midline (Dickson and Gilestro, 2006). However there is still controversy in the search to find a clear *comm* analogue in vertebrates, although robo3 is seen as a candidate in vertebrates for antagonizing slit mediated repulsion prior to commissural axon crossing at the midline (Sabatier et al., 2004). This is also supported by findings that robo3 is mutated in horizontal gaze palsy and progressive scoliosis, in which there is an aberrant crossing of ipsilateral neurons and recrossing of commissural axons at the midline of hindbrain (Jen et al., 2004).

1.2.4 Netrin

The netrins are a group of secreted proteins that are closely related to laminins (Tessier-Lavigne and Goodman, 1996) that were first identified in *C. elegans* as Unc-6 (Hedgecock, et al., 1990) and then later identified in vertebrates (Serafini et al., 1994). They were initially noted for their role in guiding commissural neurons to the midline of the embryonic spinal cord (Kennedy et al., 1994). In vertebrates there are four different netrins, netrin-1, -2, -3 and -4, with netrin-1, netrin-3 and netrin-4 being found in mammals. *In vitro*, all four different netrins have been shown to affect axon outgrowth (Koch, 2000; Puschel, 1999; Serafini et al., 1994; Yin et al.,

2000), however the bulk of research on the netrin family has been focused on netrin-1. Netrin-1 has been broadly implicated in axon guidance during the development of the nervous system, beyond guiding spinal commissural neurons, including the development of the basal ganglia (Métin et al., 1997) optic nerve (Deiner and Sretavan 1999) and the corpus callosum (Fothergill, 2014). Netrin-1 has also been linked to functions in development outside of the nervous system including lung branching (Liu et al., 2004b) and mammary gland outgrowth (Hinck, 2004). Netrin-1 has the capacity to act bi-functionally as either a chemo-attractant or as a chemo-repellent, depending on the composition of the receptor population of the target (Kennedy, et al., 1994). The interaction between netrin-1 and the receptor, deleted in colorectal cancer (DCC) has been found to mediate growth cone extension and attraction to the netrin-1 source (Chan et al., 1996), while the interaction between netrin-1 and uncoordinated protein 5 (UNC5) has been associated with repulsion and growth cone collapse (Leonardo et al., 1997). Other netrin receptors found to mediate axon guidance include Down syndrome cell adhesion molecule (DSCAM) (Ly et al., 2008) and neogenin (Wilson and Key, 2006). The different domains of netrin-1 consist of a Laminin VI-like domain, a Laminin V-like domain composed of three Epidermal growth factor (EGF) repeats, and a C-terminal domain with homology to complement components C3, C4, and C5 (Fig. 1A) (Kruger et al., 2004). Netrin-1 signaling remodels the actin cytoskeleton of the growth cone through regulating Rho GTPases. Netrin-1 interacting with DCC leads to filopodia and lamellipodia formation through the activation of Rac1 (Antoine-Bertrand et al., 2011b) while RhoA and Rac1 activity has been implicated in netrin-1/UNC5a mediated repulsion (Picard et al., 2009).

1.3 Role of DCC in development

As implied by its name, DCC is implicated in carcinogenesis, functioning as a tumor suppressor gene prone to deletion in colorectal cancer (Fearon et al., 1990). The deletion of DCC has been directly linked to tumor cell metastasis (Krimpenfort et al., 2012) and DCC expression has been found to be reduced in a variety of cancers, including prostate (Latil et al., 2003), breast (Ho et al., 1999), endometrial (Gima et al., 1994), ovarian (Saegusa, et al., 2000), esophageal (Huang, et al., 1992), testicular (Strohmeyer et al., 1997), glial (Reyes-Mugica et al., 1997), hematologic (Porfiri, et, al., 1993) and neuroblastomal tumors (Kong et al., 1997; Mehlen and Fearon, 2004). It has been suggested that the link between the deletion of DCC and cancer is due to DCC deletion making the tumor cells insensitive to the netrin-1 dependent apoptotic pathway (Mazelin et al., 2004). In this model DCC acts as a dependence receptor, in which DCC by default induces apoptosis unless DCC interacting with netrin-1. When netrin-1 is absent, pro-apoptotic signaling induces the cleavage of DCC at aspartic acid 1290 by caspases (Castets et al., 2012). However, the presence of netrin-1 prevents the cleavage of DCC, and promotes cell survival (Castets et al., 2012). Thus it is suggested that DCC acts as a late gatekeeper, which restricts tumor growth.

However the main focus in the research of DCC has been its role in axon guidance. Early in development, DCC expression is largely limited to the developing CNS. The initiation of DCC expression correlates to the commencement of neurogenesis in the CNS of mouse embryos and elevated levels of DCC persist in all areas of the CNS that are actively undergoing neurogenesis. Eventually, by embryonic day 18.5 (E18.5) DCC expression becomes restricted to structures that maintain active neurogenesis postnatally, such as the cerebellum, the hippocampus and the olfactory bulb (Gad et al., 1997). A deficiency in DCC expression during

embryonic development stops the development of both cerebral and hippocampal commissures in mice (Fazeli et al., 1997). DCC is also expressed in the spinal cord during embryonic development, particularly in commissural neurons and deletion of DCC in embryonic mice results in defects in commissural axon projection that are even more severe than those seen in netrin-1 deficiencies (Fazeli et al., 1997).

1.4 Netrin-1/DCC Interaction

DCC is part of the immunoglobulin superfamily of proteins. The extracellular domain of DCC is composed of 4 N-terminal immunoglobulin like (Ig-like) domains, which are arranged in a horseshoe arrangement, and this horseshoe structure has been found to be essential for DCC's ability to mediate axon outgrowth (Chen et al., 2013). The Ig-like domains are followed by 6 fibronectin III (FN) domains, a transmembrane region, and a C-terminal cytosolic region containing 3 conserved regions: P1, P2 and P3 (Fig. 1A) (Keino-Masu et al., 1996). Upon its interaction with netrin-1 through its extracellular region, DCC homodimerizes by way of its cytoplasmic P3 region (Stein et al., 2001). When UNC5 is also present, DCC and UNC5 heterodimerize through the interaction of the cytoplasmic P1 region of DCC and the cytoplasmic DCC-binding motif of UNC5 (Hong et al., 1999). The FN domains of DCC proximal to the transmembrane region of DCC have long been known to be the region of the protein that interacts with netrin-1 (Geisbrecht et al., 2003). However, exactly how netrin-1 and DCC associate is a much newer discovery. Each netrin-1 molecule interacts with two DCC receptors (Xu et al., 2014). The FN5 domain of one of the DCC receptor interacts with the EGFR-3 domain (in the laminin-like V domain) of the netrin-1 molecule and the other DCC receptor binds to the laminin-like VI domain of netrin-1 via its fibronectin 4 domain (Xu et al., 2014).

There is also evidence that the sixth fibronectin domain plays a role in the netrin-1-DCC interaction. (Finci et al., 2014).

1.5 Signaling pathways mediated by DCC/netrin-1

Subsequent to netrin-1 interacting with DCC, a series of biochemical signaling cascades are mediated through the cytoplasmic tail of DCC. These pathways lead to gene transcription, cytoskeletal rearrangement and changes in DCC receptor localization that are ultimately necessary for netrin-1/DCC mediated axon outgrowth in neurons (Fig 1B). The following section presents an overview of our current knowledge concerning the key signaling pathways downstream of DCC/netrin-1.

1.5.1 Rho GTPases

The netrin-1/DCC pathway ultimately exerts its influence on axon outgrowth through affecting the activity of different RhoGTPases, which in turn modify the cytoskeletal dynamics at the growth cone, particularly through actin polymerization. In cultured Swiss 3T3 fibroblasts cells expressing DCC, netrin-1 exposure was found to mediate actin reorganization through modulating Rac1 activity (Li et al., 2002b). In commissural neurons it has also been shown that netrin-1 inhibits RhoA activity in a DCC-dependent manner (Moore et al., 2008). Over the past two decades the pathways leading from the interaction between netrin-1 and DCC to the activation of these GTPases and the subsequent cytoskeletal rearrangement have become steadily elucidated.

1.5.2 Fyn

Upon binding to netrin-1, the intracellular domain of DCC rapidly becomes phosphorylated on serine, threonine and tyrosine residues (Meriane et al., 2004). One of these sites, tyrosine 1418 has been shown to be essential to DCC induced activation of Rac1 and to overall neurite outgrowth. Y1418 is phosphorylated by the Src kinase Fyn, which itself is phosphorylated in response to netrin-1 interacting with DCC (Liu et al., 2004). A knockdown of Fyn has been shown, *in vivo*, to impair DCC induced neurite outgrowth in response to netrin-1 (Meriane et al., 2004).

1.5.3 Nck, Pak1 and Trio

Subsequent to DCC interacting with netrin-1, the adaptor protein, Nck couples with DCC via its SH3 domain (Li et al., 2002a). Nck then forms a complex with the serine and threonine kinase, p21-activated kinase 1 (PAK1) binding to its SH3 domain while another protein, Trio binds to PAK1 via its spectrin domains in its N-terminus (Briançon-Marjollet et al., 2008). Trio is a multidomain protein that contains two GEF domains, the first (GEFD1) activates Rac1 and RhoG, while the second GEF domain acts on RhoA. Complete deletion of Trio is embryonic lethal in mice between E15.5 and birth. This implies that Trio is required for late embryonic development, and it is theorized that it is involved in the formation of fetal skeletal muscle and neural tissue (O'Brien et al., 2000). Trio's GEF activity has been shown to be required for netrin-1/DCC-induced neurite outgrowth (Briançon-Marjollet et al., 2008). Trio is also phosphorylated by Fyn on Y2622. Mutant forms of Trio lacking this phosphorylation site retain their GEF activity towards Rac1, however netrin-1-induced Rac1 activation is impaired and, hence netrin-1/DCC-induced neurite outgrowth is also impaired (DeGeer et al., 2013). In

addition, Y2622 phosphorylation also appears to be required for proper transport of DCC to the surface of the growth cone (DeGeer et al., 2013).

1.5.4 DOCK180

A second GEF was found to mediate Rac1 activation downstream of netrin-1/DCC and that is dedicator of cytokinesis protein 1 (DOCK180). DOCK180 has previously been implicated in cytoskeletal reorganization by way of the activation of Rac1 through its involvement in integrin signaling through the CrkII-p130CAS complex (Kiyokawa, et al., 1998). It has since been classified as part of the CDM subfamily of GEFs, which are characterized by their ability to activate Rac1 (Li et al., 2008). DOCK180 was found to be directly associated with netrin-1 and inhibition of DOCK180 drastically reduced netrin-1/DCC dependent stimulation of Rac1 and partially reduced Cdc42 activation (Li, et al., 2008). Furthermore knocking down DOCK180 reduced netrin-1/DCC induced neurite outgrowth in cultured neurons and *in vivo* it was shown that DOCK180 activity was required for the projection of commissural neurons in the neural tube (Li, et al., 2008).

1.5.5 FAK, Src, PI3K and WASP

Focal adhesion kinase or FAK (also known as protein tyrosine kinase 2 or PTK2) has also been identified as playing a role in the netrin-1/DCC pathway (Liu et al., 2004a; Li et al., 2004; Ren et al., 2004). FAK is a protein tyrosine kinase, which has previously been shown to be involved in a variety of cellular processes including, adhesion, spreading, migration, survival, cell cycle progression and proliferation (Liu et al., 2004a). Tyrosine phosphorylation of FAK has

previously been shown to be implicated in integrin induced cell migration. Upon phosphorylation in response to netrin-1, FAK can provide specific binding sites for several different proteins including the Src family kinases, forming a complex that can act on downstream substrates (Liu et al., 2004a). The interaction of netrin-1 with DCC causes FAK and DCC to form a complex and induces the phosphorylation of FAK on tyrosine residues and this phosphorylation has been found to be dependent on Src family proteins. Dominant negative FAK has been shown to reduce growth cone sensitivity to netrin-1 (Liu et al., 2004a). The phosphorylation of FAK by Src creates a binding site for the adaptor protein growth factor receptor-bound protein 2 (Grb2) (Schlaepfer and Hunter, 1996) and it is theorized that the interaction between FAK and Grb2 might provide a link between netrin-1/DCC to the mitogen activated protein kinase (MAPK) cascade (Round and Stein, 2007). Extracellular-signal-regulated kinase 1/2 (ERK1/2) itself has been shown to be phosphorylated in response to the interaction of netrin-1 with DCC (Forcet et al., 2002). This phosphorylation of ERK1/2 has been shown to be essential for mediating the effects of netrin-1 stimulation as blocking the phosphorylation of ERK1/2 has been shown to attenuate netrin-1/DCC induced neurite outgrowth (Forcet et al., 2002).

It is also thought that FAK might recruit and activate phosphatidylinositol 3-kinase (PI3K) downstream of netrin-1/DCC (Chen and Guan, 1994) because inhibition of PI3K has been shown to disrupt netrin-1's chemoattractive capability and because PI3K activates Abnormal cell migration protein 10 (MIG10), a *C. elegans* homolog of mammalian lamellipodin, which stimulates lamellipodial outgrowth in response to netrin-1 (Chang et al., 2006). Netrin-1 stimulation of DCC expressing cortical neurons also induces the phosphorylation of protein kinase B (Akt), which is a downstream kinase of the phosphatidylinositol 3-kinase pathway (Xie

et al., 2005). FAK has also been found to interact with neural Wiskott–Aldrich syndrome protein (N-WASP), with FAK activating N-WASP by phosphorylating it on Y256 and this phosphorylation causing its nuclear localization by way of nuclear importin NPI-1, from where N-WASP is able to activate Cdc42 (Wu et al., 2003). N-WASP, which is itself also a downstream effector of Cdc42 that regulates actin polymerization, has been found to be recruited to into a complex with DCC and the activity of N-WASP has been shown to be required for netrin-1/DCC mediated filopodia formation in the growth cone (Shekarabi, 2005).

1.5.6 PLC, PIP2 and calcium signaling

Phosphoinositides and phospholipase C (PLC) also appear to play a role in netrin-1/DCC signaling (Xie et al., 2005). Phosphoinositides (e.g. PIP2) are a group of phospholipids in cell membranes that can act either as precursors of second messengers such as inositol 1,4,5-trisphosphate (IP3) and diacylglycerol or they can act directly by interacting with proteins that either contain an actin-binding or pleckstrin homology domain to affect their spatiotemporal distribution and/or activity (Yin and Janmey, 2003). PIP2 also act as a cofactor for small GTP-binding proteins (e.g. Arf) and act to modify the actin cytoskeleton (Yin and Janmey, 2003). In the nervous system, PIP2 plays a role in membrane trafficking at the synapse (Xie et al., 2005). It has been found that netrin-1 stimulation of DCC expressing cortical neurons induces PIP2 hydrolysis via phosphorylating PLC on tyrosine residues and this is required for proper netrin-1/DCC induced neurite outgrowth. This is demonstrated by both PLC activation and PIP2 hydrolysis increasing upon netrin-1 stimulation, and through the inhibition of PLC (through the use of a tyrosine phosphorylation inhibitor) causing the attenuation of netrin-1 stimulated neurite outgrowth (Xie et al., 2005). Furthermore, PLC activity has been implicated in nerve growth

factor and netrin-1/DCC induced growth cone guidance of *Xenopus* spinal neurons (Ming et al., 1999). It has also been found that IP3 receptor-mediated calcium release from intracellular reserves plays a role in the axon outgrowth in the dorsal root ganglia of chick embryos (Takei et al., 1998). There is also evidence that netrin-1 stimulation, in cultured primary neurons, induces calcineurin dependent nuclear localization of nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 4 (NFATC4) and subsequent activation of NFAT-mediated gene transcription and that this calcineurin-NFAT signaling is essential for proper netrin-1-induced neurite outgrowth (Graef et al., 2003). It is therefore theorized that there is a pathway consisting of netrin-1/DCC causing phosphorylation of PLC, inducing PIP2 hydrolysis enhancing IP3 production, elevating the intracellular calcium concentration, and thus stimulating calcineurin-NFAT signaling leading to neurite outgrowth (Xie et al., 2005).

1.5.7 Localization of DCC to lipid rafts

Proper netrin-1/DCC signaling relies on the DCC receptor being localized to the appropriate microdomains within the cell membrane, namely cholesterol and sphingolipid-enriched membrane microdomains (lipid rafts) (Guirland et al., 2004). The amount of DCC targeted to lipid rafts is only a comparatively small fraction of the total DCC in the cell, however it is the predominate location of DCC on the surface of both cell lines and cortical neurons (Petrie et al., 2009). The importance of the localization of DCC to lipid rafts is demonstrated by findings that netrin-1 stimulates DCC association with lipid rafts and that by disrupting lipid raft integrity, netrin-1-induced growth cone attraction and repulsion can be abolished (Guirland et al., 2004). In addition, by disrupting the lipid rafts asymmetrically, it is possible to induce opposite turning responses in *Xenopus* growth cones treated with netrin-1 (Guirland et al., 2004). Fyn has

also been found to localize to lipid rafts and it has been found that this population of Fyn is capable of interacting with the DCC also localized within the lipid rafts, with palmitoylation acting as a common mechanism to target both proteins to lipid rafts (Petrie et al., 2009). However, despite the experimental evidence pointing to the functional importance of lipid rafts for netrin-1/DCC signaling (Guirland et al., 2004), it appears that DCC found in the non-raft portion of the plasma membrane is actually the site of the propagation of early signaling events in response to netrin-1 (Petrie et al., 2009).

1.5.8 ERM Proteins

Ezrin–radixin–moesin (ERM) proteins are a group of conserved molecules that act to link the plasma membrane to the cytoskeleton in the netrin-1/DCC pathway (Antoine-Bertrand, et al., 2011a). They have been found to play a variety of roles including being involved in the formation of microvilli, cell–cell junctions, membrane ruffles, as well as to regulate substrate adhesion and motility (Gautreau, et al., 2000). ERM proteins, including the brain-specific homologue merlin, are expressed throughout the CNS during development and their activity is associated with growth cone dynamics, neurite outgrowth, and branching (Antoine-Bertrand, et al., 2011a). They all have a similar domain structure that includes an N-terminal four-point-one, ezrin, radixin, moesin (FERM) domain, responsible for membrane targeting, an alpha helical linker region, and a C-terminal F-actin binding domain (Antoine-Bertrand, et al., 2011a).

The FERM domain and the F-actin binding domain strongly interact with each other in an intramolecular fashion, which keeps the ERM proteins in a dormant form in the cytosol, in which binding sites for membrane components and F-actin are blocked. It has been found that this interaction can be interrupted by the phosphorylation of a conserved threonine residue (T567) in

ezrin (Gautreau, et al., 2000). A phospho-null mutant form of T567 also showed a poor association with the cytoskeleton and an inability to interact with F-actin, being unable to induce the formation of lamellipodia, membrane ruffles, and tufts of microvilli (Gautreau, et al., 2000). This intramolecular interaction also allows ERM proteins to form oligomers when in this inactive state, with ezrin dimers, trimers, tetramers, and higher order oligomers assembled in a head to tail sequence, being identified in purified placental microvilli (Berryman et al., 1995). A phospho-mimic mutant of the site (T567D) was found to show a drastically reduced amount of oligomers at the plasma membrane, and this monomeric T567D ezrin was able to associate with the actin cytoskeleton and consequently induce actin-rich membrane projections. This implies that the phosphorylation of this threonine site acts as a mechanism to control a conversion from oligomers to monomers, which then act as plasma membrane–actin cytoskeleton linkers (Gautreau, et al., 2000). It was later found that the phosphorylation T567 is also critical for translocating the ERM proteins from the cytoplasm to the plasma membrane, where the their FERM domain are then able to binds to membrane bound proteins such as cell-surface receptors (McClatchey and Fehon, 2009).

It was found that upon stimulation with netrin-1, ezrin interacts with DCC via its FERM domain and that this results in the activation of ezrin via phosphorylation and the accumulation of phosphorylated ezrin at the growth cone (Antoine-Bertrand, et al., 2011a). Phosphorylation of DCC at tyrosine 1418, previously determined to be due to Fyn (Meriane et al., 2004), has been shown to be required for netrin-1 mediated activation of ERM proteins (Antoine-Bertrand et al., 2011a). The functional importance of the activation of ezrin in netrin-1/DCC mediated neurite outgrowth is confirmed by evidence that the impairment of ERM activity inhibits netrin-1 mediated axon outgrowth in cortical neurons (Antoine-Bertrand et al., 2011a).

1.5.9 ADF/cofilin

There is evidence that netrin-1-induced growth cone guidance works in part by influencing actin depolymerizing factor (ADF)/cofilin activity (Marsick, et. al., 2010). Actin depolymerizing factor and cofilin (AC) family proteins are key regulators of cytoskeletal mechanics acting to remodel actin filaments by enhancing assembly/disassembly dynamics (Marsick, et. al., 2010). AC activity is controlled by affecting the phosphorylation status of a conserved residue, serine-3 (Marsick, et al., 2010). Upon phosphorylation of this residue, which can be achieved by multiple kinases including LIM kinase 1 and LIM kinase 2, AC is inactivated (Toshima et al., 2001), while dephosphorylation of this residue via multiple phosphatases results in activation of AC (Ohta, et al., 2003). A further method of controlling AC proteins is by inhibition via phosphatidylinositol phosphates in a pH-dependent manner (Frantz et al., 2008). The inactive form of AC is found to be reduced in response to netrin-1 stimulation, supporting its role in netrin-1-mediated axon guidance (Marsick, et al., 2010). Furthermore, directly increasing AC activity mimics many of the effects of netrin-1 stimulation, inducing growth cone protrusion and F-actin polymerization, while reducing AC activity reduces axon-turning responses to netrin-1 (Marsick, et al., 2010). It has therefore been theorized that netrin-1 differentially activates AC in the growth cone, activating it preferentially on the side of the growth cone proximal to the netrin-1 source causing asymmetrical F-actin polymerization and growth cone turning (Marsick, et al., 2010).

1.6 Rationale and Hypothesis

Phosphorylation has been shown to be a common mechanism to regulate netrin-1/DCC-mediated neurite outgrowth at wide variety of levels within the pathway. Phosphorylation has

been shown to control the activity of GEFs downstream of netrin-1, such as Trio (DeGeer, 2013), and to activate kinases, such as FAK (Xie et al., 2005), which upon phosphorylation, it phosphorylates and activates other proteins such as those in the MAP kinase pathway (Round and Stein, 2007) and N-WASP (Wu et al., 2003) leading to neurite outgrowth through either causing downstream protein transcription or by directly affecting the actin cytoskeleton. Phosphorylation in the netrin-1/DCC pathway has also been shown to be key for controlling signal propagation through secondary messengers, with the phosphorylation of PLC upon netrin-1 stimulation being essential for the hydrolysis of PIP2 into active IP3 (Xie et al., 2005). The physical link between the plasma membrane and the actin cytoskeleton has even been found to be controlled by netrin-1-induced phosphorylation of the ERM proteins (Antoine-Bertrand et al., 2011a). It has been shown that phosphorylation of DCC itself is a key step in netrin-1 mediated axon outgrowth, with the phosphorylation of Y1418 being essential (Meriane et al., 2004). However DCC is known to be phosphorylated extensively upon netrin-1 stimulation on serine and threonine residues as well, but the role of phosphorylation on these residues is yet to be determined (Meriane et al., 2004). Using a proteomic approach we have identified several serine and threonine phosphorylation sites on the cytoplasmic tail of DCC by mass spectrometry analysis, including S1178, S1182 and T1219. We have also generated a several phospho-null DCC mutants lacking these phosphorylation sites as well as a series of DCC deletion mutants lacking different portions of the cytoplasmic tail of DCC (Fig. 2). The basis of this thesis is to use these DCC mutants to analyze the role of the cytoplasmic tail of DCC and in particular the role of these serine and threonine phosphorylation sites in netrin-1/DCC-mediated neurite outgrowth. Through the analysis of these residues, we aim to elucidate the role for serine and threonine phosphorylation on the cytoplasmic tail of DCC upon netrin-1 stimulation.

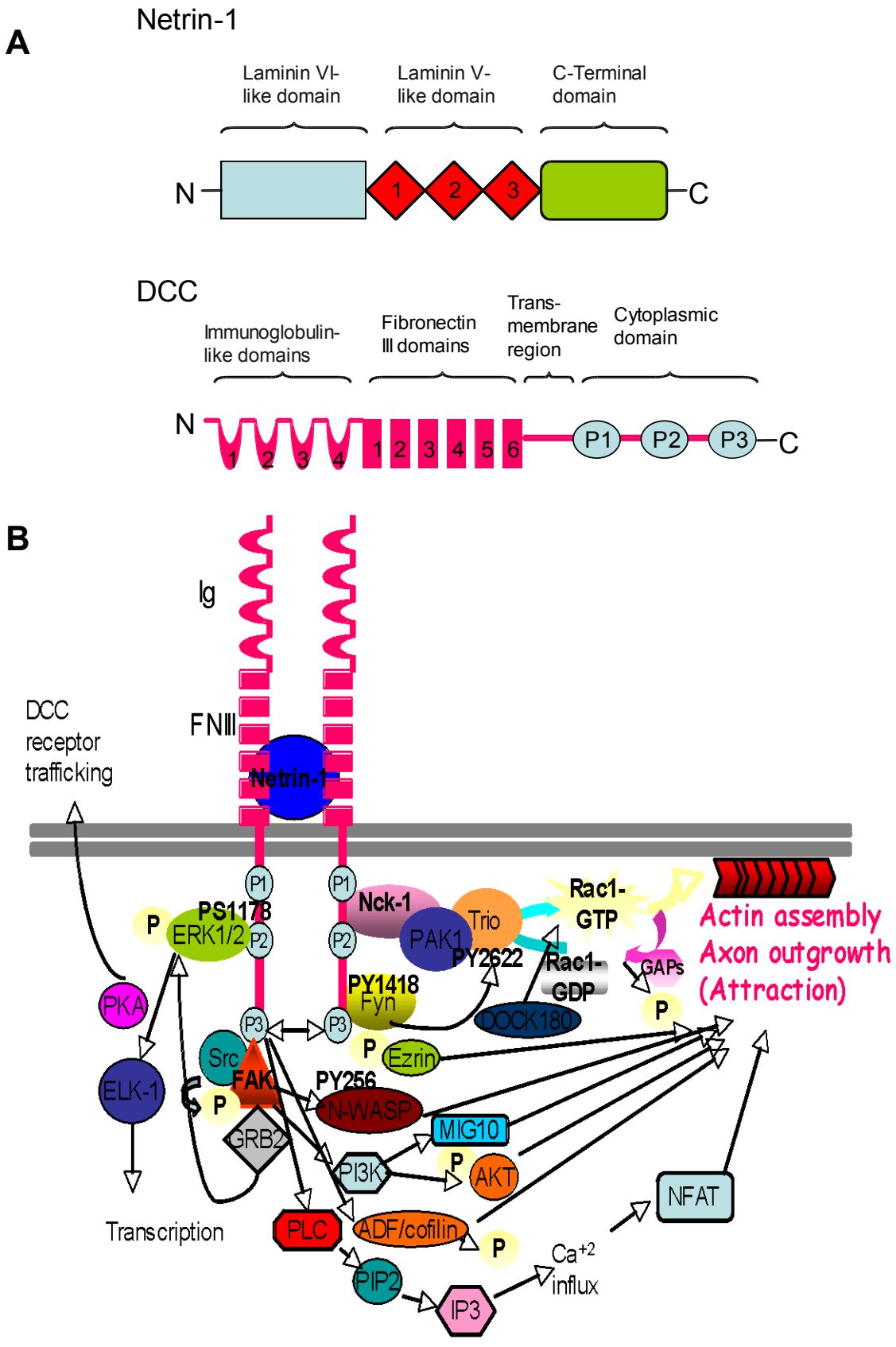
A
Netrin-1
Laminin VI-like domain
Laminin V-like domain
C-Terminal domain
N
1
2
3
C
DCC
Immunoglobulin-like domains
Fibronectin III domains
Trans-membrane region
Cytoplasmic domain
N
1 2 3 4
1 2 3 4 5 6
P1
P2
P3
C
B
Ig
FNIII
DCC receptor trafficking
Netrin-1
P1
P2
P3
PS1178
ERK1/2
Nck-1
PAK1
Trio
Rac1-GTP
PY2622
Rac1-GDP
GAPs
Actin assembly
Axon outgrowth
(Attraction)
PY1418
Fyn
DOCK180
PKA
Src
FAK
PY256
Ezrin
ELK-1
N-WASP
MIG10
GRB2
PI3K
AKT
NFAT
Transcription
PLC
ADF/cofilin
PIP2
IP3
Ca+2 influx

Figure 1. Netrin-1/DCC-mediated signaling pathways in neurons. (A) Netrin-1 is composed of a Laminin VI-like domain, a Laminin V-like domain containing three Epidermal growth factor (EGF) repeats, and a C-terminal domain. A DCC receptor is composed of an extracellular domain containing of 4 immunoglobulin-like domains and 6 fibronectin-like (FN) domains, a transmembrane domain and a cytoplasmic domain containing three conserved regions (termed P1, P2 and P3) (Keino-Masu, 1996). Each netrin-1 ligand interacts with two DCC receptors through their FN4, FN5 and FN6 domains (Xu et al., 2014; Finci et al., 2014). (B) Upon interaction with Netrin-1, the DCC receptors dimerize through their P3 regions (Xu et al., 2014) and the intracellular domain of DCC rapidly becomes phosphorylated on serine, threonine and tyrosine residues, critically tyrosine 1418, which is phosphorylated by the Src kinase Fyn (Meriane, 2004). The adaptor protein, Nck couples with DCC and forms a complex with PAK1 and Trio (Briançon-Marjollet, et al., 2008). Trio is subsequently phosphorylated by Fyn at Y2622 where it then acts as a GEF for Rac1, stimulating axon outgrowth (DeGeer, 2013). DOCK180 is activated in response to Netrin-1 and also acts as a GEF for Rac1 (Li, et al., 2008). FAK is recruited to DCC where it is then phosphorylated by Src, creating a binding site for Grb2, which then interacts with the MAPK pathway (Round and Stein, 2007). FAK also recruits PI3K, which stimulates actin reorganization through MIG10 (Chang et al., 2006) and Akt (Xie et al., 2005). FAK also activates N-WASP through phosphorylating it on Y256, which then acts to regulate actin polymerization (Wu et al., 2003). ERK1/2 is phosphorylated in response the netrin-1 and acts through Elk-1 to stimulate transcription (Forcet et al., 2002). ERK1/2 also phosphorylates DCC, and an ERK1/2 phosphorylation consensus site is located at S1178 (Ma et al., 2010). Netrin-1/DCC also causes the phosphorylation of PLC, inducing PIP2 hydrolysis enhancing IP3 production, elevating the intracellular calcium concentration and stimulating calcineurin-NFAT signaling, leading to neurite outgrowth (Xie et al., 2005). DCC also interacts with ezrin, activating it through phosphorylation leading to the accumulation of phosphorylated ezrin at the growth cone, which is necessary for netrin-1-mediated axon outgrowth (Antoine-Bertrand, et al., 2011a). Netrin-1 stimulation causes the dephosphorylation of ADF/cofilin, activating it, causing F-actin polymerization (Marsick, et al., 2010). DCC receptor trafficking to the cell surface has been found to be mediated by PKA activity (Bouchard et al., 2004), which acts to regulate the sensitivity of Netrin-1, induced chemoattraction (Moore and Kennedy, 2006).

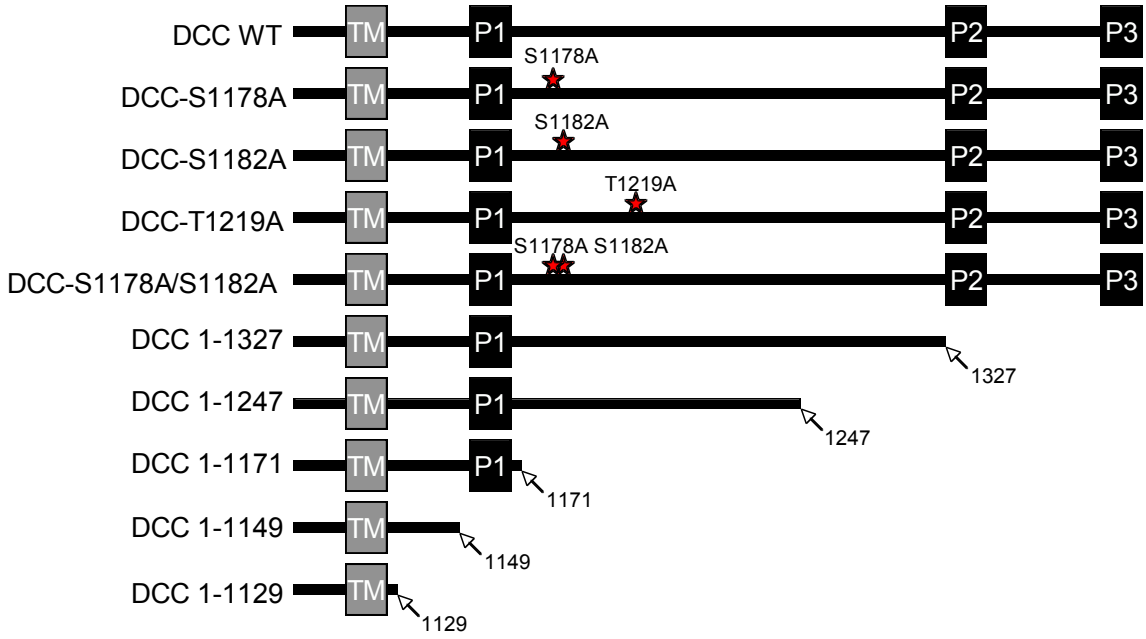

Figure 2. **Schematic of DCC and DCC mutant proteins**. WT, wild type; TM, transmembrane region; P1, conserved region 1; P2, conserved region 2; P3, conserved region 3. Phosphorylation sites that were modified are marked with stars.

Chapter 2

Materials and Methods

Table 1. Antibodies. The antibodies used in these experiments are the following.

Antibody name	Company	CAT#
Mouse monoclonal anti-DCC	Calbiochem	OP45
Rabbit polyclonal anti-Pp44/42 (phospho-ERK1/2)	Cell Signaling Technologies	9101
Rabbit monoclonal anti-p44/42 (ERK1/2)	Cell Signaling Technologies	4695
Rabbit polyclonal anti-phospho-FAK (Y861)	Life Technologies	44626G
Rabbit polyclonal anti PAK (N20)	Santa Cruz Biotechnology	SC882
Mouse polyclonal anti-Myc-Tag	Cell Signaling Technologies	2272
Anti-mouse IgG HRP linked whole cell antibody	GE Healthcare	LNA931V/AG
Anti-rabbit IgG HRP linked whole cell antibody	GE Healthcare	LNA934V/AG

Cell culture

Unless otherwise indicated, N1E-115 and HEK-293 cells were cultured on Nunc^{TM} cell culture dishes in Dulbecco's Modification Eagle Medium (Millipore) supplemented with 10% Fetal Bovine Serum (FBS), 0.75mg/ml penicillin and 0.1mg/ml streptomycin. All cells were maintained at 37°C with 5% CO_2.

Plasmid constructs

DCC C-terminal deletion mutants were generated by conventional PCR using PRK5 encoding DCC full-length plasmid with the various primers (Tcherkezian et al., 2010). The phospho-null mutants were created as described previously (Meriane et al., 2004). Briefly, the serine and threonine residues located at 1178, 1182 and 1219 of the intracellular domain of full-length DCC in pRK5 vector (Li et al., 2002b) were substituted with alanine residues using a PCR oligonucleotide-directed mutagenesis, according to standard protocols.

Creation of plasmid stocks

Samples from bacterial stocks were added to 200ml of LB media containing a 1/1000 dilution of 100mg/ml ampicillin (if plasmids had a PRK5 vector) or 1/1000 dilution of 50mg/ml kanamycin (if plasmids had a GFP vector) and incubated at 37°C for 16 hours. Plasmid DNA was purified using a Life Technologies PureLink® HiPure Plasmid Maxiprep Kit using a protocol that was conducted as previously (Martin et al., 2007).

Netrin-1 purification

Netrin-1 was purified as described previously (Serafini, et al., 1996). Briefly, HEK-293 cells expressing netrin-1 were cultured until 100% confluent in P150 cell culture dishes in culture media containing DMEM supplemented with 10% FBS, 250mg/ml hygromycin B and 200mg/ml G418 sulfate. The cell culture media containing the secreted netrin-1 was collected and NaCl was added to a final concentration of 0.5M. 10X PBS was added until the pH of the media was 7.5. Protease inhibitors were added and the cell debris was spun down and then concentrated and filtered. Fast Protein Liquid Chromatography (FPLC) was then performed. The supernatant was loaded onto a HiTrap^{TM} Heparin column and eluted with a gradient of PBS NaCl from 0.6M to 2M, pH 7.4. The elution was harvested in 2ml fractions. The fractions containing netrin-1 were then pooled and 10 µl aliquots containing 500 µg/ml netrin-1 were created.

In-vitro kinase assay and phospho-amino acid analysis

An in vitro kinase assay was performed as has been described previously (Meriane, 2004). Briefly, activated ERK-1 was incubated with GST-tagged DCC-C (cytoplasmic tail), myelin basic protein (MBP) as a positive control, in the presence of 10 μ Ci of [γ -32P]ATP/ml. The products were resolved by SDS-PAGE, and phosphorylated substrates were detected by autoradiography. GST-DCC-C phosphorylated by ERK-1 was subjected to phosphoamino acid analysis using previously described methods (Meriane et al., 2004).

Mass spectrometry

N1E-115 cells were transfected with DCC and IP DCC were subjected to SDS-PAGE and the bands corresponding to Coomassie blue-stained DCC were removed and sent to mass spectrometry facilities at McGill-Genome Quebec Innovation Center. After trypsin digestion, phosphopeptides were enriched on an iminodiacetic acid-modified sepharose beads with immobilized Al^{3+} ions and processed by LC-MS/MS and analyzed by Multiple Reaction Monitoring (MRM).

ProQ phospho-protein stain

N1E-115 cells were transfected with PRK5 empty vector, wild-type DCC, DCC-S1178A, DCC-S1182A, DCC-T1210A, DCC-T1219A and DCC-1-1121 and immunoprecipitated using mouse anti-extracellular DCC antibodies. ProQ phospho-protein staining was conducted as described previously (Caspersen et al., 2007) and a silver-stain was used to detect total proteins.

Neurite outgrowth assay

Neurite outgrowth was analyzed as described previously (DeGeer, et al., 2013). Briefly, N1E-115 neuroblastoma cells were plated on laminin coated coverslips at a density of 125 000 cells per coverslip in P35 cell culture dishes. The next day they were transfected overnight (18 hours) with 3 µ g of plasmid DNA (PRK5-empty vector, wild-type DCC, or one of the different truncation or phospho-null mutants) using Lipofectamine® 2000. The cells were then fixed using 3.7% Formaldehyde and blocked with 0.25% BSA in PBS. They were then incubated with primary antibody (anti-extracellular DCC) in a 1/500 dilution with 0.1% BSA in PBS for 1 hour and incubated in secondary antibody (1/1000 dilution of Alexa488 and 1/10000 dilution of Phalloidin) for 45 minutes. The coverslips were then mounted on slides using Prolong®. The cells were visualized using an Olympus IX81®-ZDC2 Zero Drift Inverted Microscope System and neurite outgrowth was quantified. A neurite was defined as an extension from the cell that was as long as or longer then the cell body. The number of transfected cells that expressed neurites was compared to the number of transfected cells that did not express neurites for each condition.

ERK1/2 and FAK Western blots

Netrin-1 stimulation and Western blots were conducted as described previously (Forcet et al., 2002). Briefly, HEK-293 cells were plated in P100 cell culture dishes. The next day the cells were transfected for four hours with 4 µ g of plasmid DNA (wild-type DCC, or one of the different truncation or phospho-null mutants, with two plates being transfected per condition) using 40 µ l of PEI. The cells were then serum starved overnight (16 hours) and 1 plate from

each transfected condition was subsequently stimulated with 500ng/ml of netrin-1 for 5 minutes. Protein samples (50 µg) were separated using SDS-PAGE and transferred to a nitrocellulose membrane, which was incubated with rabbit anti-phospho ERK (1:1000), rabbit anti-phospho-FAK (1:1000) and mouse anti-extracellular DCC antibody (1:1000). Immunoreactivity was visualized following incubation with horseradish peroxidase-conjugated secondary antibodies using the chemiluminescence reagent, Immobilon™ Western. The blots were then stripped and re-probed with rabbit anti-ERK (1:1000) and visualized using the same method described above.

Biotinylation assay

HEK-293 cells were plated in P100 cell culture dishes. The next day the cells were transfected for four hours with 4 µg plasmid DNA (PRK5-empty vector, wild-type DCC, DCC-1-1120, DCC-T1219A, DCC-S1178A/S1182A) using 40 µl of PEI. The next day the cells were washed three times with PBS containing 1mM $MgCl_2$ and 1mM$CaCl_2$ and incubated for 20 minutes at 4C with 5mg/ml sulfo-NHS-biotin in PBS. Cells were then washed two times with 10 mM glycine in PBS to remove and quench any unreacted biotin. Cells were then washed two times with in PBS. Cells were then lysed with RIPA buffer for 20 minutes at 4C and harvested. 50 µl of Immobilized Streptavidin Agarose was added and the lysates and they were rotated for 2 hours at 4C. The samples were washed 3 times in cold RIPA buffer before addition of 50 µl of 5x sample blue buffer. Proteins were subjected to SDS/PAGE and Western blotting using mouse anti-extracellular DCC antibody (1:1000) and visualized as above.

Phos-Tag Blots

HEK-293 cells were plated in P100 cell culture dishes. The next day the cells were transfected for four hours. The transfection conditions for the PAK1 Phos-Tag experiments were 1 plate for each condition transfected with either: 8 µ gPRK5 + 80 µ l of PEI or 4 µ gPRK5 + 4 µ g Myc-PAK1+ 80 µ l of PEI or 4 µ g CA-Rac1 + 4 µ g Myc-PAK1+ 80 µ l of PEI. The transfection conditions for the DCC Phos-Tag experiments were four plates transfected with 2 µ gPRK5+ 4 µ g wild-type DCC + 60 µ l of PEI and one plate transfected with 2 µ gCA-Fyn + 4 µ g wild-type DCC + 60 µ l of PEI. The cells in the DCC experiments were then serum starved overnight (16 hours) and stimulated with 500ng/ml of netrin-1 for 5, 10 or 15 minutes (except for one unstimulated DCC transfected control plate and the DCC+CAFyn condition). In both the PAK1 and the DCC experiments, the cells were then washed twice with cold Hepes Buffer Solution (HBS), lysed and proteins were subjected to Phos-Tag infused SDS-PAGE (100mM Phos-Tag, 100mM $MnCl_2$ 5% acrylamide for PAK1 blots, 75mM, 100mM $MnCl_2$, 3.5% acrylamide for DCC blots). The gels were then washed twice in transfer buffer containing1mM EDTA for 20 minutes to remove the Mn^{2+} ions from the gel, and were subsequently washed in transfer buffer for 10 minutes. Western blotting was performed using mouse anti-extracellular DCC antibody (1:1000) for the DCC blots and mouse anti-myc (1:1000) for the PAK blots and visualized as described above.

Statistical analysis

ProQ Phospho-protein stain: Blots were quantified using a densitometer. DCC phosphorylation was expressed as a function of ProQ phospho-protein stained DCC versus silver stained DCC.

Statistical significance was assessed through the use of an unpaired Student's T-test. The threshold for significance was p=0.05. All data are represented as mean +/- SEM.

Neurite outgrowth assay: Statistical analysis comparing neurite outgrowth between conditions was performed through the use of an unpaired Student's T-test. The threshold for significance was p=0.05. All data are represented as mean +/- SEM.

ERK1/2 Western blots: Blots were quantified using Image J. ERK1/2 phosphorylation was expressed as a function of phospho-ERK1/2 versus total ERK1/2. Statistical analysis compared the difference in phospho-ERK1/2/total ERK1/2 in between the netrin-1 stimulated and unstimulated conditions for either wild-type DCC or each of the various mutants. Statistical significance was assessed through the use of an unpaired Student's T-test. The threshold for significance was p=0.05. All data are represented as mean +/- SEM.

Chapter 3

Results

3.0 Analysis of the effects of the deletion of the cytoplasmic domain of DCC on netrin-1-mediated neurite outgrowth

3.0.1 Truncated forms of DCC show reduced neurite outgrowth in N1E-115 Cells

To examine the role of the intracellular domain of DCC on netrin-1-mediated neurite outgrowth we used a series of DCC deletion mutants, each lacking a portion of the cytosolic domain. DCC -1-1129, DCC-1-1149, DCC-1-1171, DCC-1-1247 and DCC-1-1327 deletion mutants (Tcherkezian, 2010) were transfected into N1E-115 neuroblastoma cells. N1E-115 cells were ideal for these experiments since they are able to grow neurites and they endogenously express netrin-1, however they do not express DCC, which negates the need of employing a method such as siRNA to block endogenous DCC (Ly et al., 2008). Furthermore, N1E-115 cells are known to express an increased number of neurites when transfected with wild-type DCC and have been previously used as a model for analyzing netrin-1/DCC mediated neurite outgrowth (Li et al., 2002, DeGeer et al., 2012). The N1E-115 cells were plated on laminin-coated coverslips and transfected overnight with the deletion mutant DNA using Lipofectamine® 2000. The cells were then immuno-stained for DCC (using an anti-extracellular DCC antibody) and for actin, using phalloidin. They were visualized using an immunofluorescence microscope and the number of transfected cells where neurite outgrowth was induced was counted and compared with the number of transfected cells where neurite outgrowth was not induced. In general the shorter the deletion mutant was, the more reduced the neurite outgrowth was, with the DCC-1-1129, DCC-1-1149 and DCC-1-1171 mutants each showing a significant reduction in neurite outgrowth, while the DCC-1-1247 and DCC-1-1327 mutants did not show a significant reduction in neurite outgrowth (Fig. 3).

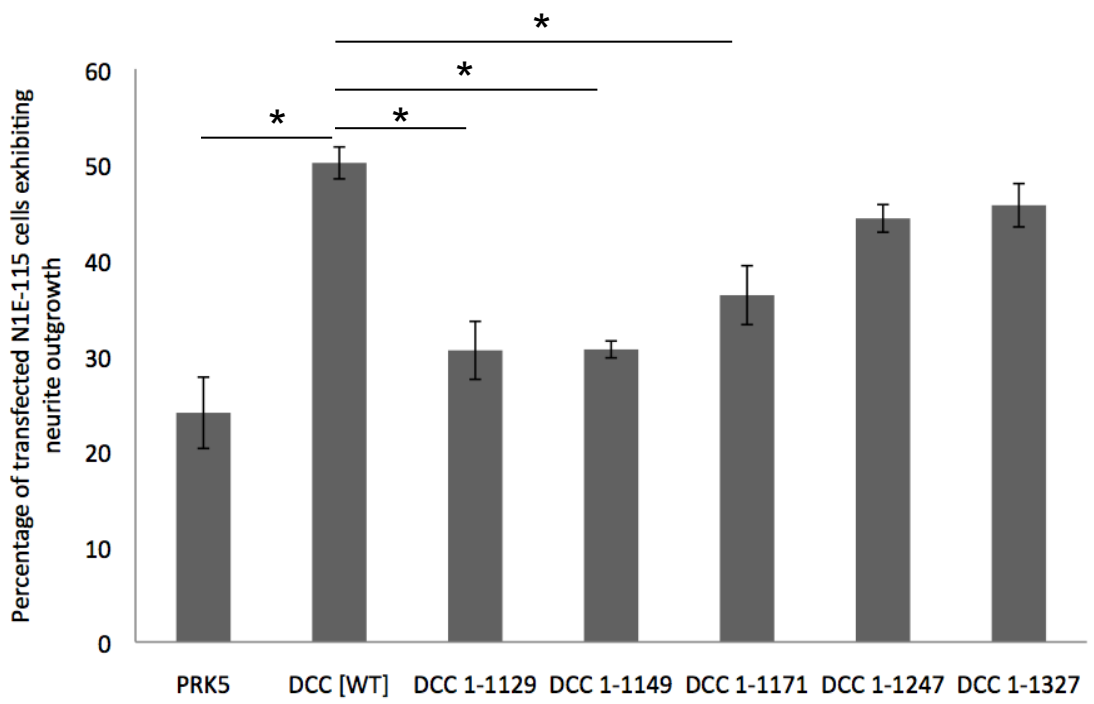

Figure 3. The shorter DCC deletion mutants show reduced netrin-1-mediated neurite outgrowth. N1E-115 cells were transfected with either wild-type DCC (DCC [WT]) or with one of several truncation mutant DCC plasmids. Neurite outgrowth was assessed by immunofluorescence and the percentage (%) of transfected cells exhibiting neurite outgrowth was quantified. n=3, * = $p < 0.05$.

3.0.2 Truncated forms of DCC show reduced ERK1/2 activation

It has been previously shown that ERK1/2 phosphorylation and FAK phosphorylation occur downstream of DCC upon netrin-1 stimulation and these events are required for netrin-1/DCC-mediated neurite outgrowth (Forcet et al., 2002; Round and Stein, 2007). Therefore we attempted to test whether the deletion of different parts of the cytoplasmic tail of DCC would affect the phosphorylation of ERK1/2 and FAK upon netrin-1 stimulation. In order to test this, HEK-293 cells were transfected with either wild-type DCC, DCC-1-1129, DCC-1-1149, DCC-1-1171, DCC-1-1247 or DCC-1-1327 plasmids. The next day the cells were stimulated with netrin-1 for 5 minutes. The cells were then lysed and loaded onto a 7.5% SDS PAGE gel, and transferred overnight. They were then analyzed by western blotting using antibodies for extracellular DCC, phospho-ERK1/2 and total ERK1/2. The cells transfected with DCC-1-1129, DCC-1-1149, DCC-1-1171, DCC-1-1247 or DCC-1-1327 all failed to elicit a netrin-1 dependent induction of ERK1/2 phosphorylation as seen in the wild-type DCC control (Fig. 4).

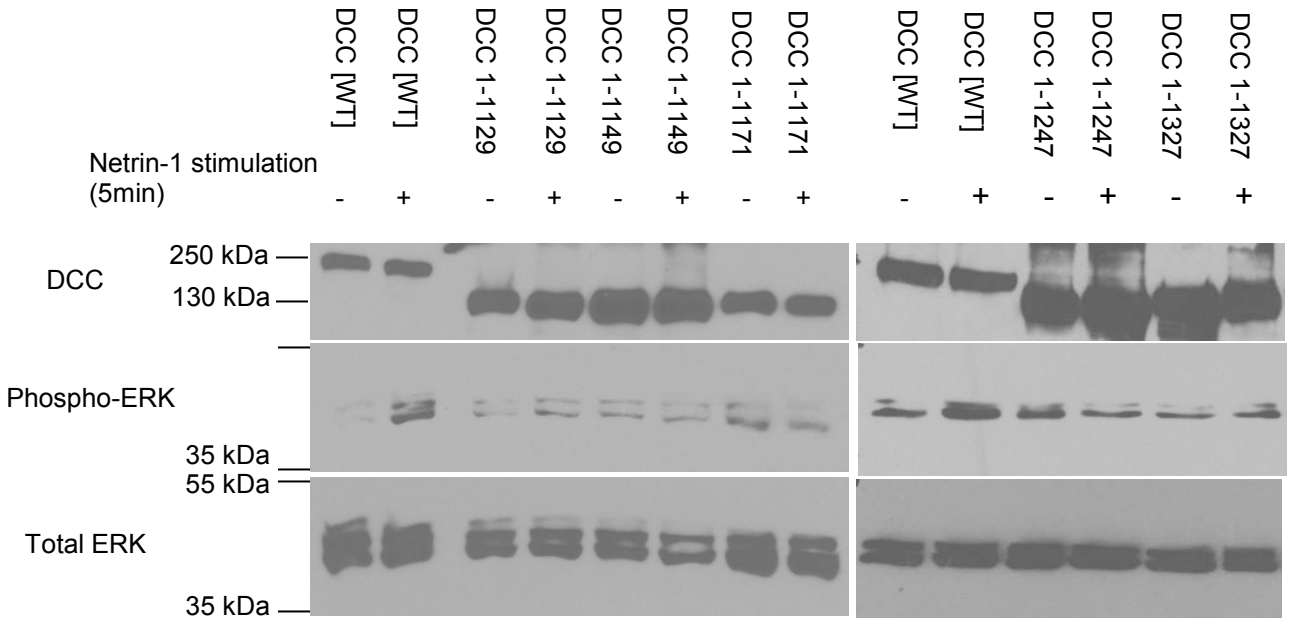

Figure 4. The deletion mutants show reduced ERK1/2 activation in response to netrin-1 stimulation. HEK-293 cells were transfected with either wild-type DCC [WT] or with one of several DCC deletion mutant plasmids. They were then serum starved for 16 hours and subsequently stimulated with 500ng/ml of netrin-1 for 5 minutes. The proteins from cells extracts were resolved by SDS-PAGE and detected by Western blotting using the appropriate antibodies.

3.1 Analysis of the role of DCC phosphorylation on serine and threonine residues in response to netrin-1

3.1.1 Serine and threonine residues in the cytosolic domain of DCC are phosphorylated upon exposure to netrin-1

We have previously determined that DCC is mainly phosphorylated *in vivo* on serine, threonine and tyrosine residues in embryonic commissural neurons in response to netrin-1, and that tyrosine phosphorylation plays a key role in netrin-1-induced neurite outgrowth (Meriane et al., 2004). However the purpose of the phosphorylation of the serine and threonine residues has yet to be determined. Four in vivo Ser/Thr phosphorylation sites were identified in the cytoplasmic tail of DCC: S1178, S1182, T1210, and T1219, through the use of mass spectrometry (Piché, unpublished) (Fig. 5). The S1178 phosphorylation site was found to correspond to an ERK1/2 consensus phosphorylation site (Ma et al., 2010; Tcherkezian, unpublished) (Fig. 6A), and we have found that the cytoplasmic C-terminal domain of DCC is phosphorylated *in vitro* by activated ERK1/2, mainly on serines (Tcherkezian, unpublished) (Fig. 6C). This implies that ERK1/2 may phosphorylate DCC *in vivo*. Phospho-null mutants of each of the identified phosphorylation sites were generated by replacing each serine and threonine with an alanine residue (Luangrath, unpublished).

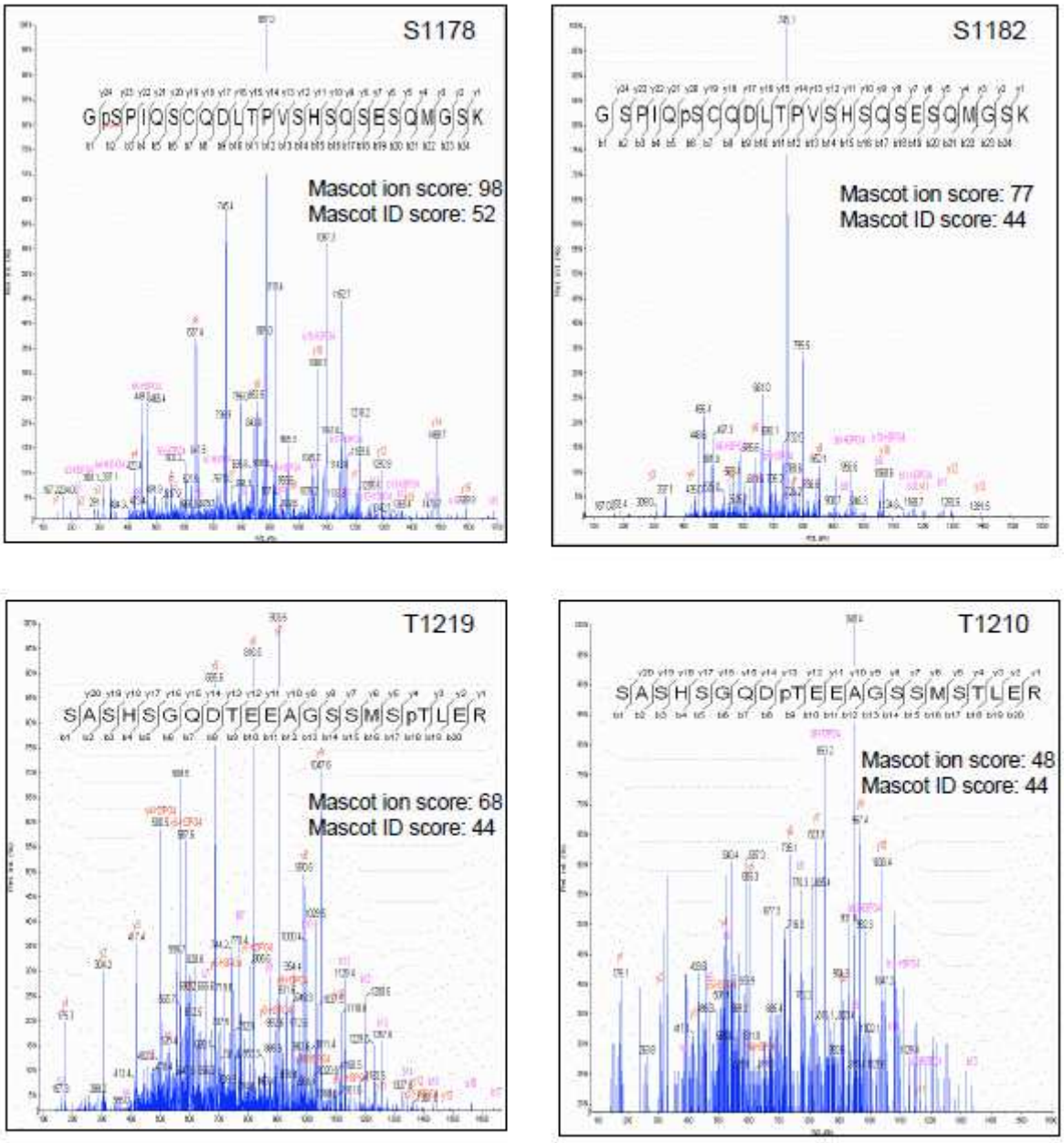

Figure 5. DCC is phosphorylated on several serine and threonine residues. N1E-115 neuroblastoma cells overexpressing DCC were analyzed by mass spectrometry and 4 phosphorylation sites in the cytoplasmic tail of DCC, between the conserved regions P1 and P2 were identified. Performed by Chantal Piché.

A

ERK 1/2 Consensus phosphorylation site	1171TDPAGRG**SP**IQSCODLT1187
ERK-D binding domain	1220ERSLAARRATRTKLM1234

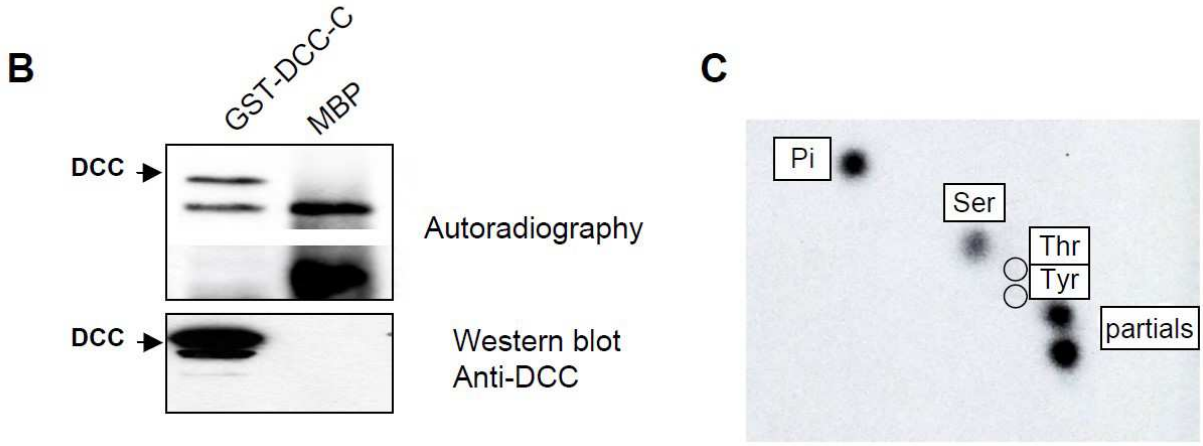

Figure 6. ERK-1 phosphorylates DCC. (A) S1178 is a consensus phosphorylation site of ERK1/2. The potential phosphorylation site within the consensus motif (bold highlighted) and the ERK binding site (underlined). (B) An in vitro kinase assay was performed by incubating activated ERK-1 with GST-tagged DCC-C (cytoplasmic tail), myelin basic protein (MBP) as a positive control, in the presence of 10 μ Ci of [γ -32P]ATP/ml. The products were resolved by SDS-PAGE, and phosphorylated substrates were detected by autoradiography (upper panel). Western blot with monoclonal anti-DCC (BD Biosciences) (lower panel). (C) GST-DCC-C phosphorylated by ERK-1 was subjected to phosphoamino acid analysis. Performed by Joseph Tcherkezian.

3.1.2 Phosphorylation of DCC at specific intracellular serine and threonine residues affects total DCC phosphorylation

We then wanted to analyze whether the different phospho-null mutants affected the overall phosphorylation level of DCC. In order to determine this, the mutants were expressed in N1E-115 cells and immunoprecipitated using anti-extracellular DCC antibodies (intracellular DCC antibodies were avoided in case the mutations affected antibody binding). ProQ phospho-protein staining was then employed to detect phosphate groups on Tyr, Ser, and Thr residues. This was followed by silver staining to detect the total protein levels (Luangrath, unpublished) (Fig. 7A). The S1182A and T1219A mutants both showed a ratio of phospho-protein to total protein that was not statistically different from the wild-type DCC, but the S1178A and T1210A mutants both showed a statistically significant reduction in the ratio of phospho-protein to total protein compared to wild-type DCC (Fig. 7B). A DCC truncation mutant lacking all four phosphorylation sites (1-1171) also showed a reduction in the ratio of phospho-proteins to total proteins compared to wild-type DCC (Fig. 7B). The expression of the T1210A mutant led to a truncated protein of 141.4 kDa, as opposed to full-length protein of 183.2 kDa. We discovered that the T1210A mutant was recognized by anti-extracellular but not anti-intracellular DCC antibodies during western blotting and that the mutant protein was secreted in the media, both of which suggested that the cytoplasmic tail of the mutant DCC is truncated and that the extracellular domain is shredded. This data suggested that T1210 may be important in the regulation of DCC proteolysis and could be required for proper expression of DCC at the cell surface, which was later demonstrated to be the case when biotinylation experiments demonstrated a reduction of T1210A DCC at the cell membrane, compared to wild-type DCC (Duquette, unpublished).

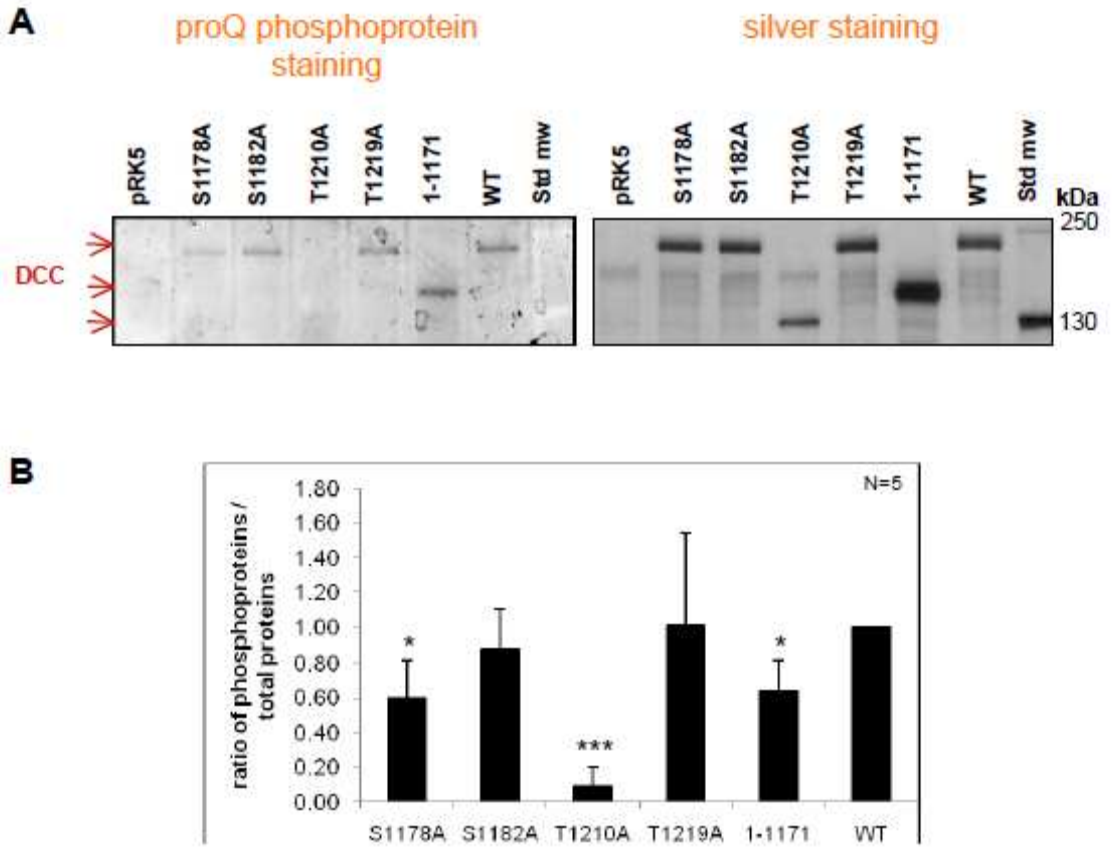

Figure 7. DCC-S1178 is phosphorylated in N1E-115 cells. (A) DCC WT or DCC point mutants were immunoprecipitated (IP) from N1E-115 cells transfected with PRK5 and PRK5-DCC constructs as indicated. Immunoprecipitates were resolved by SDS-PAGE, and phosphorylated DCC was revealed by ProQ phospho-protein staining and subsequently by silver staining to reveal total protein. (B) Quantification of the ratio of phosphorylated DCC on total DCC. * = $p < 0.05$; *** = $p < 0.001$. Performed by Vilayphone Luangrath.

3.1.3 Phosphorylated serine and threonine residues on the intracellular domain of DCC affect neurite outgrowth

We have previously demonstrated that phosphorylation of the cytosolic domain of DCC is required for netrin-1-mediated neurite outgrowth (Meriane et al., 2004). Of particular importance is phosphorylation of DCC at tyrosine 1418 by Fyn. When phosphorylation at the site is abolished, it has been shown to inhibit netrin-1-mediated neurite outgrowth in N1E-115 neuroblastoma cells through inhibiting netrin-1-induced Rac1 activation (Meriane et al., 2004). The role of the phosphorylated serine and threonine residues was tested in a similar fashion, using N1E-115 neuroblastoma cells and then neurite outgrowth was analyzed in the same manner as with the deletion mutants **(**Fig. 8**)**. The S1178A, S1182A, T1219A and the S1178A/S1182A double mutant transfected cells all showed a significant reduction in neurite outgrowth compared to the wild-type DCC transfected cells ($n=4$, $p<0.05$). Preliminary data from experiments involving the transfection of N1E-115 cells with a T1210V phospho-null mutant and analyzed in a similar manner as with the other phospho-mutants also demonstrated a reduction in neurite outgrowth (data not shown).

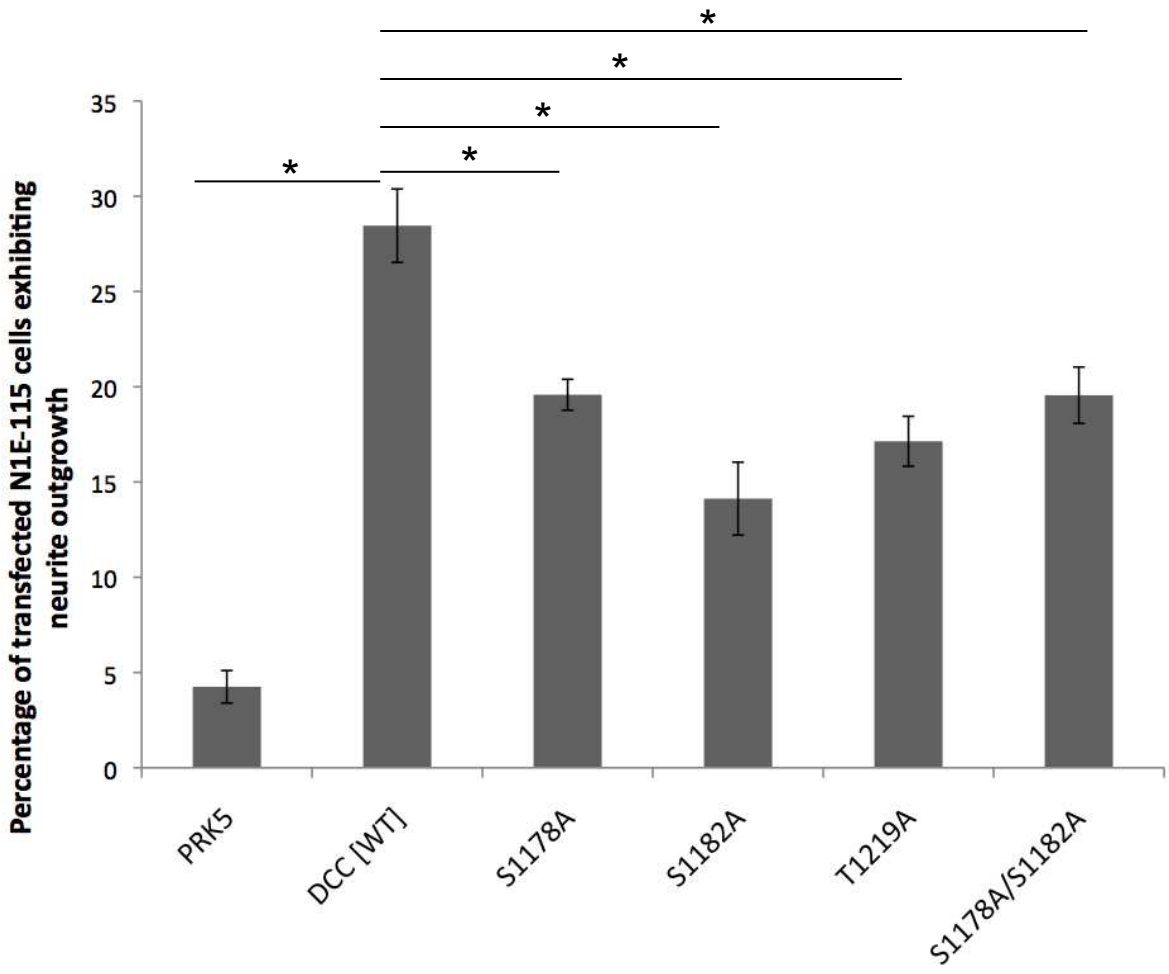

Figure 8. Phosphorylation of DCC at DCC-S1178, DCC-S1182 and DCC-T1219 is necessary for netrin-1-mediated neurite outgrowth in N1E-115 cells. N1E-115 cells were transfected with either wild-type DCC or with one of several phospho-null mutant DCC plasmids. Neurite outgrowth was assessed by immunofluorescence and the percentage (%) of transfected cells exhibiting neurite outgrowth was quantified. n=4, * =p< 0.05.

3.1.4 Inhibition of cytoplasmic DCC phosphorylation on serine and threonine residues did not affect netrin-1-mediated ERK1/2 and FAK phosphorylation

We endeavored to test whether the different DCC phosphorylation mutants affected the phosphorylation of ERK1/2 and FAK upon netrin-1 stimulation. In order to analyze this, HEK-293 cells were transfected with either one of the different phospho-null mutants or wild-type DCC. The next day the cells were stimulated with netrin-1 for 5 minutes, which had previously been shown to be an optimal time point for examining ERK and FAK phosphorylation in response to netrin-1 (Forcet et al., 2002; Liu et al., 2004a). The cells were then lysed, subjected to SDS-PAGE, and transferred overnight. They were then analyzed by western blotting using antibodies for extracellular DCC, phospho-ERK1/2, Total ERK1/2 and phospho-FAK. The S1178A, S1182A, T1219A and the S1178A/S1182A double mutant transfected cells all showed an increased level of both ERK and FAK phosphorylation upon netrin-1 stimulation that was not significantly different from what was observed in the wild-type DCC expressing cells (Fig. 9).

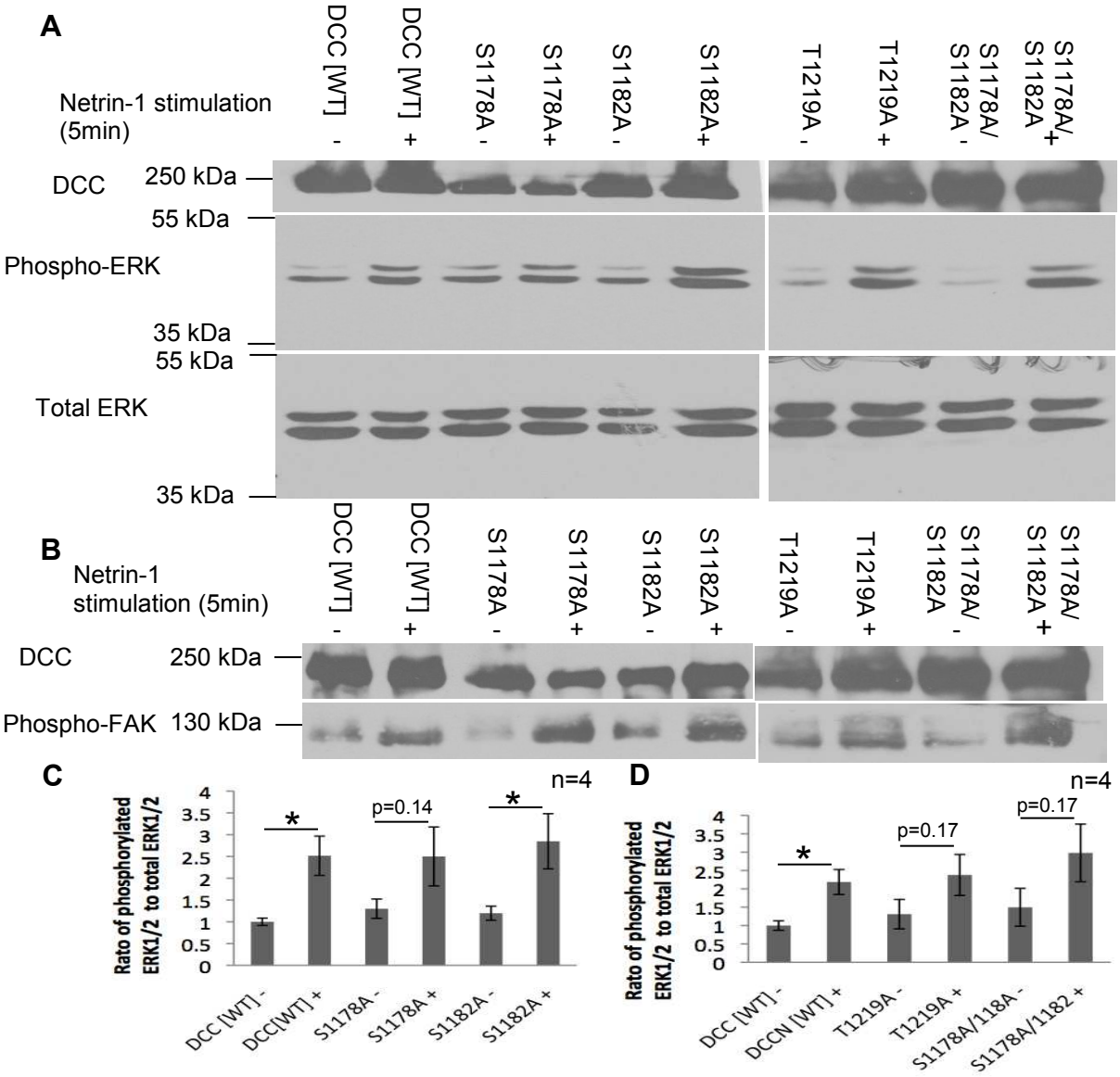

Figure 9. Serine and threonine phosphorylation on the cytoplasmic tail of DCC does not affect ERK-1/2 or FAK phosphorylation downstream of DCC. (A) and (B) HEK-293 cells were transfected with either wild type DCC [WT] or with one of several phospho-null mutant DCC DNAs. They were then serum starved for 16 hours and subsequently stimulated with 500ng/ml of netrin-1 for 5 minutes. The proteins from cells extracts were resolved by SDS-PAGE and detected by western blotting using the appropriate antibodies.(C) and (D) Densitometry was performed using Image J. n=4, *= $p < 0.05$.

3.1.5 Effects of phospho-null mutants on cell surface expression of DCC

In order to identify if the reduced neurite outgrowth observed in N1E-115 cells transfected with the different DCC phospho-null mutants was due to a reduced level of cell surface DCC expression, a biotinylation assay was performed. HEK-293 cells were transfected with either an empty vector (PRK5) control, wild-type DCC, a DCC truncation mutant (1-1120) that has previously been shown to have reduced expression at the cell surface (Fig. 10A), T1219A or S1178A/1182A. Biotinylated proteins were then pulled down using Streptavidin Agarose resin, and were immuno-blotted using an extracellular DCC antibody. All three of the tested mutants showed a reduced amount of signal relative to the wild-type DCC control (Fig. 10B), suggesting that the T1219, S1178 and S1182 sites may play a role in DCC expression at the cell surface.

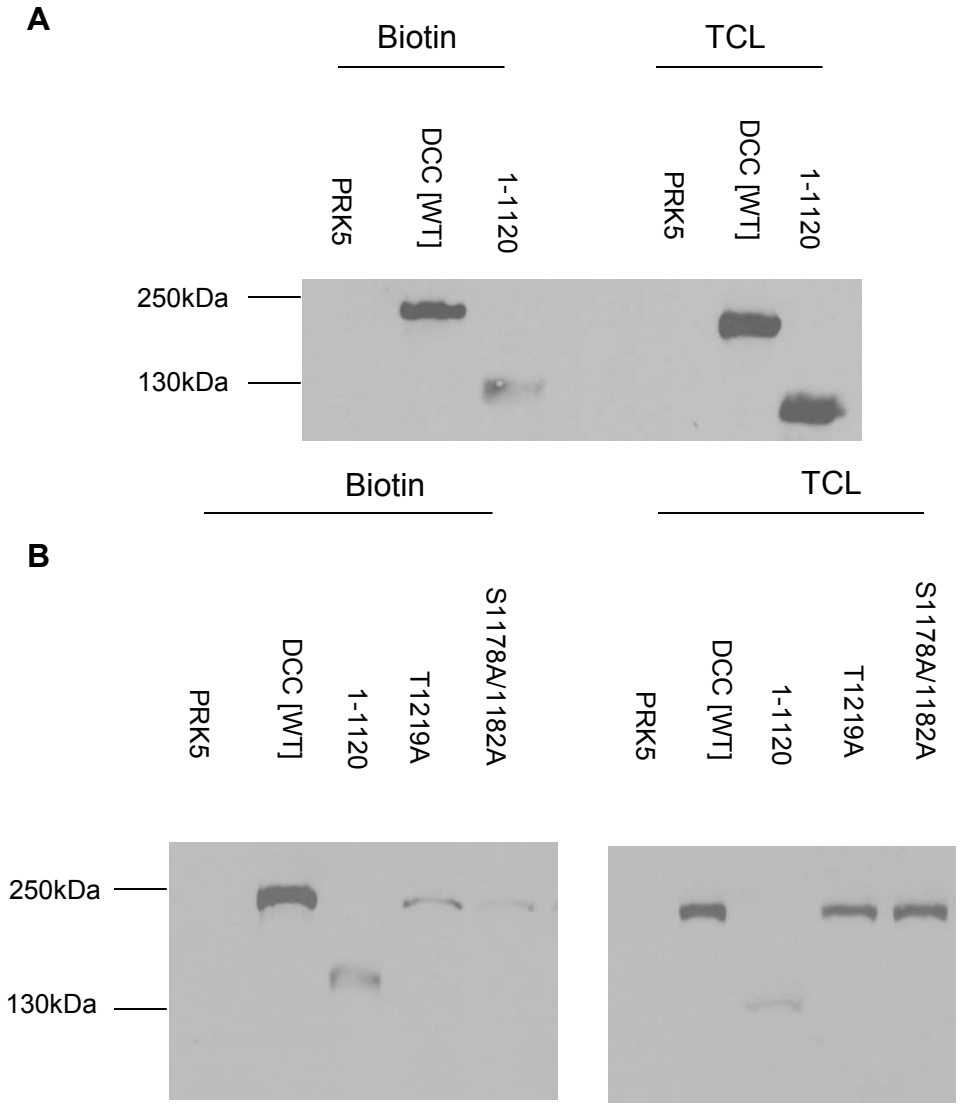

Figure 10. The phosho-null mutants show reduced cell surface expression of DCC. HEK-293 cells were transfected with either wild type DCC [WT] or with one of several mutant DCC plasmids. Cell surface proteins were then labelled with biotin by washing the cells with 5ml of 0.5mg/ml of biotin dissolved in PBS. Biotinylated proteins were then pulled down using Streptavidin Agarose resin and DCC expression was analyzed using an extracellular DCC antibody.

3.2 Analysis of DCC phosphorylation using the Phos-Tag system

3.2.1 Analyzing netrin-1-induced phosphorylation of DCC using a Phos-Tag gel

We next wanted to analyze DCC phosphorylation upon netrin-1 stimulation using a Phos-Tag system. The Phos-Tag system is a method for analyzing phsopho-proteins using western blotting (Kinoshita et al., 2009). The Phos-Tag product contains a compound that can bind specifically to the negative charge of a phosphate ion, using 2 metallic cations (eg. Mn^{2+} or Zn^{2+}), thus allowing it to bind preferentially to phosphorylated proteins. The Phos-Tag solution is added to the acrylamide solution prior to casting the gel in SDS-PAGE. The Phos-Tag bound phospho-proteins will subsequently migrate at a reduced rate through the gel, relative to unphosphorylated proteins, which when blotted, results in a separate band containing the phospho-protein above the band containing the non-phosphorylated protein. Although the Phos-Tag system was originally designed to analyze smaller proteins (under 150kDa) (Kinoshita et al., 2009) than DCC (183.2 kDa), such as PAK1 (60.6 kDa) (Fig. 11A) we were nonetheless able to assess DCC phosphorylation, clearly showing enhanced DCC phosphorylation in response to netrin-1, at three different time-points and to constitutively active Fyn (Fig. 11B).

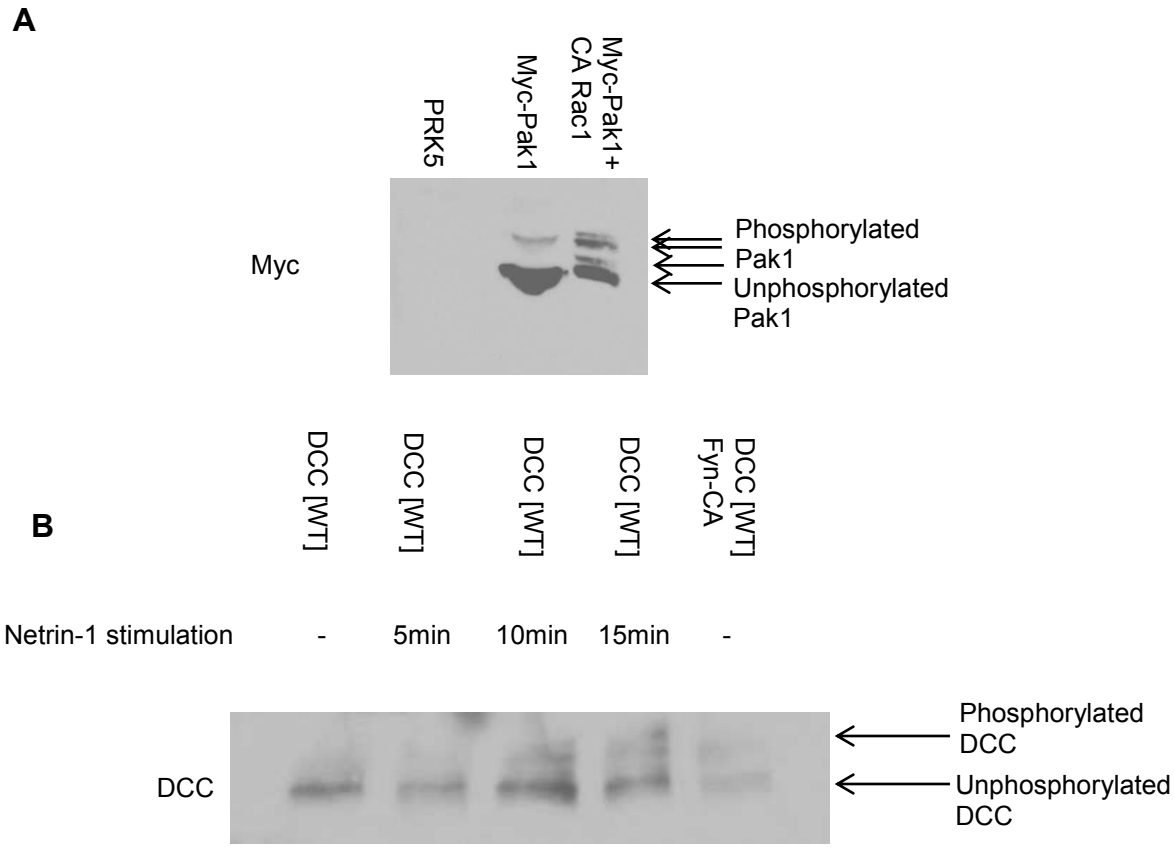

Figure 11. Phos-Tag can be used to separate phosphorylated proteins from unphosphorylated proteins. (A) HEK-293 cells were transfected with either a PRK5 empty vector or Myc-tagged Pak1 alone or with constitutively active Rac1. The proteins from cells extracts were resolved using a 5% SDS-PAGE containing 100uM of Phos-Tag product and detected by western blotting using anti-Myc. (B) HEK-293 cells were transfected with either wild type DCC [WT] alone or with constitutively active Fyn. They were then serum starved for 16 hours and subsequently stimulated with 500ng/ml of netrin-1 for several different time periods. The proteins from cells extracts were resolved using a 3.5% SDS-PAGE containing 75uM of Phos-Tag product and detected by western blotting using an anti-extracellular DCC antibody.

Chapter 4

General Discussion and Conclusions

4.1 Summary of original findings

Throughout this thesis we have characterized the functional relevance of different regions of the cytoplasmic tail of DCC and the role of specific serine and threonine phosphorylation sites located in this domain. Firstly, by using N1E-115 cells, the effects of different DCC deletion and phosphorylation mutants on netrin-1mediated neurite outgrowth was analyzed. Secondly, we investigated the effects of these different mutants on downstream signaling and DCC receptor localization.

1. In N1E-115 cells transfected with DCC deletion mutants of various lengths, the shorter deletion mutants (DCC-1-1129, DCC-1-1149, DCC-1-1171) all demonstrated significantly reduced netrin-1-mediated neurite outgrowth while the longer deletion mutants (DCC-1-1247, DCC-1-1327) did not demonstrate a statistically significant reduction in netrin-1-mediated neurite outgrowth relative to the wild-type DCC transfected controls. In a similar set of experiments, each of the three serine and threonine phospho-null mutants (DCC-S1178A, DCC-S1182A, DCC-T1219A) and a double mutant (DCC-S1178A/S1182A) demonstrated a statistically significant reduction in netrin-1-mediated neurite outgrowth relative to wild-type DCC transfected cells.

2. In HEK-293 cells stimulated with netrin-1 all of the different DCC deletion mutants demonstrated impaired netrin-1-mediated ERK1/2 phosphorylation (DCC-1-1129, DCC-1-1149, DCC-1-1171, DCC-1-1247, DCC-1-1327) relative to wild-type-DCC. In similar experiments all of the DCC phospho-null mutants (DCC-S1178A, DCC-S1182A, DCC-T1219A) and a double mutant (DCC-S1178A/S1182A) demonstrated a level of ERK1/2

and FAK phosphorylation that was similar to wild-type-DCC. In biotinylation experiments, HEK-293 cells transfected with the DCC-T1219A and DCC-S1178A/S1182A mutants showed a reduced amount of DCC at the cell surface relative to wild-type-DCC transfected cells.

4.2 Clinical importance of Netrin-1/DCC mediated axon guidance

There is an increasing amount of research that suggests that aberrant netrin-1 mediated axon guidance plays a role in the development of several neurological diseases. Congenital mirror movements, which are contralateral movements that mirror voluntary movements, have previously been linked to mutations in the *DCC* gene (Srour et al., 2010). Congenital mirror movements are often a result of defects in midline crossing of neurons in the developing central nervous system. In particular, frame-shift mutations causing truncated forms of DCC that fail to bind to netrin-1 have been linked to congenital mirror movements (Srour et al., 2010). Small nucleotide polymorphisms within the genes encoding netrin-1 and DCC have also been linked to a multitude of neurological diseases, including schizophrenia, Parkinson's, Alzheimer's diseases, and amyotrophic lateral sclerosis (ALS) (Grant et al., 2012; Lesnick et al., 2008; Schmidt et al., 2009; Lin et al., 2009). Furthermore, netrin-1 and DCC have been shown to be potential therapeutic targets in the treatment of spinal cord injuries (Curinga and Smith, 2008). As a result it is essential to understand the molecular mechanisms and downstream signaling that govern the netrin-1/DCC chemoattractive pathway.

4.3 The role of the intracellular domain of DCC in Netrin-1 mediated neurite outgrowth

The N1E-115 cells transfected with the shorter deletion mutants (DCC-1-1129, DCC-1-1149 and DCC-1-1171) all showed a significant reduction in neurite outgrowth compared to the wild-type DCC transfected cells (Table 2). However cells transfected with the longer deletion mutants (DCC-1-1247 and DCC-1-1327) did not show a significant reduction in neurite outgrowth (Table 2). The DCC-1-1129 and DCC-1-1149 mutants both lack all three of the conserved intracellular domains (P1, P2 and P3), while the DCC-1-1171, DCC-1-1247 and DCC-1-1327 mutants possess an intact P1 domain, but lack a P2 and P3 domain. This suggests that the section of the intracellular domain of DCC that is necessary for proper netrin-1 mediated neurite outgrowth is located in the region between the P1 domain and the P2 domain, in particular between residues 1171 and 1247. These results were unexpected because a longer deletion mutant (DCC-1-1421) lacking only the P3 domain has been previously expressed in N1E-115 cells and was found to have a small but statistically significant reduction in neurite outgrowth relative to wild-type DCC (DeGeer et al., 2012). This suggests that the P3 domain could possibly have a subtle role in netrin-1 mediated neurite outgrowth, but that the effect of its absence in DCC-1-1247 and DCC-1-1327 transfected cells was too small to reach statistical significance in our experiments. Interestingly all three of the analyzed phosphorylation sites (S1178, S1182, T1219) are located between residues 1171 and 1247. The fact that all three of the deletion mutants that lacked these three phosphorylation sites showed reduced neurite outgrowth, while the two deletion mutants that had these sites intact did not show a significant reduction in neurite outgrowth, reinforces our findings with the different phospho-null mutants and supports the theory that these serine and threonine residues are necessary for netrin-1 mediated neurite outgrowth. A potential future experiment would be to generate a

S1178A/S1182A/T1219A triple mutant and transfect it into N1E115 cells to see if there is a further decrease in neurite outgrowth relative to the single mutants.

4.4 The role of ERK1/2 activity in Netrin-1 mediated neurite outgrowth

There are previous experiments suggesting that ERK1/2 phosphorylation downstream of netrin-1 stimulation is required for netrin-1 mediated neurite outgrowth (Forcet et al., 2002). However our data does not entirely support this theory. All of the deletion mutants that were analyzed showed a reduction in ERK1/2 activity, regardless of whether they had a reduced induction of neurite outgrowth in response to netrin-1 or not (Table 2). This suggests that the reduction in neurite outgrowth seen in the 1-1129, 1-1149 and 1-1171 mutants was not a result of the inability of those deletion mutants to induce ERK1/2 phosphorylation. Furthermore, despite showing no effect on netrin-1 meditated ERK1/2 induction, each of the S1178, S1182, and T1219 phosphorylation sites appears to be necessary for proper netrin-1-mediated neurite outgrowth. Together, these findings contradict previous research that suggests that the phosphorylation of ERK1/2 downstream of netrin-1/DCC is necessary for netrin-1 dependent neurite outgrowth (Forcet et al., 2002). The conclusion that ERK1/2 phosphorylation is necessary for netrin-1 mediated outgrowth was based upon experiments that analyzed ERK1/2's involvement in this pathway through an indirect manner, by studying the effects of a MEK1 and MEK2 inhibitor; U0126 on inhibiting axon outgrowth in rat commissural neurons treated with netrin-1 (Forcet et al., 2002). It was also found that the turning response of *Xenopus* spinal neurons stimulated with netrin-1 was antagonized by U0126 exposure (Forcet et al., 2002). However, even though U0126 blocked ERK1/2 activation in response to netrin-1, it did not completely block netrin-1 mediated axon turning in the *Xenopus* spinal neurons (Forcet et al.,

2002) suggesting that ERK1/2 activity is not entirely necessary for netrin-1 function. Forcet et al. (2002) also found that a DCC deletion mutant that lacked its intracellular domain showed reduced axon outgrowth and an inability to phosphorylate ERK1/2 upon netrin-1 stimulation, however this mutant had a stop codon at residue 1121. Therefore it lacked almost the entire intracellular domain, which would presumably have affected nearly all signaling events downstream of netrin-1/DCC, so it would be impossible to prove that the reduction in axon outgrowth that this mutant demonstrated is specifically linked to its effect on ERK1/2 phosphorylation. Furthermore, as our biotinylation experiments performed using the 1-1120 deletion mutant have demonstrated (Fig. 9A), this particular mutant analyzed by Forcet et al. (2002) would not have reached the cell membrane and it is likely that any reduction seen in axon outgrowth in response to netrin-1 with this mutant would have been due to reduced expression of DCC at the cell surface. There is also evidence that ERK1/2 phosphorylation plays a role in downstream gene transcription in response to netrin-1 stimulation, with the transcription factor Elk-1 being activated in response to netrin-1 in HEK-293 cells, an effect that was abolished when the wild-type DCC transfected cells were treated with U0126 and was not present in cells transfected with the DCC truncation mutant lacking the cytosolic domain (Forcet et al., 2002). Further evidence has been gathered showing that ERK1/2 is activated downstream of netrin-1, confirming an increase in ERK1/2 activity within 5 minutes of netrin-1 stimulation in the growth cones of *Xenopus* retinal neurons (Campbell and Holt, 2003). In addition, there is evidence suggesting ERK1/2 plays a role in netrin-1 mediated growth cone repulsion as well, with treatment of *Xenopus* retinal neuron growth cones with U0126 causing a significant attenuation of netrin-1 mediated growth cone collapse (Campbell and Holt, 2003). Of note, ERK1/2 activation upon netrin-1 stimulation has been shown to be necessary for other roles of netrin-1

outside of axon outgrowth including angiogenesis, through inducing the production of nitric oxide upon netrin-1 stimulation of DCC expressing endothelial cells (Nguyen and Cai, 2006).

It has been found that DCC interacts with ERK2 in a direct manner and that this interaction is dependent on its P1 domain (Ma et al., 2010). Furthermore, through analyzing the binding between ERK2 and different DCC deletion mutants, it has been found that there are two ERK2 binding sites on the intracellular domain of DCC. The first and stronger binding site is between residues 1120-1212 and the second is between residues 1212-1326 (Ma et al., 2010). Since all three of the deletion mutants that demonstrated impaired netrin-1 mediated neurite outgrowth (DCC-1-1129, DCC-1-1149 and DCC-1-1171) (Table 2) lacked both of these ERK2 binding sites, it is therefore not surprising that they also showed reduced netrin-1 mediated ERK1/2 phosphorylation. ERK2 is known to phosphorylate DCC upon netrin-1 stimulation and interestingly, one of the phosphorylation sites we analyzed, S1178 was found to be phosphorylated by ERK2 (Ma et al., 2010). However, our findings indicate that this site, as well as the S1182 and T1219 sites does not play a role in netrin-1 mediated ERK1/2 signaling downstream of DCC (Table 2).

ERK1/2 phosphorylation has been heavily studied in relation to the netrin-1 pathway in the context of netrin-1's role in axon guidance (Forcet et al., 2002; Campbell and Holt, 2003; Ma et al., 2010). However, examination of the literature suggests that our findings that it is possible for netrin-1-mediated neurite outgrowth to be inhibited without having an effect on ERK1/2 or FAK phosphorylation, as seen with the DCC-S1178A, DCC-S1182A, DCC-T1219A and DCC-S1178A/S1182A mutants (Table 2) and conversely that it is possible to inhibit netrin-1 mediated neurite outgrowth while maintaining ERK1/2 phosphorylation as seen with the DCC-1-1247, DCC-1-1327 mutants (Table 2) appears to be novel. These results suggest that the area of the of

the cytoplasmic tail of DCC necessary for netrin-1-mediated neurite outgrowth and the area of the cytoplasmic tail of DCC that is necessary for netrin-1-induced ERK1/2 phosphorylation may be separate and that the role of ERK1/2 phosphorylation in the netrin-1/DCC pathway has yet to be fully elucidated.

To further study the role of ERK1/2 in netrin-1-mediated neurite outgrowth, one experiment we could perform would be to transfect N1E-115 cells with wild-type DCC, as we have previously (Fig. 8) and then expose them to U0126 and analyze whether there is a reduction in neurite outgrowth in the U0126 exposed cells compared to unexposed wildytpe DCC transfected control cells, as would be predicted by previous experiments in the field (Forcet et al, 2002). We could also perform a similar experiment using our phospho-null mutants in order to determine if through inhibiting ERK1/2 phosphorylation with U0126 there was a further reduction in their ability to induce netrin-1-mediated neurite outgrowth. Another potential future experiment would be to further analyze the effects of the longer deletion mutants that did show a reduction in ERK1/2 phosphorylation, but did not show a statistically significant reduction in neurite outgrowth. We could test whether the reduction in ERK1/2 phosphorylation observed in the 1-1247 and 1-1327 deletion mutants had other effects on netrin-1 mediated growth cone guidance, such as analyzing whether these deletion mutants had an effect on netrin-1-induced growth cone turning. To study this we could use a similar turning assay as used by Forcet et al. (2002). However as rat spinal neurons endogeneously express DCC we would have to employ a method such as siRNA to block the endogenous DCC (Ly et al., 2008). Through such experiments, we would aim to have a better understanding of the precise role of ERK1/2 phosphorylation in the netrin-1/DCC signaling.

4.5 The effect of the phosphorylation of the cytoplasmic domain of DCC on DCC cell surface expression

Our biotinylation experiments showed that HEK-293 cells tranfected with either the DCC-T1219A mutant or the DCC-S1178A/S1182A double mutant each showed a reduction in cell surface DCC relative to cells transfected with wild-type DCC (Table 2). This suggests that these three phosphorylation sites may play a role in the expression of DCC at the surface of the growth cone, and the reduction in neurite outgrowth seen in N1E-115 cells transfected with the phospho-null mutants relative to the wild-type DCC transfected controls (Table 2) suggests that this result is functionally important. What is particularly interesting about this result is that despite the reduced cell surface expression of DCC seen in the phospho-null mutants, downstream ERK1/2 and FAK phosphorylation remained intact (Table 2). This could be due to one of several factors. First, even the reduced amount of DCC that reaches the cell surface in the phospho-null mutant transfected cells could be a sufficient quantity for proper downstream activation of the ERK1/2 and FAK pathways, while being an insufficient amount of DCC at the cell surface to get proper neurite outgrowth in response to netrin-1. The second possibility is that FAK and ERK1/2 activation could be occurring through the endocytosed population of DCC receptors as well as the receptors that remain at the cell surface subsequent to netrin-1 stimulation. Previous research studying the insulin pathway, has demonstrated that downstream signaling can occur from receptors that are internalized upon ligand binding (Di Guglielmo et al., 1998). It is possible that the ERK1/2 and FAK signaling we observed in the cells expressing the phospho-null mutants could have been due to receptors that were internalized subsequent to netrin-1 stimulation, but still functioned to activate ERK1/2 and FAK, thus compensating for the lower amount of receptors at the cell surface that were activating ERK1/2 and FAK.

The next step in analyzing how these phosphorylation sites affect DCC cell surface expression would be to study the trafficking of DCC in live cell conditions. In order to further characterize the cell surface expression of the phospho-null mutants we could employ live cell imaging using a fluorescent tag to visualize the transport of the DCC receptor from the intracellular pool of DCC to the cell membrane. In order to achieve this each of the DCC mutant proteins will be subcloned into a mCherry-DCC vector, which has previously been used to analyze DCC expression in neurons (Winkle et al., 2014). Previous research has demonstrated that upon netrin-1 stimulation, the amount of DCC localized to the cell surface in cortical growth cones increases (DeGeer, et al., 2013). Therefore we could electroporate neurons with mCherry-DCC or a mCherry tagged form of each mutant and then stimulate the cells with netrin-1 for a range of times. Next, we could perform time-lapse imaging to follow the localization of mCherry-DCC or the mutants in response to netrin-1. In addition, we could transfect neurons with mCherry-DCC or our mCherry tagged phospho-null mutants, fix them and then measure the fluorescence intensity in the cell bodies, axons, and growth cones (DeGeer, et al., 2015).

If we can confirm that these phosphorylation sites do indeed affect DCC cell surface expression in live cell conditions with neurons, the next objective would be to analyze how this occurs. It has been previously discovered in rat cortical neurons that DCC expression is enhanced at the growth cone upon netrin-1 stimulation and that protein kinase A (PKA) activity is critical for this netrin-1 mediated translocation of DCC from an intracellular vesicular pool to the growth cone (Bouchard et al., 2004). Furthermore, PKA activity has been shown to regulate the sensitivity of netrin-1-induced chemoattraction in rat spinal commissural axons, although netrin-1 stimulation does not actually modulate PKA activity (Moore and Kennedy, 2006). Therefore there is the possibility that these phosphorylation sites could play a role in affecting

PKA activity and through this cause a reduction in DCC expression at the cell surface. Therefore potential future experiments could analyze whether HEK-293 cells transfected with the phospho-null mutants show reduced PKA activity relative to wild-type DCC transfected cells. Through these experiments we would aim to analyze whether the reduction in DCC surface expression seen with the T1219A and S1178A/S1182A phospho-null mutants is mediated through affecting PKA activity.

4.6 Analyzing phosphorylation on the intracellular region of DCC in response to Netrin-1 using a Phos-Tag system

The analysis of the phosphorylation of the different DCC phospho-null mutants upon netrin-1 stimulation using Pro-Q phospho-protein staining suggested that the S1182 and T1219 sites are not phosphorylated by netrin-1, while the S1178 and the T1210 sites are (Fig. 7). However this data is weakened by the fact that the Pro-Q phospho-protein staining results were based on comparing the total phosphorylation of all threonine, tyrosine and serine residues of the cells expressing the different mutants with the total phosphorylation of all threonine, tyrosine and serine residues of the cells expressing wild-type DCC. Therefore to be able to infer that a particular site is being phosphorylated in response to netrin-1 using Pro-Q phospho-protein staining, the elimination of that site (such as is the case with phospho-null mutants) would have to cause a significant reduction in the total amount of phosphorylation of DCC upon netrin-1 stimulation. Since DCC is heavily phosphorylated upon netrin-1 stimulation (Meriane, 2004), this means that a site that is comparatively infrequently phosphorylated upon netrin-1 stimulation, but may still play a functional role, could be missed by Pro-Q phospho-protein stain analysis. Thus we are unable to conclude that the S1182 or T1219 sites are not phosphorylated

upon netrin-1 stimulation, we can only state that the loss of these phosphorylation sites dos not significantly reduce the total amount of netrin-1 dependent phosphorylation of DCC.

One method to circumvent this sensitivity problem is to employ a Phos-Tag system to analyze DCC phosphorylation. Phos-Tag is a method that allows for the analysis protein phosphorylation by physically separating the phosphorylated and unphosphorylated proteins loaded on an acylamide gel. The Phos-Tag molecule contains two positively charge cations (usually zinc or manganese), which bind selectively to the negative charge on phosphate groups. When samples containing phosphorylated proteins are run on a Phos-Tag infused gel, the Phos-Tag molecules will selectively bind to the phosphorylated proteins and slow their progress through the gel. This will produce a blot in which the lowest band will be the non-phosphorylated protein, whereas the bands above it will be the different phosphorylated species of the protein (Fig. 11). This allows for not just comparing the amount of phosphorylated DCC to unphosphorylated DCC to infer phosphorylation of a particular phospho-site, as with Pro-Q, but to directly analyze phosphorylation at a specific site on DCC upon netrin-1 stimulation. Furthermore, when combined with 2D gel analysis, this process will allow for even the separation of proteins phosphorylated on different sites, but with the same raw amount of phosphorylation (Kimura et al, 2010). Since all the different phosphorylation species of DCC would be separated out into a series of bands, we could analyze the phosphorylation status of the different phospho-null mutants by comparing the differences between the banding pattern of the mutants and wild-type DCC. As a result we should be able to detect the loss of a phosphorylation site that is only infrequently phosphorylated by netrin-1 as a difference in the banding pattern of the mutant compared to wild-type DCC. Therefore we could analyze the DCC-S1182A and DCC-T1219A mutants to determine if they are in fact phosphorylated upon netrin-1, but at a

frequency that was below the threshold for detection by the Pro-Q phospho-protein stain. Furthermore we could analyze the phosphorylation status of the DCC-S1178A/S1182A double mutant and the DCC-S1178A/S1182A/T1219A triple mutant we would plan to create. Through employing a Phos-Tag system, we hope to generate a more complete understanding of whether these serine and threonine sites in the cytoplasmic domain of DCC are phosphorylated in response to netrin-1.

4.7 General conclusions

In this thesis we have investigated the role of the cytoplasmic domain of DCC in netrin-1-mediated neurite outgrowth. First, through using a series of DCC cytoplasmic-tail deletion mutants, we identified the region of the cytoplasmic tail of DCC (between residues 1171 and 1247) that appears to be necessary for netrin-1 mediated neurite outgrowth. Secondly we discovered that several phosphorylation sites (S1178, S1182 and T1219) located within this region of the cytoplasmic tail are required for netrin-1 mediated neurite outgrowth.

Finally, we have identified serine and threonine phosphorylation on the cytoplasmic tail of DCC as an important regulator of DCC expression at the cell membrane, suggesting a mechanism behind the role of the phosphorylation sites in netrin-1-mediated neurite outgrowth. With increasing amounts of research linking netrin-1 and DCC dysfunction to various neurodegenerative diseases (Grant et al., 2012; Lesnick et al., 2008; Schmidt et al., 2009; Lin et al., 2009) and with both proteins being looked to as potential therapeutic targets for treating nerve damage (Curinga and Smith, 2008), a proper understanding of how the cytoplasmic tail of DCC mediates the downstream signaling events that are critical to netrin-1 functioning appears to be of increasing clinical relevance.

Table 2. Summary of results. Summary of the effects of the DCC-1-1129, DCC-1-1149, DCC-1-1171, DCC-1-1247, DCC-1-1327 deletion mutants as well as the DCC-S1178A, DCC-S1182A, DCC-T1219A and DCC-S1178A/S1182A phospho-null mutants on netrin-1 mediated neurite outgrowth, netrin-1-induced ERK1/2 and FAK phosphorylation and DCC cell surface expression.

Mutant	**Reduced neurite outgrowth**	**Reduced ERK1/2 phosphorylation**	**Reduced FAK phosphorylation**	**Reduced DCC expression at the cell surface**
1-1129	yes	yes	not tested	not tested
1-1149	yes	yes	not tested	not tested
1-1171	yes	yes	not tested	not tested
1-1247	no	yes	not tested	not tested
1-1327	no	yes	not tested	not tested
S1178A	yes	no	no	not tested
S1182A	yes	no	no	not tested
T1219A	yes	no	no	yes
S1178A/S1182A	yes	no	no	yes

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